extension unit of contrast file., Including - (CH2) rC (O) NRb (CH2CH2O) r- (seeing the 150th page the 1st section in 1 description of contrast file). ., It is thus clear that wherein contained - NH- (CH2CH2O) 2-., - (CH2) 2Cchar(39)s (O) structure., Those skilled in the art carry out conventional the transformation in order to obtain the said structure of this claim to above-mentioned structure easily., Its technological effect can be expected.

Claim 15-18 is quoted to claim 19. Further prescribing a limit to - NH- (CH2) n1-La-Lb-Lc-char(39)s structure. Yet. 1 selection of still having instructed multiple extension unit of contrast file., Including - (CH2) rC (O) NRb (CH2CH2O) r- (seeing the 150th page the 1st section in 1 description of contrast file). ., It is thus clear that wherein contained - NH-., -(CH2)rC(O)- - CH2CH2O-char(39)s structure., Those skilled in the art carry out conventional the transformation in order to obtain this claim several kinds of said structures to above-mentioned structure easily., Its technological effect can be expected.

Claim 20-21 all further prescribes a limit to specific joint design. Yet. The extension unit has been instructed to contrast file 1., The selection of amino acid unit., And DXchar(39)s enlightenment has been given to select., Those skilled in the art obtain the said antibody of claim 20-21 - medicine conjugate through limited experiment easily., Antibody - medicine conjugate that contrast file 1 provided has the technological effect of dwindling internal tumour volume equally., The said conjugate of claim 20-21 does not possess unexpected technological effect (seeing 1 embodiment part of contrast file). .

In sum. When its claim of quoting does not possess creativeness., It does not possess outstanding substantive characteristics and remarkable progress to subordinate to claim 16-21 yet., Be not conform to the regulation of the 22nd the 3rd section of patent law.

6、Claim 22-29 is not conform to the regulation of the 22nd the 3rd section of patent law.

Claim 22-24 quotes claim 1 or 15 respectively. The further average linking number of prescribing a limit to 1 kind of medicine - joint design of selection for 1 antibody. Yet. Contrast file 1 still discloses a 1-4 medicine module and has been connected (seeing the 151st page the 3rd section in 1 description of contrast file). with antibody. Those skilled in the art adjust and verify its curative effect to medicine - joint design for the average linking number of 1 antibody easily on the basis of contrast file 1.

Claim 1 or 15 is quoted to claim 25. Further prescribe a limit to the function of antibody. Yet. Contrast file 1 still discloses treats like CD22char(39)s antibody to B cell surface mark through using., Including using the antibody (seeing the 63rd page the 1st section in 1 description of contrast file). that the coupling has the cell toxic agent., It is thus clear that having instructed, contrast file 1 adopts said antibody - the medicine conjugate of antibody preparation

that has discernment target cell characteristic.

Claim 1 or 15 is quoted to claim 26. Further prescribe a limit to the type of target cell. Yet. Contrast file 1 discloses the target cell that is directed against for B cell malignant tumour (seeing the 63rd page the 1st section in 1 description of contrast file). .

Claim 27-29 quotes claim 1 or 15 respectively. Further prescribe a limit to the type of antibody. Yet. Contrast file 1 still discloses treats like CD22char(39)s antibody to B cell surface mark through using., Including using the antibody (seeing the 63rd page the 1st section in 1 description of contrast file). that the coupling has the cell toxic agent., And other antibody all are the antibody that is directed against the known target in this field., Those skilled in the art select according to specific disease type and therapeutic schedule easily., Its target effect can be expected.

In sum. When its claim of quoting does not possess creativeness., It does not possess outstanding substantive characteristics and remarkable progress to subordinate to claim 22-29 yet., Be not conform to the regulation of the 22nd the 3rd section of patent law.

7、Claim 30-33 is not conform to the regulation of the 22nd the 3rd section of patent law.

Claim 30 medicine of asking for protection. It contains 1 or 15 said antibody - medicine conjugate or its salt of claim. Aforementioned examination reports have been commented claim 1 or 15 in detail and have not been possessed creative reason. Contrast file 1 still discloses provides the Pharmaceutical composition (seeing the 39th page the 1st section in 1 description of contrast file). who contains any the aforesaid immune conjugate and pharmaceutically acceptable carrier., Those skilled in the art expect preparing into the medicine with the antibody of claim 1 or 15 - medicine conjugate or its salt easily., Its technological effect can be expected.

Claim 31 ask for protection antineoplastic and / or anticancer drugs. Aforementioned examination reports have been commented claim 1 or 15 in detail and have not been possessed creative reason. Contrast file 1 still discloses provides the Pharmaceutical composition that contains any the aforesaid immune conjugate and pharmaceutically acceptable carrier., And use said Pharmaceutical composition in order to treat B cell proliferation nature disease for the individuality., Said B cell proliferation nature disease is selected from the lymthoma., (seeing the 39th page the 1st section in 1 description of contrast file). such as leukaemia., Those skilled in the art expect preparing into tumour medicine and / or anticancer drugs with the antibody of claim 1 or 15 - medicine conjugate or its salt easily., Its technological effect can be expected.

Claim 31 is quoted to claim 32. Further prescribe a limit to the type of cancer. Yet. Contrast file 1 still discloses said cancer and has still included breast cancer., (seeing the 62nd page of last section in 1 description of contrast

file). such as kidney cancer., Those skilled in the art expect coming the different kinds of cancer of treatment through the type of replacement antibody and medicine easily., Its technological effect can be expected.

Claim 33 pharmaceutical composition of asking for protection. Aforementioned examination reports have been commented claim 1 or 15 in detail and have not been possessed creative reason. Contrast file 1 still discloses provides the Pharmaceutical composition (seeing the 39th page the 1st section in 1 description of contrast file). who contains any the aforesaid immune conjugate and pharmaceutically acceptable carrier., Those skilled in the art expect preparing into the pharmaceutical composition with the antibody of claim 1 or 15 - medicine conjugate or its salt easily., Its technological effect can be expected.

In sum. The known general knowledge that combines this field on the basis of contrast file 1 technical scheme in order to obtain claim 30-33 and ask for protection., Conspicuous as far as those skilled in the art., Claim 30-33 does not possess outstanding substantive characteristics and remarkable progress., Be not conform to the regulation of the 22nd the 3rd section of patent law.

The applicant should answer the problem that the this notice book proposed in the appointed answer time limit at the this notice book one by one. Answer the amend the patent application document in case of necessity., Otherwise this application will be difficult to get the Green Light. The applicant should accord with the regulation of the 33rd patent law to the modification of applying for the file. The scope that must not exceed the record of former description and claims.

Name of the Examiner:李翠莹
Examiner Code:779438

中华人民共和国国家知识产权局

100020

北京市朝阳区东三环中路 1 号环球金融中心办公楼东楼 20 层 北京市
金杜律师事务所
杨宏军 牛蔚然

发文日：

2016 年 11 月 01 日

申请号或专利号：**201380053256.2**　　发文序号：**2016102700941030**

申请人或专利权人：第一三共株式会社

发明创造名称：抗体－药物偶联物

第 一 次 审 查 意 见 通 知 书

（进入国家阶段的 PCT 申请）

1. ☒应申请人提出的实质审查请求，根据专利法第 35 条第 1 款的规定，国家知识产权局对上述发明专利申请进行实质审查。
   ☐根据专利法第 35 条第 2 款的规定，国家知识产权局决定自行对上述发明专利申请进行实质审查。
2. ☒申请人要求以其在：
   JP 专利局的申请日 2012 年 10 月 11 日为优先权日。
3. ☐经审查，申请人于_____提交的修改文件，不符合专利法实施细则第 51 条第 1 款的规定，不予接受。
4. ☐审查是针对原始提交的国际申请的中文文本或中文译文进行的。
   ☒审查是针对下列申请文件进行的：
   依据专利合作条约第 28 条或者第 41 条提交的修改的权利要求第 1-49 项；
   2015 年 4 月 10 日提交的说明书附图第 1-22 幅、说明书摘要、说明书第 1-7, 22-2581 段、说明书核苷酸和氨基酸序列表；
   2015 年 9 月 6 日提交的说明书第 8-21 段。
5. ☒本通知书引用下列对比文献（其编号在今后的审查过程中继续沿用）

编号	文 件 号 或 名 称	公 开 日 期 （或抵触申请的申请日）
1	CN 101490087A	20090722

6. 审查的结论性意见：
   关于说明书：
   ☐申请的内容属于专利法第 5 条规定的不授予专利权的范围。
   ☐说明书不符合专利法第 26 条第 3 款的规定。
   ☐说明书不符合专利法第 33 条的规定。
   ☐说明书的撰写不符合专利法实施细则第 17 条的规定。
   ☐_____
   关于权利要求书：
   ☐权利要求_____不符合专利法第 2 条第 2 款的规定。
   ☐权利要求_____不符合专利法第 9 条第 1 款的规定。
   ☐权利要求_____不具备专利法第 22 条第 2 款规定的新颖性。

纸件申请，回函请寄：100088 北京市海淀区蓟门桥西土城路 6 号　国家知识产权局专利局受理处收
电子申请，应当通过电子专利申请系统以电子文件形式提交相关文件。除另有规定外，以纸件等其他形式提交的文件视为未提交。

中 华 人 民 共 和 国 国 家 知 识 产 权 局

☒权利要求 1-33 不具备专利法第 22 条第 3 款规定的创造性。
☐权利要求_____不具备专利法第 22 条第 4 款规定的实用性。
☒权利要求 34 属于专利法第 25 条规定的不授予专利权的范围。
☐权利要求_____不符合专利法第 26 条第 4 款的规定。
☒权利要求 1-49（具体参见通知书正文）不符合专利法第 31 条第 1 款的规定。
☐权利要求_____不符合专利法第 33 条的规定。
☐权利要求_____不符合专利法实施细则第 19 条的规定。
☐权利要求_____不符合专利法实施细则第 20 条的规定。
☐权利要求_____不符合专利法实施细则第 21 条的规定。
☐权利要求_____不符合专利法实施细则第 22 条的规定。
☐_____

☐申请不符合专利法第 26 条第 5 款或者实施细则第 26 条的规定。
☐申请不符合专利法第 20 条第 1 款的规定。
☐分案申请不符合专利法实施细则第 43 条第 1 款的规定。

上述结论性意见的具体分析见本通知书的正文部分。

7．基于上述结论性意见，审查员认为：

☐申请人应当按照通知书正文部分提出的要求，对申请文件进行修改。

☒申请人应当在意见陈述书中论述其专利申请可以被授予专利权的理由，并对通知书正文部分中指出的不符合规定之处进行修改，否则将不能授予专利权。

☐专利申请中没有可以被授予专利权的实质性内容，如果申请人没有陈述理由或者陈述理由不充分，其申请将被驳回。

☐_____

8．申请人应注意下列事项：

（1）根据专利法第 37 条的规定，申请人应当在收到本通知书之日起的 4 个月内陈述意见，如果申请人无正当理由逾期不答复，其申请将被视为撤回。

（2）申请人对其申请的修改应当符合专利法第 33 条的规定，不得超出原说明书和权利要求书记载的范围，同时申请人对专利申请文件进行的修改应当符合专利法实施细则第 51 条第 3 款的规定，按照本通知书的要求进行修改。

（3）申请人的意见陈述书和／或修改文本应当邮寄或递交国家知识产权局专利局受理处，凡未邮寄或递交给受理处的文件不具备法律效力。

（4）未经预约，申请人和／或代理人不得前来国家知识产权局与审查员举行会晤。

9．本通知书正文部分共有 7 页，并附有下列附件：

☐引用的对比文件的复印件共_____份_____页。

☐_____

审查员：李翠莹　　联系电话：020-28950489　　审查部门：专利审查协作广东中心医药生物发明审查部

纸件申请，回函请寄：100088 北京市海淀区蓟门桥西土城路 6 号　国家知识产权局专利局受理处收
电子申请，应当通过电子专利申请系统以电子文件形式提交相关文件。除另有规定外，以纸件等其他形式提交的文件视为未提交。

中华人民共和国国家知识产权局

第一次审查意见通知书

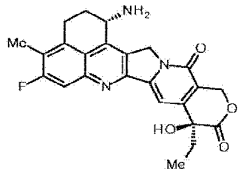
（进入国家阶段的 PCT 申请）

申请号:2013800532562

该申请涉及抗体-药物偶联物。经审查，现得出如下审查意见：

一. 单一性

该申请涉及如下 3 组发明：

（1）权利要求 1-34，涉及抗体-药物偶联物、相关的药物、抗癌药、药物组合物以及治疗方法，其中的药物是抗肿瘤性化合物  ，所示的抗肿瘤性化合物与抗体经由下式：$-L^1-L^2-L^P-NH-(CH_2)n^1-L^a-L^b-L^c-$ 所示的结构的接头连接而成的，其中，抗体连接于 L^1 的末端，抗肿瘤性化合物以 1 位的氨基的氮原子为连接部位、连接于 L^c 的末端其中 L^1 表示-(琥珀酰亚胺-3-基-N)-$(CH_2)n^2$-C(=O)-、$-CH_2$-C(=O)-NH-$(CH_2)n^3$-C(=O)-、-C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-、或 -C(=O)-$(CH_2)n^4$-C(=O)-，其中，n^2 表示 2～8 的整数，n^3 表示 1～8 的整数，n^4 表示 1～8 的整数；

（2）权利要求 35-48，涉及药物-接头中间体化合物 Q-$(CH_2)n^Q$-C(=O)-$L^{2a}-L^P$-NH-$(CH_2)n^1-L^a-L^b-L^c$-(NH-DX)，其中的 Q 表示(马来酰亚胺-N-基)-、HS-、X-CH2-C(=O)-NH-、或（吡咯烷-2,5-二酮-N-基)-O-C(=O)-，X 表示溴原子或碘原子，nQ 表示 2～8 的整数；

（3）权利要求 49 涉及-$L^1-L^2-L^P$-NH-$(CH_2)n^1-L^a-L^b-L^c$- 所示的接头，其用于得到经由接头将药物与抗体连接而成的抗体－药物偶联物，其中 L^1 表示-(琥珀酰亚胺-3-基-N)-$(CH_2)n^2$-C(=O)-、$-CH_2$-C(=O)-NH-$(CH_2)n^3$-C(=O)-、-C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-、或-C(=O)-$(CH_2)n^4$-C(=O)-，其中，n^2 表示 2～8 的整数，n^3 表示 1～8 的整数，n^4 表示 1～8 的整数。

上述 3 组发明的技术方案之间仅仅具有相同或相应的技术特征“用于将药物与抗体连接的接头结构”。然而，对比文件 1（CN101490087 A，2009.07.22）已公开了一种接头结构：“接头”(L) 为可用于连接一个或多个药物模块(D)和抗体单元(Ab)而形成通式 I 的抗体-药物偶联物(ADC)的双功能或多功能模块(参见对比文件 1 说明书第 148 页倒数第 1 段)，可见上述相同或相应的技术特征“用于将药物与抗体连接的接头结构”不是对现有技术作出贡献的技术特征，即不是特定技术特征。因此，涉及“用于将药物与抗体连接的接头结构”的技术方案之间没有相同或相应的特定技术特征，不属于一个总的发明构思，在技术上互不关联，因此不具备单一性，不符合专利法第 31 条第 1 款的规定。

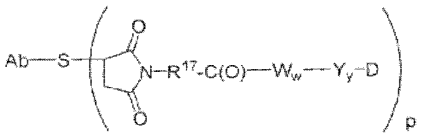
二. 针对第一组发明的审查意见如下：

1、权利要求 34 属于专利法第 25 条第 1 款第（3）项所述的疾病的诊断和治疗方法，不能被授予专利权

权利要求 34 请求保护肿瘤及/或癌的治疗方法，该方法是为使有生命的人体或动物体恢复或获得健康或减少痛苦，进行阻断、缓解或者消除病因或病灶的过程，属于利法第 25 条第 1 款第（3）项所述的疾病的诊断和治疗方法，因而不能被授予专利权。

2、 权利要求 1 不符合专利法第 22 条第 3 款的规定

权利要求 1 请求保护一种抗体-药物偶联物。当 n^1 为 1、L^1 为-(琥珀酰亚胺-3-基-N)-$(CH_2)n^2$-C(=O)-、n^2 为 5、L^2 和 L^a 及 L^b 为单键、L^c 为-C(=O)-时，对比文件 1 公开了如下的结构：



II，其中 R^{17} 为 $(CH_2)_5$，Ab 为抗体单元，D 为药物模块，Ww 为氨基酸单元，Yy 为间隔基单元（参见对比文件 1 说明书第 148 页倒数第 1 段、第 149 页倒数第 3-11 行、第 151 页倒数第 3 段至第 152 页第 1 段）。权利要求 1 请求保护的技术方案与对比文件 1 公开的技术内容相比，区别在于（1）权利要求 1 限定了药物的具体类型；（2）权利要求 1 限定了药物与接头连接的方式；（2）权利要求 1 还限定了 L^p 和 L^c 的具体结构。权利要求 1 实际解决的技术问题是提供另一种抗体-药物偶联物。

对于区别特征（1），对比文件 1 公开了本发明提供包含偶联有细胞毒剂的抗体的免疫偶联物或抗体-药物偶联物，所述细胞毒剂包括化疗剂；并列举了多种化疗剂，包括喜树碱的类似物托泊替康、伊立替康（参见对比文件 1 说明书第 22 页倒数第 2 段、第 72 页第 3 段）。虽然对比文件 1 未公开权利要求 1 所述的抗肿瘤性化合物，但权利要求 1 的抗肿瘤性化合物也是本领域常见的喜树碱衍生物，本领域技术人员容易想到根据癌症的类型、具体治疗方案，通过有限的试验选择合适的抗肿瘤性化合物用于制备所述的抗体-药物偶联物，其增强药物疗效的技术效果是可以预期的。

对于区别特征（2）-（3），对比文件 1 已公开了 Ww 为二肽-七肽单元，以及间隔物单元可以选择-Gly-Gly-或 Gly （参见对比文件 1 说明书第 153 页倒数第 8 行、第 155 页第 3 段）。可见，当对比文件 1 式 II 中的 Yy 为-Gly-Gly-或-Gly-时，相当于权利要求 1 中的 L^c 为-C(=O)-，并使得抗肿瘤性化合物以 1 位的氨基的氮原子连接于 L^c 末端。可见，本领域技术人员在对比文件 1 公开的技术内容中容易选择 L^p 和 L^c 的具体结构，其连接抗体和药物的技术效果是可以预期的。

综上所述，在对比文件 1 的基础上结合本领域的公知常识以获得权利要求 1 请求保护的技术方案，对本领域技术人员而言是显而易见的，权利要求 1 不具备突出的实质性特点和显著的进步，不符合专利法第 22 条第 3 款的规定。

纸件申请，回函请寄：100088 北京市海淀区蓟门桥西土城路 6 号 国家知识产权局专利局受理处收
电子申请，应当通过电子专利申请系统以电子文件形式提交相关文件。除另有规定外，以纸件等其他形式提交的文件视为未提交。

中华人民共和国国家知识产权局

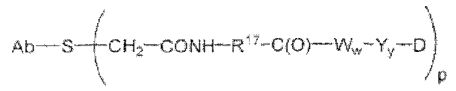
3、 权利要求 2-14 不符合专利法第 22 条第 3 款的规定

权利要求 2 引用权利要求 1，进一步限定 L^p 的肽残基。然而，对比文件 1 还公开了氨基酸单元包括二肽、三肽、四肽、或五肽。例示性的二肽包括：缬氨酸-瓜氨酸(vc 或 val-cit)；丙氨酸-苯丙氨酸(af 或 ala-phe)。例示性的三肽包括：甘氨酸-缬氨酸-瓜氨酸(gly-val-cit)和甘氨酸-甘氨酸-甘氨酸(gly-gly-gly)。构成氨基酸接头构 件的氨基酸残基可以包括天然存在的氨基酸残基，以及次要氨基酸和非天然存在氨基酸类似物，诸如瓜氨酸（参见对比文件 1 说明书第 154 页倒数第 3 段）。可见，对比文件 1 公开了氨基酸单元的肽可以选自苯丙氨酸、甘氨酸、缬氨酸、瓜氨酸，而其他类型的氨基酸也是氨基酸接头结构的常规选择。

权利要求 3 引用权利要求 1，进一步限定接头的结构。然而，对比文件 1 已公开了 L^a 及 L^b 为单键、L^c 为 -C(=O)-，以及 L^p 可以为四肽（参见权利要求 2 的审查意见）。

权利要求 4 引用权利要求 1，进一步限定接头的结构。然而，对比文件 1 已公开了 L^a 及 L^b 为单键、L^c 为 -C(=O)-，以及 L^p 可以为四肽（参见权利要求 2 的审查意见），虽然对比文件 1 未公开 GGFG 的四肽残基，但其公开了所述的氨基酸单元可以选自 G 和 F，本领域技术人员容易通过有限的试验获得 GGFG 所示的氨基酸单元，其连接效果是可以预期的。

权利要求 5 引用权利要求 1，进一步限定 L^1 的结构。然而，当 n^2 为 5 时，对比文件 1 已公开了结构-(琥珀酰亚胺-3-基-N)-$(CH_2)n^2$-C(=O)-，对比文件 1 还公开了结构



III，其中 R17 为-$(CH_2)_5$-（参见对比文件 1 说明书第 152 页倒数第 2 行、第 153 页倒数第 8 行），即公开了结构-CH_2-C(=O)-NH-$(CH_2)n^3$-C(=O)-。

权利要求 6 引用权利要求 1，进一步限定 L^1 的结构。请参见权利要求 5 的审查意见。

权利要求 7 引用权利要求 1，进一步限定 L^1 的结构。然而，对比文件 1 已教导了多种延伸物单元的选择，其中包括-$(CH_2CH_2O)_r$-（参见对比文件 1 说明书第 150 页第 1 段），当 r>1 时，即包含了-C(=O)-$(CH_2)n^4$-C(=O)-的结构。

权利要求 8 引用权利要求 1-6，进一步限定 n^2 为 2-5 的整数，L^2 为单键。然而，对比文件 1 已公开了 n^2 为 5，L^2 为单键（参见前面的审查意见）。

权利要求 9 引用权利要求 1-6，进一步限定 n^2 和 L^2。然而，对比文件 1 已公开了为 5（参见前面的审查意见）。对比文件 1 还教导了多种延伸物单元的选择，包括-$(CH_2)_rC(O)NR_b(CH_2CH_2O)_r$-（参见对比文件 1 说明书第 150 页第 1 段），当 r=3 时，即包含了-NH-$(CH_2CH_2O)n^5$-CH_2-CH_2-C(=O)-的结构。

权利要求 10-11 分别引用权利要求 1-7，进一步限定-NH-$(CH_2)n^1$-L^a-L^b-L^c-的原子个数。然而，对比文件 1 已公开了的 L^c 多种选择（参见对比文件 1 说明书第 155 页第 2-3 段），本领域技术人员通过有限的试验容易

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电子申请，应当通过电子专利申请系统以电子文件形式提交相关文件。除另有规定外，以纸件等其他形式提交的文件视为未提交。

获得权利要求 10-11 所述原子数目的链长的部分结构，其技术效果是可以预期的。

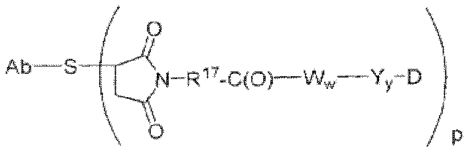
权利要求 12 引用权利要求 1-7，进一步限定-NH-(CH$_2$)$_n$^{1}-L^a-L^b-L^c-的结构。然而，对比文件 1 还教导了多种延伸物单元的选择，包括-(CH$_2$)$_r$C(O)NR$_b$(CH$_2$CH$_2$O)$_r$-（参见对比文件 1 说明书第 150 页第 1 段），可见其中包含了-NH-、-(CH$_2$)$_r$C(O)-、-CH$_2$CH$_2$O-的结构，本领域技术人员容易对上述结构进行常规改造以获得该权利要求所述的几种结构，其技术效果是可以预期的。

权利要求 13-14 均进一步限定具体的接头结构。然而，对比文件 1 已教导了延伸物单元、氨基酸单元的选择，以及给予了选择 DX 的启示，本领域技术人员通过有限的试验容易获得权利要求 13-14 所述的抗体-药物偶联物，对比文件 1 提供的抗体-药物偶联物同样具有缩小体内肿瘤体积的技术效果，权利要求 13-14 所述的偶联物并未具备预料不到的技术效果（参见对比文件 1 实施例部分）。

综上所述，当其引用的权利要求不具备创造性时，从属权利要求 2-14 也不具备突出的实质性特点和显著的进步，不符合专利法第 22 条第 3 款的规定。

4、 权利要求 15 不符合专利法第 22 条第 3 款的规定

权利要求 15 请求保护一种抗体-药物偶联物。当 n^{1}为 1、n^{2}为 5、L^{2}和 L^{a}及 L^{b}为单键时，对比文件 1 公开了如下的结构：



II，其中 R^{17}为-(CH$_2$)$_5$-，Ab 为抗体单元，D 为药物模块，Ww 为氨基酸单元，Yy 为间隔基单元（参见对比文件 1 说明书第 148 页倒数第 1 段、第 149 页倒数第 3-11 行、第 151 页倒数第 3 段至第 152 页第 1 段），可见对比文件 1 已公开了-(琥珀酰亚胺-3-基-N)-(CH$_2$)$_5$-C(O)-经由硫醚键连接至抗体，以及-(琥珀酰亚胺-3-基-N)-的 3 位与抗体连接，在 1 位的氮原子上与包含该结构的接头结构内的亚甲基连接。权利要求 15 请求保护的技术方案与对比文件 1 公开的技术内容相比，区别在于（1）权利要求 15 限定了药物的具体类型；（2）权利要求 15 还限定-(琥珀酰亚胺-3-基-N)-(CH$_2$)$_n$^{2}-C(=O)-经由存在于抗体的铰链部的二硫键部分中形成的硫醚键而连接至抗体，以及 L^{p}和 L^{c}的具体结构。权利要求 15 实际解决的技术问题是提供另一种抗体 药物偶联物。

对于区别特征（1），对比文件 1 公开了本发明提供包含偶联有细胞毒剂的抗体的免疫偶联物或抗体-药物偶联物，所述细胞毒剂包括化疗剂；并列举了多种化疗剂，包括洗漱间的类似物托泊替康、伊立替康（参见对比文件 1 说明书第 22 页倒数第 2 段、第 72 页第 3 段）。虽然对比文件 1 未公开权利要求 15 所述的抗肿瘤性化合物，但权利要求 15 的抗肿瘤性化合物也是本领域常见的喜树碱衍生物，本领域技术人员容易想到根据癌症的类型、具体治疗方案，通过有限的试验选择合适的抗肿瘤性化合物用于制备所述的抗体-药物偶联物，其增强药物疗效的技术效果是可以预期的。

对于区别特征（2），对比文件 1 已公开了 L^1 的结构以及其通过硫醚键而连接至抗体，本领域技术人员为了避免空间位阻影响抗体的 Fab 和 Fc 区域发挥其功能，容易想到将接头与铰链区的二硫键部分连接，其技术效果是可以预期的；对比文件 1 还公开了 Ww 为二肽-七肽单元，以及间隔物单元可以选择-Gly-Gly-或-Gly-（参见对比文件 1 说明书第 153 页倒数第 8 行、第 155 页第 3 段）。可见，当对比文件 1 式 II 中的 Yy 为-Gly-Gly-或-Gly-时，相当于权利要求 1 中的 L^c 为-C(=O)-，并使得抗肿瘤性化合物以 1 位的氨基的氮原子连接于 L^c 末端。可见，本领域技术人员在对比文件 1 公开的技术内容中容易选择 L^p 和 L^c 的具体结构，其连接抗体和药物的技术效果是可以预期的。

综上所述，在对比文件 1 的基础上结合本领域的公知常识以获得权利要求 15 请求保护的技术方案，对本领域技术人员而言是显而易见的，权利要求 15 不具备突出的实质性特点和显著的进步，不符合专利法第 22 条第 3 款的规定。

5、 权利要求 16-21 不符合专利法第 22 条第 3 款的规定

权利要求 16 引用权利要求 15，进一步限定 n^2、L^2 、L^a、L^b 的结构。然而，对比文件 1 已公开了 n^2 为 5，L^2 为单键（参见前面的审查意见）。对比文件 1 还公开了多种延伸物单元的选择，包括 $-(CH_2)_rC(O)NR_b(CH_2CH_2O)_r-CH_2-$（参见对比文件 1 说明书第 150 页第 1 段），当 r 为 1 时，其包括了该权利要求限定的第二种结构。

权利要求 17 引用权利要求 15，进一步限定 n^2、L^2 的结构。然而，对比文件 1 已公开了 n^2 为 5，L^2 为单键（参见前面的审查意见）。

权利要求 18 引用权利要求 15，进一步限定 n^2、L^2 的结构。对比文件 1 已公开了 n^2 为 5，（参见前面的审查意见）。然而，对比文件 1 还教导了多种延伸物单元的选择，包括 $-(CH_2)_rC(O)NR_b(CH_2CH_2O)_r-$（参见对比文件 1 说明书第 150 页第 1 段），可见其中包含了-NH- $(CH_2CH_2O)_2-$、$-(CH_2)_2C(O)-$的结构，本领域技术人员容易对上述结构进行常规改造以获得该权利要求所述的结构，其技术效果是可以预期的。

权利要求 19 引用权利要求 15-18，进一步限定 $-NH-(CH_2)n^1-L^a-L^b-L^c-$的结构。然而，对比文件 1 还教导了多种延伸物单元的选择，包括 $-(CH_2)_rC(O)NR_b(CH_2CH_2O)_r-$（参见对比文件 1 说明书第 150 页第 1 段），可见其中包含了-NH-、$-(CH_2)_rC(O)-$、$-CH_2CH_2O-$的结构，本领域技术人员容易对上述结构进行常规改造以获得该权利要求所述的几种结构，其技术效果是可以预期的。

权利要求 20-21 均进一步限定具体的接头结构。然而，对比文件 1 已教导了延伸物单元、氨基酸单元的选择，以及给予了选择 DX 的启示，本领域技术人员通过有限的试验容易获得权利要求 20-21 所述的抗体-药物偶联物，对比文件 1 提供的抗体-药物偶联物同样具有缩小体内肿瘤体积的技术效果，权利要求 20-21 所述的偶联物并未具备预料不到的技术效果（参见对比文件 1 实施例部分）。

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电子申请，应当通过电子专利申请系统以电子文件形式提交相关文件。除另有规定外，以纸件等其他形式提交的文件视为未提交。

综上所述，当其引用的权利要求不具备创造性时，从属权利要求 16-21 也不具备突出的实质性特点和显著的进步，不符合专利法第 22 条第 3 款的规定。

6、 权利要求 22-29 不符合专利法第 22 条第 3 款的规定

权利要求 22-24 分别引用权利要求 1 或 15，进一步限定选择的 1 种药物－接头结构相对于 1 抗体的平均连接数。然而，对比文件 1 还公开了 1-4 个药物模块与抗体连接（参见对比文件 1 说明书第 151 页第 3 段）。本领域技术人员在对比文件 1 的基础上容易对药物－接头结构相对于 1 抗体的平均连接数进行调整并验证其疗效。

权利要求 25 引用权利要求 1 或 15，进一步限定抗体的功能。然而，对比文件 1 还公开了通过施用针对 B 细胞表面标志物如 CD22 的抗体来治疗，包括施用偶联有细胞毒剂的抗体（参见对比文件 1 说明书第 63 页第 1 段），可见对比文件 1 已教导了采用具有识别靶细胞特性的抗体制备所述的抗体-药物偶联物。

权利要求 26 引用权利要求 1 或 15，进一步限定靶标细胞的类型。然而，对比文件 1 已公开了针对的靶细胞为 B 细胞恶性肿瘤（参见对比文件 1 说明书第 63 页第 1 段）。

权利要求 27-29 分别引用权利要求 1 或 15，进一步限定抗体的类型。然而，对比文件 1 还公开了通过施用针对 B 细胞表面标志物如 CD22 的抗体来治疗，包括施用偶联有细胞毒剂的抗体（参见对比文件 1 说明书第 63 页第 1 段），而其他抗体均是针对本领域公知的靶标的抗体，本领域技术人员容易根据具体的疾病类型和治疗方案进行选择，其靶向效果是可以预期的。

综上所述，当其引用的权利要求不具备创造性时，从属权利要求 22-29 也不具备突出的实质性特点和显著的进步，不符合专利法第 22 条第 3 款的规定。

7、 权利要求 30-33 不符合专利法第 22 条第 3 款的规定

权利要求 30 请求保护药物，其含有权利要求 1 或 15 所述的抗体－药物偶联物或其盐。前述审查意见已详细评述了权利要求 1 或 15 不具备创造性的理由，对比文件 1 还公开了提供包含任何上述免疫偶联物和药学上可接受的载体的药用组合物（参见对比文件 1 说明书第 39 页第 1 段），本领域技术人员容易想到将权利要求 1 或 15 的抗体-药物偶联物或其盐制备成药物，其技术效果是可以预期的。

权利要求 31 请求保护抗肿瘤药及/或抗癌药。前述审查意见已详细评述了权利要求 1 或 15 不具备创造性的理由，对比文件 1 还公开了提供包含任何上述免疫偶联物和药学上可接受的载体的药用组合物，并给个体施用所述药用组合物以治疗 B 细胞增殖性疾病，所述 B 细胞增殖性疾病选自淋巴瘤、白血病等（参见对比文件 1 说明书第 39 页第 1 段），本领域技术人员容易想到将权利要求 1 或 15 的抗体-药物偶联物或其盐制备成肿瘤药及/或抗癌药，其技术效果是可以预期的。

权利要求 32 引用权利要求 31，进一步限定癌症的类型。然而，对比文件 1 还公开了所述癌症还包括乳腺癌、肾癌等（参见对比文件 1 说明书第 62 页最后一段），本领域技术人员容易想到通过替换抗体和药物的类型来治疗不同类型的癌症，其技术效果是可以预期的。

权利要求 33 请求保护药物组合物。前述审查意见已详细评述了权利要求 1 或 15 不具备创造性的理由，对比文件 1 还公开了提供包含任何上述免疫偶联物和药学上可接受的载体的药用组合物（参见对比文件 1 说明书第 39 页第 1 段），本领域技术人员容易想到将权利要求 1 或 15 的抗体-药物偶联物或其盐制备成药物组合物，其技术效果是可以预期的。

综上所述，在对比文件 1 的基础上结合本领域的公知常识以获得权利要求 30-33 请求保护的技术方案，对本领域技术人员而言是显而易见的，权利要求 30-33 不具备突出的实质性特点和显著的进步，不符合专利法第 22 条第 3 款的规定。

申请人应当在本通知书指定的答复期限内对本通知书提出的问题逐一进行答复，必要时应修改专利申请文件，否则该申请将难以获得批准。申请人对申请文件的修改应当符合专利法第 33 条的规定，不得超出原说明书和权利要求书记载的范围。

审查员姓名:李翠莹
审查员代码:779438

中华人民共和国国家知识产权局

检索报告

申请号：2013800532562	申请日：20131010	首次检索
申请人：第一三共株式会社	最早的优先权日：20121011	
权利要求项数：49	说明书段数：2581	
审查员确定的 IPC 分类号：C07K16/28,A61K39/395,A61P35/00,A61K31/4745,A61K31/48		
检索记录信息：C07K 16/28，A61K 39/395，A61P 35/00，A61K 31/4745，A61K 31/48，CNABS,CNTXT,sipoabs,dwpi，WOTXT,CNKI, GOOGLE，第一三共株式会社，抗体，药物，偶联，肿瘤，接头，antibody，medicine，link，tumor，caner，(琥珀酰亚胺-3-基-N)-(CH2)n2-C，CH2-C(=O)-NH-(CH2)n3-C(=O)，C(=O)-cyc.Hex(1,4)-CH2-(N-ly-3-diminiccuS)，C(=O)-(CH2)n4-C(=O)，NH-(CH2-CH2-O)n5-CH2-CH2-C(=O)，S-(CH2)n6-C(=O)，单键，C(=O)-NH，-NR1-(CH2)n7，O，氢原子，烷基、-(CH2)n8-COOH，(CH2)n9-OH，CH2-或-C(=O)，琥珀酰亚胺-3-基-N，N-ly-3-diminiccuS，1，4-亚环己基，GGFG，anticancer，drug，antibody，linker，B7-H3，依莎替康		

相关专利文献

类型	国别以及代码[11]给出的文献号	代码[43]或[45]给出的日期	IPC 分类号	相关的段落和/或图号	涉及的权利要求

X	CN101490087A	20090722	C07K16/28	说明书第 22 页倒数第 2 段、第 39 页第 1 段、第 62 页最后一段、第 63 页第 1 段、第 72 页第 3 段、第 148 页倒数第 1 段、第 149 页倒数第 3-11 行、第 150 页第 1 段、第 151 页第 3 段和倒数第 3 段至第 152 页第 1 段和倒数第 2 行、第 153 页倒数第 8 行、第 154 页倒数第 3 段、第 155 页第 2-3 段，实施例部分	1-33
A	WO2006065533A2	20060622		全文	1-49
A	WO03043583A2	20030530	A61K	全文	1-49
A	JPH1192405A	19990406	A61K47/48	全文	1-49
A	JPH1095802A	19980414	A61K31/47	全文	1-49
A	WO2002000734P1	20020103	A61K	全文	1-49

中华人民共和国国家知识产权局

相关非专利文献					
类型	书名（包括版本号和卷号）	出版日期	作者姓名和出版者名称	相关页数	涉及的权利要求
类型	期刊或文摘名称（包括卷号和期号）	发行日期	作者姓名和文章标题	相关页数	涉及的权利要求
A	《世界临床药物》 第 33 卷 第 3 期	20120310	梁文等 肿瘤靶向抗体偶联物的研究进展	1-5	1-49

表格填写说明事项：

1．审查员实际检索领域的 IPC 分类号应当填写到大组和 / 或小组所在的分类位置。

2．期刊或其它定期出版物的名称可以使用符合一般公认的国际惯例的缩写名称。

3．相关文件的类型说明：

X：一篇文件影响新颖性或创造性；

Y：与本报告中的另外的 Y 类文件组合而影响创造性；

A：背景技术文件；

R：任何单位或个人在申请日向专利局提交的、属于同样的发明创造的专利或专利申请文件。

P：中间文件，其公开日在申请的申请日与所要求的优先权日之间的文件；

E：抵触申请。

审 查 员：李翠莹　　审查部门：专利审查协作广东中心医药生物发明审查部

2016 年 10 月 19 日

国 家 知 识 产 权 局

100020

北京市朝阳区东三环中路 1 号环球金融中心办公楼东楼 20 层 北京市金杜律师事务所
杨宏军(58785048) 牛蔚然(010-58785588)

发文日：

2019 年 11 月 08 日

申请号或专利号：**201580019138.9**　　发文序号：**2019102401175650**

申请人或专利权人： 第一三共株式会社,第一三共欧洲有限公司

发明创造名称： 抗 HER3 抗体-药物偶联物

授 予 发 明 专 利 权 通 知 书

（进入国家阶段的 PCT 申请）

1. 根据专利法第 39 条及实施细则第 54 条的规定，上述发明专利申请经实质审查，没有发现驳回理由，现作出授予专利权的通知。

申请人收到本通知书后，还应当依照办理登记手续通知书的内容办理登记手续。

申请人按期办理登记手续后，国家知识产权局将作出授予专利权的决定，颁发发明专利证书，并予以登记和公告。期满未办理登记手续的，视为放弃取得专利权的权利。

2. 授予专利权的上述发明专利申请是以下列申请文件为基础的：

☐原始提交的国际申请的中文文本或中文译文进行的。

☒针对下列申请文件进行的：

2016 年 10 月 10 日提交的说明书第 1-2630 段、说明书附图、说明书摘要、说明书核苷酸和氨基酸序列表；
2019 年 10 月 16 日提交的权利要求第 1-24 项。

3. 授予专利权的上述发明专利申请的名称：

☒未变更。

☐由__变更为上述发明创造名称。

4. ☐申请人于_____年_____月_____日提交专利号为_____的“放弃专利权声明”，经审查：

☐进入放弃专利权的程序。

☐未进入放弃专利权的程序。理由是：申请人声明放弃的专利与本发明专利申请不属于相同的发明创造。

5. ☐审查员依职权对申请文件修改如下：

6. 在本通知书发出后收到的申请人主动修改的申请文件，不予考虑。

审查员：朱宁

审查部门：专利审查协作北京中心医药生物发明审查部

联系电话：010-53961956

纸件申请，回函请寄：100088 北京市海淀区蓟门桥西土城路 6 号　国家知识产权局专利局受理处收

电子申请，应当通过电子专利申请系统以电子文件形式提交相关文件。除另有规定外，以纸件等其他形式提交的文件视为未提交。

国 家 知 识 产 权 局

100020

北京市朝阳区东三环中路 1 号环球金融中心办公楼东楼 20 层 北京市金杜律师事务所
杨宏军(58785048) 牛蔚然(010-58785588)

发文日：

2019 年 11 月 08 日

申请号或专利号：**201580019138.9**　　发文序号：**2019110500084210**

申请人或专利权人： 第一三共株式会社 第一三共欧洲有限公司

发明创造名称： 抗 HER3 抗体-药物偶联物

办 理 登 记 手 续 通 知 书

根据专利法实施细则第 54 条及国家知识产权局第 272 号公告的规定，申请人应当于 2020 年 01 月 23 日之前缴纳以下费用：

第 5 年度年费	1200.0 元	无费减 （减缴标记）
专利证书印花税	5.0 元	
共计	1205.0 元	

附已缴费用情况： 年费 0.0 元，专利证书印花税 0.0 元。

申请人按期缴纳上述费用的，国家知识产权局将在专利登记簿上登记专利权的授予，颁发专利证书，并予以公告。专利权自公告之日起生效。

申请人期满未缴纳或者未缴足上述费用的，视为放弃取得专利权的权利。

提示：

专利费用可以通过网上缴费、邮局或银行汇款缴纳，也可以到国家知识产权局面缴。

网上缴费：电子申请注册用户可登陆 http://cponline.cnipa.gov.cn，并按照相关要求使用网上缴费系统缴纳。

邮局汇款：收款人姓名：国家知识产权局专利局收费处，商户客户号：110000860。

银行汇款：开户银行：中信银行北京知春路支行；户名：国家知识产权局专利局；账号：7111710182600166032。

汇款时应当准确写明申请号、费用名称（或简称）及分项金额。未写明申请号和费用名称（或简称）的视为未办理缴费手续。了解更多详细信息及要求，请登陆 http://www.cnipa.gov.cn 查询。

按照财税[2019]13 号及京财税[2019]196 号通知，增值税小规模纳税人减半按 2.5 元缴纳印花税。

审 查 员：自动审查　　审查部门：专利局初审及流程管理部

联系电话：010-62084704

纸件申请，回函请寄：100088 北京市海淀区蓟门桥西土城路 6 号 国家知识产权局专利局受理处收

电子申请，应当通过电子专利申请系统以电子文件形式提交相关文件。除另有规定外，以纸件等其他形式提交的文件视为未提交。

国 家 知 识 产 权 局

检 索 报 告

申请号：2015800191389	申请日：20150410	补充检索
申请人：第一三共株式会社	最早的优先权日：20140410	
权利要求项数：24	说明书段数：2630+30	
审查员确定的 IPC 分类号：A61K 39/395,A61K 47/48,A61K 45/06,A61P 35/00,C07K 16/40,C07K 16/28,C07K 16/30		
检索记录信息：the anti-her3 antibody patritumab abrogates cetuximab resistance mediated by heregulin in colorectal cancer cells：google 数据库中，检索关键词是 her3、antibody。		

相 关 专 利 文 献

类型	国别以及代码[11]给出的文献号	代码[43]或[45]给出的日期	IPC 分类号	相关的段落和 / 或图号	涉及的权利要求

国 家 知 识 产 权 局

相 关 非 专 利 文 献					
类型	书名（包括版本号和卷号）	出版日期	作者姓名和出版者名称	相关页数	涉及的权利要求
类型	期刊或文摘名称 （包括卷号和期号）	发行日期	作者姓名和文章标题	相关页数	涉及的权利要求
A	《oncotarget》 第 5 卷 第 23 期	20141231	hisato kawakami 等 the anti-her3 antibody patritumab abrogates cetuximab resistance mediated by heregulin in colorectal cancer cells	11847-11856	1-24

表格填写说明事项：

1．审查员实际检索领域的 IPC 分类号应当填写到大组和 / 或小组所在的分类位置。

2．期刊或其它定期出版物的名称可以使用符合一般公认的国际惯例的缩写名称。

3．相关文件的类型说明：

X：一篇文件影响新颖性或创造性；

Y：与本报告中的另外的 Y 类文件组合而影响创造性；

A：背景技术文件；

R：任何单位或个人在申请日向专利局提交的、属于同样的发明创造的专利或专利申请文件。

P：中间文件，其公开日在申请的申请日与所要求的优先权日之间的文件；

E：抵触申请。

审 查 员：朱宁　　审查部门：专利审查协作北京中心医药生物发明审查部

2019 年 10 月 17 日

国 家 知 识 产 权 局

100020

北京市朝阳区东三环中路 1 号环球金融中心办公楼东楼 20 层 北京市金杜律师事务所

杨宏军(58785048) 牛蔚然(010-58785588)

发文日：

2019 年 08 月 20 日

申请号或专利号：**201480071134.0**　　发文序号：**2019081501186420**

申请人或专利权人： 第一三共株式会社,北海道公立大学法人札幌医科大学

发明创造名称： 抗 TROP2 抗体－药物偶联物

第 二 次 审 查 意 见 通 知 书

1. ☒审查员已经收到申请人于 2019 年 06 月 21 日提交的意见陈述书，在此基础上审查员对上述专利申请继续进行实质审查。

☐根据国家知识产权局于_____年_____月_____日作出的复审决定，审查员对上述专利申请继续进行实质审查。

☐_____

2. ☐经审查，申请人于_____提交的修改文件，不符合专利法实施细则第 51 条第 3 款的规定，不予接受。

3. 继续审查是针对下列申请文件进行的：

☐上述意见陈述书中所附的经修改的申请文件。

☒前次审查意见通知书所针对的申请文件以及上述意见陈述书中所附的经修改的申请文件替换文件。

☐前次审查意见通知书所针对的申请文件。

☐上述复审决定所确定的申请文件。

☐_____

4. ☐本通知书未引用新的对比文件。

☒本通知书引用下列对比文件(其编号续前，并在今后的审查过程中继续沿用)：

编号	文 件 号 或 名 称	公开日期 (或抵触申请的申请日)
1	US 7041818B2	20060509
2	Thomas M. Cardillo 等, Humanized Anti-Trop-2 IgG-SN-38 Conjugate for Effective Treatment of Diverse Epithelial Cancers: Preclinical Studies in Human Cancer Xenograft Models and Monkeys. Clin Cancer Res, 17(10), 3157-3169, 2011 年 05 月 15	20110515
3	CN 1764478A	20060426
4	CN 103313390A	201309018

5. 审查的结论性意见：

关于说明书：

纸件申请，回函请寄：100088 北京市海淀区蓟门桥西土城路 6 号　国家知识产权局专利局受理处收

电子申请，应当通过电子专利申请系统以电子文件形式提交相关文件。除另有规定外，以纸件等其他形式提交的文件视为未提交。

国 家 知 识 产 权 局

☐申请的内容属于专利法第 5 条规定的不授予专利权的范围。
☐说明书不符合专利法第 26 条第 3 款的规定。
☐说明书的修改不符合专利法第 33 条的规定。
☐说明书的撰写不符合专利法实施细则第 17 条的规定。
☐_____

关于权利要求书：

☐权利要求_____不符合专利法第 2 条第 2 款的规定。
☐权利要求_____不符合专利法第 9 条第 1 款的规定。
☐权利要求_____不具备专利法第 22 条第 2 款规定的新颖性。
☒权利要求 1 4,12 21 不具备专利法第 22 条第 3 款规定的创造性。
☐权利要求_____不具备专利法第 22 条第 4 款规定的实用性。
☐权利要求_____属于专利法第 25 条规定的不授予专利权的范围。
☒权利要求 6,8 不符合专利法第 26 条第 4 款的规定。
☐权利要求_____不符合专利法第 31 条第 1 款的规定。
☐权利要求_____的修改不符合专利法第 33 条的规定。
☐权利要求_____不符合专利法实施细则第 19 条的规定。
☐权利要求_____不符合专利法实施细则第 20 条的规定。
☐权利要求_____不符合专利法实施细则第 21 条的规定。
☐权利要求_____不符合专利法实施细则第 22 条的规定。
☐_____

☐申请不符合专利法第 26 条第 5 款或者实施细则第 26 条的规定。
☐申请不符合专利法第 20 条第 1 款的规定。
☐分案申请不符合专利法实施细则第 43 条第 1 款的规定。
上述结论性意见的具体分析见本通知书的正文部分。

6.基于上述结论性意见，审查员认为：

☐申请人应当按照通知书正文部分提出的要求，对申请文件进行修改。
☒申请人应当在意见陈述书中论述其专利申请可以被授予专利权的理由，并对通知书正文部分中指出的不符合规定之处进行修改，否则将不能授予专利权。
☐专利申请中没有可以被授予专利权的实质性内容，如果申请人没有陈述理由或者陈述理由不充分，其申请将被驳回。
☐_____

7. 申请人应注意下列事项：

（1）根据专利法第 37 条的规定，申请人应在收到本通知书之日起的 2 个月内陈述意见，如果申请人无正当理由逾期不答复，其申请被视为撤回。

（2）申请人对其申请的修改应当符合专利法第 33 条的规定，不得超出原说明书和权利要求书记载的范围，同时申请人对专利申请文件进行的修改应当符合专利法实施细则第 51 条第 3 款的规定，按照本通知书的要求进行修改。

（3）申请人的意见陈述书和/或修改文本应当邮寄或递交国家知识产权局专利局受理处，凡未邮寄或递交给受理处的文件不具备法律效力。

（4）未经预约，申请人和/或代理人不得前来国家知识产权局与审查员举行会晤。

8．本通知书正文部分共有 3 页，并附有下列附件：

☐引用的对比文件的复印件共_____份_____页。
☐_____

审查员：彭郁葱　　联系电话：010-53962047　　审查部门：专利审查协作北京中心医药生物发明审查部

国 家 知 识 产 权 局

第 二 次 审 查 意 见 通 知 书

申请号:2014800711340

查员在第【1】次审查意见通知书中已经对本申请权利要求【1】不符合专利法第【26】条第【4】款的相关问题提出审查意见，基于申请人【2019】年【06】月【24】日提交的意见陈述和/或修改的申请文件，为了确保对本申请的审查更为客观、公正、全面、准确，现对本申请进行进一步检索，并提出如下审查意见。

1、权利要求 1-4，12-21 不具备创造性，不符合专利法第 26 条第 4 款的规定。

权利要求 1 请求保护抗体-药物偶联物，所述抗体为抗 Trop2 抗体，药物为依沙替康，通过含 GGFG 四肽的接头连接，并限定了接头的结构以及连接方式。对比文件 1（US7041818 B2，公开日 2006 年 05 月 09 日）公开了一种由高分子载体羧甲基葡聚糖多元醇（CM-Dex-PA）与抗肿瘤药物 DX-8951（即依沙替康，分子式（(1S,9S)－1－氨基－9－乙基－5－氟－2,3－二氢－9－羟基－4－甲基－1H,12H－苯并[de]吡喃并[3',4':6,7]吲哚嗪并[1,2－b]喹啉－10,13(9H,15H)－二 酮)）通过 GGFG 四肽间隔区偶联的药物释放系统（或称药物复合物，DDS 化合物）（参见实施例 1），对比文件 1 还公开了不同的肽间隔区，在这些间隔区中，GGFG 可以短时间游离出高浓度药物（参见表 1、第 9 栏倒数第 1-4 行、第 10 栏第 8 行），当使用接头时，对接头没有特别的限定，接头可以是-O-（CH2）n-NH-（n 为 1-16 的整数）或是-（O-CH2CH2）m-NH-（m 为 1-10 的整数）（参见第 11 栏，第 48-52 行），所述药物复合物可用于治疗肝癌（参见权利要求 18，第 5 栏第 1-8 行）。可见，对比文件 1 公开了用于治疗肝癌的通过含有 GGFG 四肽间隔区的接头连接的抗肿瘤药物依沙替康与高分子载体的高分子载体-药物偶联物。权利要求 1 与对比文件 1 相比区别在于，限定其中的高分子载体是抗 Trop2 抗体，并限定了药物-接头的具体结构及其与抗体连接的连接方式。基于上述区别技术特征，本申请解决的技术问题是提供一种抗 Trop2 抗体-药物偶联物。

对于上述区别技术特征，对比文件 2（Thomas M. Cardillo 等，Humanized Anti-Trop-2 IgG-SN-38 Conjugate for Effective Treatment of Diverse Epithelial Cancers: Preclinical Studies in Human Cancer Xenograft Models and Monkeys. Clin Cancer Res，17(10)，3157-3169，2011 年 05 月 15 日）公开了用于治疗癌症的抗体-药物偶联物：人源化抗-TROP2 抗体 hRS7-CL2-SN-38（其中 hRS7 即为本申请的优选抗体，SN-38 是与依沙替康结构非常接近的喜树碱类似物）（参见摘要、结果和结论，表 1、图 1-5）。所述接头 CL2 包含一些结构元件和一个可切割的肽，并且以马来酰亚胺基与抗体 hRS7 相连，与包含不可切割的肽的 CL2A 接头的 hRS7-CL2A-SN-38 相比，包含可切割肽 hRS7-CL2-SN-38 对肿瘤的杀伤活性更强（参见图 1）。作者指出，基于所述结果，该偶联物对于抗 系列人实体瘤具有显著效果（参见结论）。

对比文件 3（CN1764478A，公开日 2006 年 04 月 26 日）公开了葸环霉素-抗体偶联物，其中所述药物和所述抗体通过包含马来酰亚胺的接头连接，所述抗体针对肿瘤-相关抗原，所述接头连接抗体上的还原的二硫键（参见权利要求 1, 4,12），并公开了将抗体的链间二硫键还原为游离硫醇，产生二硫化物-还原的 mAbs，

然后与药物-接头相偶联（参见实施例 5）。

在对比文件 1 公开的用于治疗癌症的高分子载体-药物（依沙替康）偶联物的基础上，由于使用抗体可以将药物靶向带到靶细胞，使药物集中在肿瘤部位，且降低其他组织、器官的药物浓度，达到增效减毒的作用，并且对比文件 2 公开了人源化抗-TROP2 抗体 hRS7 与 SN-38（与依沙替康结构非常接近的喜树碱类似物）的偶联物具有很好的抗肿瘤作用，因此，为了增强药物对肿瘤相关抗原的靶向性，本领域技术人员有动机使用抗-TROP2 抗体作为依沙替康的药物载体；此外本领域技术人员知晓，接头的功能不仅是连接作用，还应维持抗体-药物偶联物在细胞外的稳定存在且进入癌细胞后能迅速水解，释放出小分子药物发挥药效，从而快速清除靶细胞，并且对比文件 2 公开了包含可切割的肽接头使得偶联物对肿瘤的杀伤活性更强，以及对比文件 1 公开了在不同的肽间隔区中，-GGFG 四肽间隔区 GGFG 可以短时间游离出高浓度药物，在此基础上，为了使得药物在靶细胞中快速释放发挥作用，本领域技术人员会想到在接头中加入-GGFG-四肽间隔区，并在对比文件 1 或 3 公开的接头序列的基础上根据需要进行常规选择获得本申请的接头序列并以对比文件 3 公开的方式与药物和抗体偶联，而具有半胱氨酸残基(含硫醇基团)的抗体可与马来酰亚胺形成硫醚连接属于本领域公知常识，并且说明书中没有表明所述接头与本申请的预料不到的技术效果之间的关联性。因此，在对比文件 1 的基础上结合对比文件 2-3 以及本领域常规技术手段获得权利要求 1 的技术方案是显而易见的，权利要求 1 不具有突出的实质性特点和显著的进步，不具备专利法第 22 条第 3 款规定的创造性。

从属权利要求 2-4 限定接头结构，所限定的接头结构均在权利要求 1 中出现过，因此，基于与权利要求 1 相同的理由，权利要求 2-4 不具备专利法第 22 条第 3 款规定的创造性。

从属权利要求 12-13 限定选选择的 1 种药物－接头结构相对于 1 抗体而言的平均连接数为 2-8 个或 3-8 个，对比文件 4（CN103313390A，公开日 2013 年 09 月 18 日）丙氨酰美登木素生物碱抗体-药物偶联物，其包含半胱氨酸改造抗体(Ab)和药物模块(D)，该抗体具有一个或多个游离半胱氨酸，并通过接头模块(L)经由该一个或多个游离半胱氨酸附着至药物模块 D；该偶联化合物具有式：Ab-(L-D)p，其中 p 为 1，2，3 或 4。可经由马来酰亚胺的硫醇反应性接头模块偶联至抗体分子的药物模块数目受到导入抗体的半胱氨酸残基数目的限制，所述抗体包含具有 1，2，3 或 4 个工程化改造的半胱氨酸（参见说明书第 176-182 段）。可见对比文件 3 公开了还可以通过半胱氨酸改造抗体选择 1 个抗体上连接的药物-接头的数目为 1-4 个，即公开了权利要求 12-13 的部分连接数，并且在此基础上本领域技术人员也可以想到选择权利要求 12-13 中限定范围的其它连接数，因此，当其引用的权利要求不具备创造性时，权利要求 12-13 也不具备专利法第 22 条第 3 款规定的创造性。

权利要求 14-15 请求保护抗肿瘤药及/或抗癌药、权利要求 16-17 请求保护医药组合物。将具有抗癌作用的抗体－药物偶联物、其盐、或它们的水合物制备成抗肿瘤药及/或抗癌药，或制备成医药组合物属于本领域常规技术手段，进一步限定其应用不会对所述抗肿瘤药及/或抗癌药、医药组合物的结构或组成造成实质性影

纸件申请，回函请寄：100088 北京市海淀区蓟门桥西土城路 6 号 国家知识产权局专利局受理处收
电子申请，应当通过电子专利申请系统以电子文件形式提交相关文件。除另有规定外，以纸件等其他形式提交的文件视为未提交。

响或改变，因此，当其引用不具备创造性的权利要求 1、12-13 时，权利要求 14-17 也不具备专利法第 22 条第 3 款规定的创造性。

权利要求 18 请求保护权利要求 1 所述抗体的制备方法，对比文件 3 还公开了所述偶联物的制备方法，其中接头首先与蒽环霉素药物偶联，从而产生蒽环霉素药物-接头偶联物，并且其中蒽环霉素药物-接头偶联物接着偶联硫醇还原的单克隆抗体或抗体片段（参见权利要求 28），并公开了将抗体的链间二硫键还原为游离硫醇，产生二硫化物-还原的 mAbs，然后与药物-接头相偶联（参见实施例 5），在此基础上，本领域技术人员容易想到在还原条件下处理抗 TROP2 抗体，之后将其与接头和药物的偶联物反应，因此，在其引用的权利要求 1 不具备创造性的情况下，权利要求 18 也不具备专利法第 22 条第 3 款规定的创造性。

权利要求 19-21 引用权利要求 18，所限定的技术方案是权利要求 48 的中出现的并列技术方案之一，因此，基于相同的理由，权利要求 19-21 也不具备专利法第 22 条第 3 款规定的创造性。

2、权利要求 6、8 得不到说明书支持，不符合专利法第 26 条第 4 款的规定。

权利要求 6 和 8 中“包含序列号 12 中第 20～140 位的氨基酸序列的重链可变区”，其中“包含”的使用，意味着所述重链可变区的氨基酸序列是不确定的，可以包含序列号 12 中第 20～140 位的氨基酸序列的 5’和 3’侧翼之外的其它氨基酸序列，而本申请说明书中相应的重链可变区“由序列号 12 中第 20～140 位的氨基酸序列组成”，在上述氨基酸序列的两侧添加其它氨基酸序列后形成的重链可变区，是否会改变原重链可变区的三维空间结构，从而改变其组成的抗体与抗原的亲和性影响发挥其原有功能的发挥，甚至是否会产生新的免疫原性都是难以预期的，因此上述“包含序列号 12 中第 20～140 位的氨基酸序列的重链可变区”的限定导致权利要求 6 和 8 得不到说明书支持，不符合专利法第 26 条第 4 款的规定。

基于相同的理由，权利要求 6 和 8 中其它多处“包含序列号……中第…..位的氨基酸序列的轻链可变区”的限定均导致权利要求 6 和 8 得不到说明书支持，不符合专利法第 26 条第 4 款的规定。

综上所述，本申请权利要求 1-4，12-21 不具备创造性，权利要求 6 和 8 得不到说明书支持。因此，本申请按照目前的文本还不能被授予专利权。如果申请人按照本通知书提出的审查意见对申请文件进行修改，克服所存在的缺陷，则本申请可望被授予专利权。对申请文件的修改应当符合专利法第 33 条的规定，不得超出原说明书和权利要求书记载的范围。

附件

第108114649號專利申請案檢索報告

1.申 請 日：	104年1月30日
2.優先權日：	2014年1月31日、2014年8月22日、2014年11月10日
3.本案國際專利分類號(IPC)：	***A61K47/50*** (2017.01) ， ***A61K31/4745*** (2006.01) ***A61K39/395*** (2006.01) ， ***A61P35/00*** (2006.01)
4.檢索國際專利分類號(IPC)範圍：	A61K/47/50,A61K/31/4745,A61K/39/395,A61P/35/00 A61K47/50 (2017.01),A61K31/4745 (2006.01),A61K39/395 (2006.01),A61P35/00 (2006.01)
5.檢索使用資料庫名稱：(關鍵詞)	中華民國專利資料庫、TIPO全球/全域專利資料庫

關聯性代碼	引用文獻資料與相關段落處	相關聯請求項
A	1. CN 102481364 A 2012/05/30 全文	1-10
A	2. CN 101023100 A 2007/08/22 全文	1-10
A	3. Burke, Patrick J., et al. "Design, synthesis, and biological evaluation of antibody drug conjugates comprised of potent camptothecin analogues." Bioconjugate chemistry 20.6 (2009): 1242-1250.	1-10

關聯性代碼說明：

X：單獨引用即足以否定發明新穎性或進步性之特別相關的文獻。

Y：結合一或多篇其他文獻後足以否定發明進步性之特別相關的文獻。

A：一般技術水準之參考文獻。

D：說明書已記載之文獻。

E：申請在前、公開／公告在後之專利文獻。

O：公開使用、販賣或展覽陳列之文件。

P：申請日與優先權日間公開之文獻。

L：其他理由引用之文獻。

完成日： 109年6月24日

簽收時間 109/06/30(2020/06/30) 08:33:02

(Machine translation)

SIR
SUPERINTENDENT OF INDUSTRY AND COMMERCE
E. S. D.

REF:	**File NC2016 / 0000187**
TITLE REQUESTED:	**"CONJUGATE OF ANTIBODY-DRUG ANTI-RECEIVER 2 OF THE FACTOR OF EPIDERMAL GROWTH (HER2) "**
PUBLICATION:	**Gazette 780 of December 30, 2016**
PRIORITY:	**JA 2014-017777 01/31/2014 JA 2014-168944 08/22/2014 JA 2014-227886 11/10/2014 APPLICANT:**
	DAIICHI SANKYO COMPANY, LIMITED
APPOINED:	**CAROLINA MERCEDES DAZA**
MONTALVO	
PROCEDURE:	**Appeal for Reposition against Resolution 12260 Of February 21 of 2018, by which a patent of invention is granted**

JUAN PABLO REYES VILLAMIZAR, of legal age, identified as it appears at the foot of my signature, practicing lawyer, holder of the professional card number 44655 of the Superior Council of the Judiciary, acting as attorney-in-fact of the company LABORATORIOS LEGRAND SA, in the subject of the reference, in a respectful manner and within the terms of the law, I file an appeal for reversal against the Resolution 12260 of February 21, 2018 by which the patent application privilege is granted to application NC2016 / 0000187.

I. PETITION

Please, Mr. Superintendent, replace in full resolution 12260 of February 21, 2018, by which the opposition presented was declared unfounded and the patent privilege requested was granted in the file of the reference, and in its replacement issued another that, with based on the arguments set out in this writing, declare that opposition founded and deny the privilege of mentioned patent, proceeding then to the definitive archiving of the file in question.

In subsidy, it is requested to order the performance of a new patentability examination to the request by the Directorate of New Creations, in which the arguments and evidence that I raise in the present appeal are considered.

II. FOUNDATIONS OF THE RESOURCE AND REASONS OF INCONFORMITY.

Resolution 12260 of February 21, 2018 issued by that Superintendence presents the granting of the patent benefit for the request of the reference, however, as set forth below, the subject matter lacks features that grant it inventive level and industrial application to the Antibody-drug conjugate claimed there:

2.1. The Technical Examiner declared our Opposition unfounded without providing technical arguments on the document US20160287722 (quoted in the opposition):i) The Examiner fails to demonstrate that document US20160287722 did not anticipate the technical characteristics claimed, ii) Resolution 12260 does not establish how document US20160287722 affected the patentability requirements of the subject matter, iii) The Examiner did not identify documents other than those provided by our opposition and, consequently, the document was not identified closer to the immunoconjugates claimed, therefore, the patentability analysis was not performed correctly.

Resolution 12260 states that the foregoing presented in the Opposition does not invalidate the matter claimed by the applicant due to:

> *"The opponent argues that the invention lacks an inventive level compared to the teachings of document US20160287722 because they disclose anti-HER2 antibody conjugated with drugs derived from campothecins.*
> *The applicant did not respond to the arguments of the opponent.*
> *This Office evaluated the document provided by the opponent and with which it bases its arguments for lack of inventive level and found that the document was published on October 6, 2016, after the priority date of the request corresponding to January 31 of 2014. For the above, and taking into account that the opponent did not provide any more technical elements this Dispatch considers the opposition unfounded.*

Precisely as stated in the resolution, it is necessary that the foregoing cited by the opposition did not prosper because:

a. Document US20160287722 was published at a date after the priority date.

Faced with this particular, we express our disagreement with the argument that declares our Opposition unfounded, because the Examiner limited himself to analyzing the date of publication of the foregoing and omitted to perform the proper technical analysis of the document US20160287722. When limited in the technical analysis of the evidence provided by the Opposition, the Examiner failed to consider our arguments and consequently did not provide his technical opinion on the relevance or not in the analysis of the inventive level of the matter claimed.

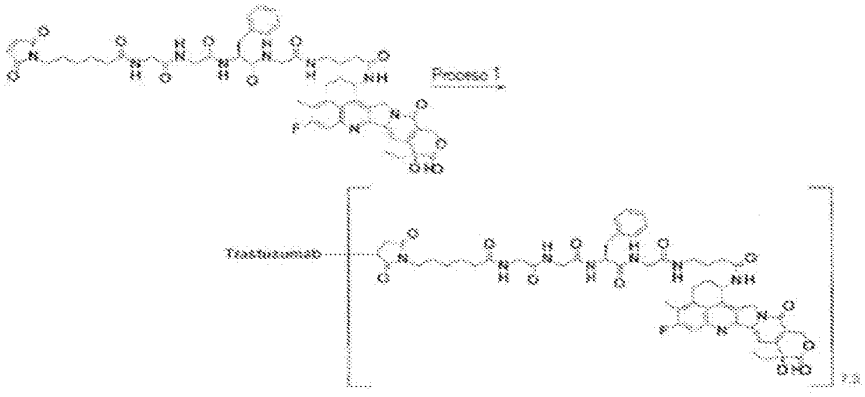
Another fact that I wish to highlight is that the Examiner did not identify other relevant documents or the state of the art document closest to the subject matter, but simply concluded in Oficio 15105 that there were no precedents related to the claim:

> «The state of the art was determined based on the date of presentation of the priority request:January 31, 2014, and did not find documents that anticipate the purpose of this request. »Highlight outside the original text taken from Oficio 15105, Exp. N ° NC2016 / 0000187, numeral 6, p. 3 out of 4

Taking into account the result of the search of the Technical Examiner-mentioned above-we respectfully present our disagreement since the application NC2016 / 0000187 refers to an antibody-drug-ADC conjugate, where the drug has an antitumor effect and is conjugated to an anti-HER2 antibody by a linker structure, (see specification, p.1, ln.5 to 9), specific form the file NC2016 / 0000187 refers to an immunoconjugate containing trastuzumab, as presented below:

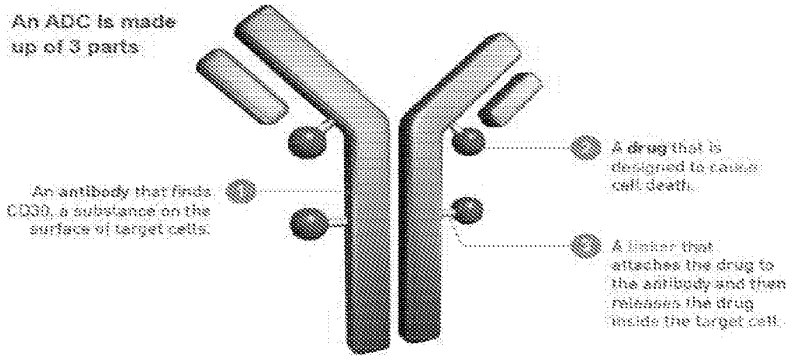
Ejemplo 3 Conjugado anticuerpo-fármaco (3)

Fórmula 29



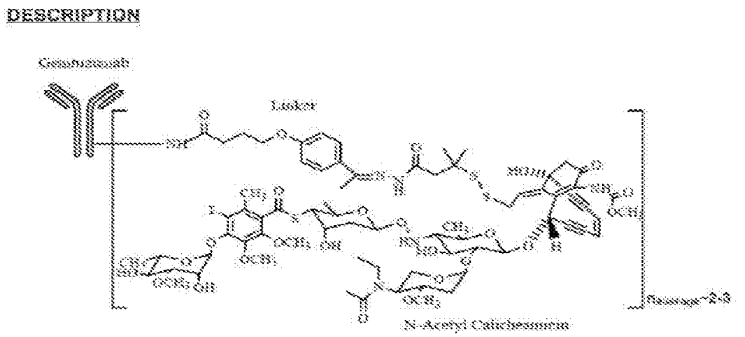
Now, immunoconjugates or ADC's are already known in the technical field

- antibody-drug conjugatesWhich consist of an antibody, a drug and a linker, as follows:



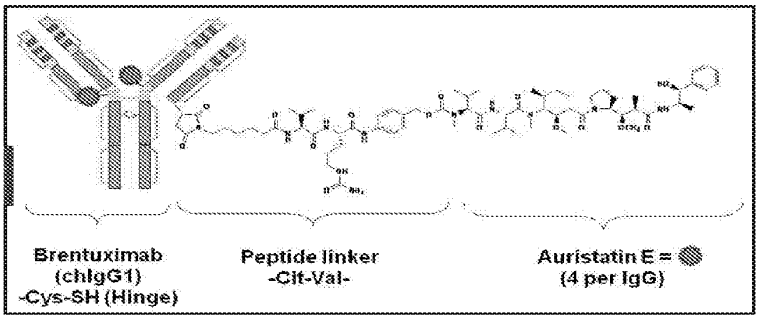
Taken from https://adcetris.com/ in cHL Consolidation/MOA,March 2018

By way of example, the Mylotarg® immunoconjugate is cited, which is composed of an anti-CD33 monoclonal antibody linked via a linker to a cytotoxic agent called N-acetyl gamma calicheamicin. Let's see:



Taken from https://www.rxlist.com/mylotarg-drug.htm,March 2018

Another example is Adcetris®1, an immunoconjugate containing the monoclonal chimeric antibody called brentuximab bound to an antimitotic agent called Vedotin2:



Taken from https://goo.gl/images/t1iSyR,March 2018

Taking into account that the file NC2016 / 0000187 is related to immunoconjugates that are well known in the technical field, we do not agree with the Technical Examiner when he argues that there are no documents anticipating the technical characteristics of the immunoconjugate claimed, moreover

1 Both Adcetris and Mylotarg were immunoconjugates mentioned in the same specification.

2 Taken from https://newdrugapprovals.org/2013/02/03/health-canada-approves-adcetris-brentuximab-vedotinfor-the-treatment-of-relapsed-or-refractory-hodgkin-lymphoma-hl-and-systemic-anaplastic-large-celllymphoma-salcl/

When the same file mentions the examples of Adcetris® and Mylotarg®3 (see page 1 of the descriptive report), therefore, it is evident that the application does have valid precedents to analyze the inventive level thereof, contrary to what has been argued by the Examiner who does not even perform the exercise of choosing the document closest to the subject matter claimed.

At this point it is concluded that the Examiner ignored the technical arguments of the opposition and omitted to study the technical matter revealed by the document US20160287722 -upported by the opposition-, likewise, it did not identify documents other than US20160287722 related to immunoconjugates or identify a nearby document to the immunoconjugate containing trastuzumab claimed, in Consequently, a due analysis of inventive level was not carried out.

2.2. The document US20160287722 is relevant for the analysis of the inventive level of the application in question:i) US20160287722 discloses immunoconjugates containing a cytotoxic drug, a binder and may contain an anti-HER-2 antibody, such as trastuzumab, ii) Document US20160287722 is a divisional of US patent US7585491 published on September 23, 2004, iii) The documents US20160287722 and US7585491, among others, anticipate the technical characteristics claimed by the file NC2016 / 0000187.

First, I would like to mention that the cover of the document cited in the opposition, US20160287722, informs the reader that the data of this application and are related to other US applications and patents published since December 2003. Let's see:

> "This application is a divisional of US patent application Ser. No. 13 / 921,747, filed Jun. 19, 2013, which was a divisional of US patent application Ser. No. 13 / 845,694 (now US Pat. No. 9,133,268), filed Mar. 18, 2013, wich was a divisional of US patent application Ser. No. 13 / 586,281 (now US Pat. No. 8,425,912), filed Aug. 15, 2012, which was a divisional of US patent application Ser. No. 12 / 512,526 (now US Pat. No. 8,268,319), filed Jul. 30, 2009, which was a divisional of US patent application Ser. No. 10 / 734,589, (now US Pat. No. 7,585,491), filed Dec. 15, 2003, which claimed

3 Which should not be admitted since the File is referring to trademarks, which reduces the clarity of the request.

Priority to US Provisional Application No. 60 / 433,017 filed Dec. 13, 2002, the contents of each of which are incorported herein by reference in their intirety. »(cover (62), and paragraph [0001])

Such patents published prior to the priority date of the file
NC2016 / 0000187 -31 January 2014-, are presented below:

- US patent US9,133,268B2, entitled Immunoconjugates with an intracellularly-cleavable linkage, by Govindan S., published on August 22, 2013
- U.S. Patent US8,425,912, entitled Immunoconjugates with an intracellularly-cleavable linkage, by Govindan S., published on December 27, 2012
- U.S. Patent No. 8,268,319, entitled Immunoconjugates with an intracellularly-cleavable linkage, by Govindan S., published on November 26, 2009
- U.S. Patent US7,585,491B2, entitled Immunoconjugates with an intracellularly-cleavable linkage, by Govindan S., published on September 23, 2004

Taking the American patent US7,585,491 as a reference hereinafter, I will proceed to demonstrate that this precedence effectively anticipated the technical characteristics of the file NC2016 / 0000187:

- US Pat. No. 7,585,491 teaches immunoconjugates containing a marker and a therapeutic part:

> *«The invention relates to therapeutic conjugates with improved ability to target various cancer cells containing a targeting moiety and a therapeutic moiety. The targeting and therapeutic moieties are linked via an acid cleavable linkage that increases therapeutic efficacy of the immunoconjugate. »(US7,585,491, Abstract)*

- It also teaches that monoclonal antibodies are used for the delivery of highly toxic drugs:

> «For many years it has been an aim of scientists in the field of specifically targeted drug therapy to use monoclonal antibodies (mAbs) for the specific delivery of toxic agents to human cancers. Conjugates of tumor-associated mAbs and suitable toxic

Agents have been developed, but have had mixed success in the therapy of cancer, and virtually no application in other diseases. The toxic agent is most commonly a chemotherapy drug, although particle-emitting radionuclides, or bacterial or plant toxins have also been conjugated to mAbs.
The advantages of using mAb-chemotherapy drug conjugates are that (a) the chemotherapy drug itself is structurally well defined; (b) the chemotherapy drug is linked to the mAb protein using very well defined conjugation chemistries, often at specific sites from the mAbs antigen binding regions; (c) mAbchemotherapy drug conjugates can be made more reproducibly than chemical conjugates involving mAbs and bacterial or plant toxins, and as such are amenable to commercial development and regulatory approval; and (d) the mAbchemotherapy drug conjugates are orders of magnitude less toxic than radionuclide mAb conjugates. »(US7,585,491, Col. 1, ln 15 to 35)

- It also reveals that the antibodies used in said conjugates can be anti-HER-2:

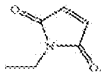
  «In a preferred embodiment of the present invention, antibodies, such mAbs, are they used to recognize or bind to markers or tumor-associated antigens that are expressed at high levels on target cells and that are expressed predominantly or only on diseased cells versus normal tissues, and antibodies that internalize rapidly. (...) Other useful antigens that may be targeted using these conjugates include HER-2 / neu, BrE3, (...) »(US7,585,491, col 8, ln 47 to 54 and ln 65 to 68)

- As for the therapeutic agent, the US patent US7,585,491 mentions that it can be CampotecinAs stated below-:

-

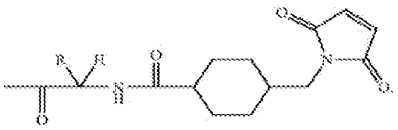
  «The chemotherapeutic agent is preferably selected from the group consisting of doxorubicin (DOX), epirubicin, morpholinodoxorubicin (morpholino-DOX), cyanomorpholino-doxorubicin (cyanomorpholino-DOX), 2-pyrrolino-doxorubicin (2PDOX), camptothecin (CPT), irinotecan (CPT-11), SN-38 (the active drug which is metabolically produced, in vivo, from the prodrug CPT-11), topotecan, taxanes, geldanamycin, ansamycins, and epothilones. Targeting moiety is a monoclonal antibody (mAb). »(US7,585,491, Col. 5, ln. 34 to 41)

- As for the binder, it mentions that it contains the maleimido group



, and that can have more than one amino acid in the internal chain of the linker, like this:

  «The targeting moiety preference links to at least one chemotherapeutic moiety, more prerably it links to about 7 to 12 said chemotherapeutic moieties. A preferred linker has the formula:



The linker optionally comprises a functional group at the N-terminus, at watersolubilizing moiety at the C-terminus, and one or more internal amino acids with side chains available for attachment to said chemotherapeutic moiety. The water-solubilizing moiety is optionally DTPA, EDTA, TTHA, benzyl-DTPA, DOTA, benzyl-DOTA, NOTE, benzyl-NOTE, or N, N'-dialkyl substituted piperazine. The functional group is optionally a thiol-reactive or an amine-reactive group. "

In summary, Govindan S, in his patent US7,585,491 (2004) discloses the structures of immunoconjugates that may optionally contain an anti-HER-2 monoclonal antibody, a cytotoxic agent similar to campothecin and a binding agent containing the maleimido group and more than one amino acid, as claimed in the Colombian application in question.

2.3. Other documents showed technical characteristics that complement the teachings of Govindan S. (2004):i) Wente et al. (2005), and Rowinsky (2005) anticipated that the compound exatecan presented a great antitumor activity, ii) Acchione et al. (2012) anticipated the factors to be considered for the choice of a linker that provides stability and a good binding profile with the therapeutic target, iii) Inoue et al. (2012), teaches the factors that must be taken into account to make the choice of the binding agent for different cases.

At this point I wish to state that the Exatecan compound - which is mentioned in the main claim granted - was already known in the state of the art as a compound with a high antitumor activity.

This is how Wente et al. (2005), in the article titled:«DE-310, a macromolecular prodrug of the topoisomerase-I-inhibitor exatecan (DX8951), in patients with operable solid tumors», presents the Exatecan compound

- DX-8951, as a synthetic analog of Campotecin with a broad antitumor activity, to which a drug delivery system called DE-310 (a conjugate) was designed that increased the accumulation of this cytotoxic at the level of the tumor tissue:

«One of the recently introduced members of camptothecin analogs, exatecan mesylate (DX-8951), is a synthetic water-soluble hexacyclic camptothecin analogue with broad anti-tumor activity ... To use the potentially beneficial effects of DX-8951 over other camptothecins and to enhance the accumulation of the drug in tumor tissues, DE-310 has been developed as a drug-delivery system. Each molecule of DX-8951 is linked to a carboxymethyldextran polyalcohol carrier via glycyl-glycyl-phenylalanyl-glycyl (GGFG) peptidyl spacer (Figure 1). Upon enzymatic cleavage, DX-8951 and glycyl-DX-8951 (G-DX-8951) are released from DE310 [20]. The carboxymethyldextran polyalcohol carrier portion of DE-310 was designed to impart greater tumor specificity to the molecule. The slow release of DX-8951 from the carrier within the tumor provides for a level of localized chemotherapeutic activity. p. 339 and 340

Taken from Wente the at., 2005

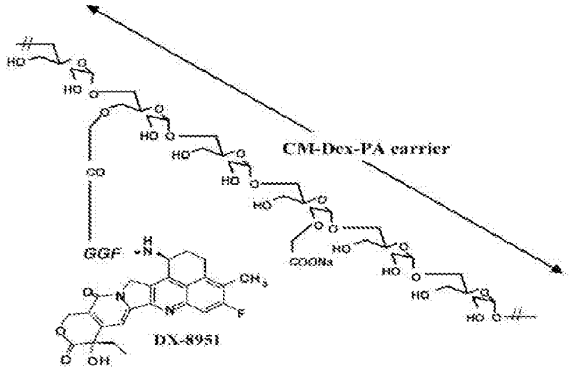


Figure 1. Chemical structure of DE-310.

Also, Rowinsky (2005), in the book «Campotecine in Cancer Therapy», in his chapter entitled:«Preclinical and Clinical Development of Exatecan (DX8951f)», published in 2005, reports that the Exatecan developed by Daiichi pharmaceutical Co., had already gone through clinical phases and that a prodrug -DE-310- has been developed for it:

«The main rationale for synthesizing extecan (DX-8951f, Daiichi Pharmaceutical Co., Ltd, Japan) was to exploit physicochemical features of the camptothecins (CPTs) anticipated to yield an increase in cost effectiveness compared with currently available CPT analogs such as topotecan and irinotecan . The overall therapeutic profile sought in these efforts was greater and broader than antitumor activity, decreased toxicity, and intrinsic activity without requiring metabolic activation, which accentuate the fundamentally large interindividual variability in the pharmacologic behavior of the CPT analogs and consequently in their antitumor and toxicologic profiles . Exatecan has completed Phase I clinical development on a broad range of schedules and is currently undergoing more focused disease-directed Phase II and Phase III evaluations. Additionally, DE-310, a unique polymer prodrug of exatecan, which was developed to achieve protracted systemic exposure to the active drug after a single dose, is also undergoing early clinical development. »p. 317 and 318

On the other hand, Inoue K., Kumazawa E., Kuga H., Susaki H., Masubuchi N., Kajimura T. (2004), in the article entitled:«CM-Dextran-PolyalcoholCamptothecin Conjugate.», Teaches the conjugate DE-310 obtained by optimizing the conjugate CM-Dex-PA-GGFG-DX-89514 and that showed superior antitumor efficacy:

> «Thus for the application of a camptothecin analog DX8951f11, methanesulfonic acid salt of DX-8951, to the conjugate system, we used the CM-Dex-PA carrier with molecular sizes higher than renal filtration threshold to avoid rapid renal excretion and consequent maintain a high plasma level of the drug conjugate. In preclinical studies, DE-310, which was obtained by optimizing the conjugate system CM-Dex-PAGGFG-DX-8951, showed a long retention of high conjugatelevels in blood, a preferential tumor-accumulation and sustained releases of both DX-8951 and glycyl DX-8951 (G-DX-8951) in tumor tissue, and resulted in exhibition *Of a superior antitumor efficacy with a high therapeutic index by single iv dose. In addition, the carrier moiety of DE-310 was shown to be depolymerized and excreted mainly into urine after IV dose of DE-310. Phase I trials of DE-310 have been going in US and Europe, while DX8951f is now in phase III. »P. 145 and 146*

Now, Acchione et al., (2012) in its article "Impact of linker and conjugation chemistry on antigen binding, Fc receptor binding and thermal stability of model antibody-drug conjugates", teaches that to choose the best linker according to each antibody, some factors must be considered. Let's see:

> «... General 'rules' about the advantages or disadvantages of specific linkers or conjugation methods are not well documented. Here we systematically evaluate the impact of linker and coupling chemistry on the properties of two model IgG1 antibodies (Fig. 1A). The results demonstrate some general trends concerning optimal linker strategies (...)
> *Overall strategyTo explore the effects of IgG1 structure, conjugation chemistry and linker on ADC properties, we exploited two model IgG1 scaffolds, with multiple linkers of variable length and hydrophobicity. The linkers were used to attach the model drug biotin to amines, reduced thiols, or oxidized carbohydrates on each IgG. In all cases, the form of biotin used for conjugation was biocytin, which allows for a facile amine coupling of the biotin. Amine and carbohydrate linkers were purchased with this biocytin attached. For clarity, we refer to the group as biotin rather than biocytin in the remainder of this report. The structures of the linkers and biotin are shown in Figure 1B. For ADC identification, the subscripts 'N', 'T' and 'C' refer to coupling to amines, thiols or carbohydrates respectively ...*
> *Of the three functional groups investigated, thiol conjugation resulted in the greatest decreases in observed Tm as monitored by DSC. Furthermore, the degree of change in Tm is correlated with the number of biotin molecules linked to the*

4 CM-Dex-PA means carboxymethyldextran polyalcohol. GGFG is the peptidyl-glycyl-glycyl-phenylalanylglycyl (GGFG) spacer and DX-8951 is the campothecin analogue.

Antibody via thiols. The degree to which the molecule is independent of the length of the linker used or its hydrophobicity when the molecule being attached to the antibody is small and slightly hydrophobic. »P. 362

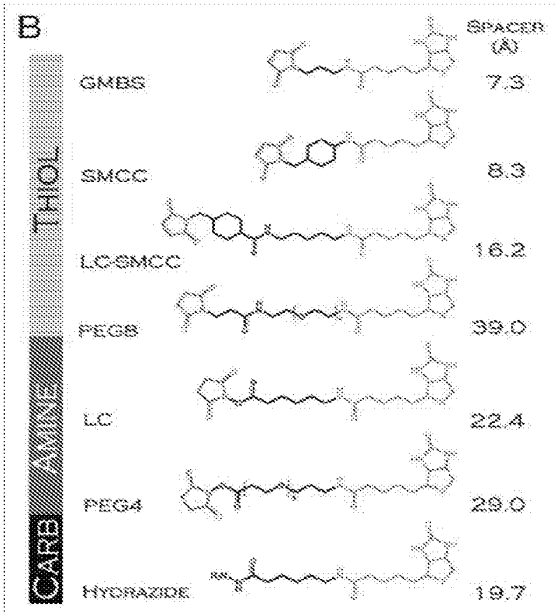


Figure 1. (A) Schematic representation of an IgG1 antibody showing sites of conjugation (thiol, amine and carbohydrate) used in generating the antibody-biotin conjugates. (B) Structure and length of linkers used in conjugating biotin (biocytin) to antibody. N-hydroxysuccinimide (blue), spacer arm (black), maleimide (violet), biotin (biocytin) (green).

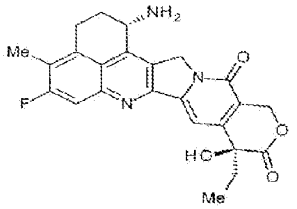
p. 363

2.4. The technical characteristics of the invention had been disclosed before the priority date and therefore a technician versed in the matter -in order to solve the technical problem posed- could have taken the suggestions of the foregoing presented here and reach the conjugates . In this way, the incorrect and superficial analysis of priorities made by the Examiner is demonstrated.

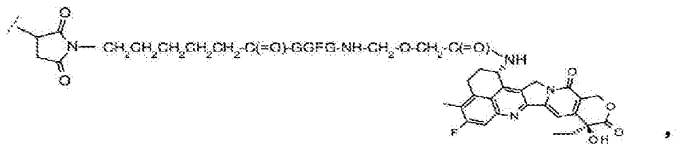
According to Resolution 12260, the main claim granted refers to an immunoconjugate with the following characteristics:

«1. An antibody-drug conjugate characterized by an antitumor compound represented by the following formula:

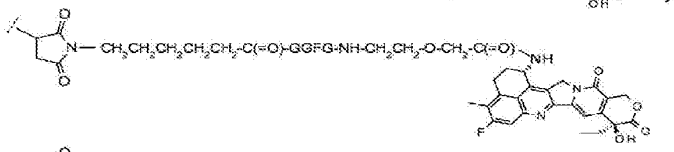
Formula 1



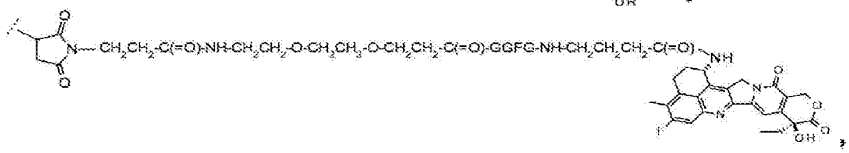
Is conjugated to an anti-HER2 antibody as in the form of a structure
Drug-linker selected from the following group:



,



,



,

Where
Said drug-linker structure is connected to an antiHER2 antibody by a thioether bond which is formed in a disulfide bond portion present in the hinge part of the anti-HER2 antibody, -GGFG represents a peptide residue of -Gly-Gly-Phe- Gly-,
Y
Wherein the anti-HER25 antibody comprises a heavy chain and light chain selected from the group:
a heavy chain consisting of an amino acid sequence consisting of positions 1 to 449 of SEQ ID NO:1 and a light chain consisting of

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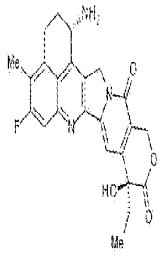
5 It is noted that file NC2016 / 0000187 refers to trastuzumab when it claims "anti-HER2 antibody", this based on the sequences "claimed" and revealed in the specification:"The term" HER2 "as used herein, is used with the same meaning as HER2 protein. Examples of the anti-HER2 antibody as used herein may include, but are not limited to, pertuzumab (International Patent Publication No. WO 01/00245) and trastuzumab (U.S. Patent No. 5821337). Trastuzumab is preferred. However, the anti HER2 antibody of the present invention is limited thereto insofar as it is an anti HER2 antibody that specifically binds to HER2, and more preferably having an internalization activity in cells expressing HER2 by binding to HER2. The term "trastuzumab" as used herein is also called HERCEPTINE (R), huMAb4D5 8, or rhuMAb4D5 8 and is a humanized antibody comprising a heavy chain consisting of an amino acid sequence consisting of amino acid residues. 1 to 449 of SEQ ID NO:1 (Figure 1) and a light chain consisting of an amino acid sequence consisting of amino acid residues 1 to 214 of SEQ ID NO:2 (Figure 2) »P 45 and 46, descriptive memory.

An amino acid sequence consisting of positions 1 to 214 of SEQ ID NO:2 and a heavy chain consisting of the amino acid sequence of SEQ ID NO:1 and a light chain consisting of the amino acid sequence of SEQ ID NO:2. »

As mentioned, US patent US7,585,491 - and its divisional US20160287722 - anticipates immunoconjugates of a monoclonal antibody to treat breast cancer, ie an antiHER-2 antibody, such as trastuzumab and a cytotoxic compound, such as campothecin, and also discloses several linker alternatives for this conjugate. Let's see:

[0003] «For many years it has been an aim of scientists in the field of specifically targeted drug therapy to use monoclonal antibodies (mAbs) for the specific delivery of toxic agents to human cancers. Conjugates of tumor-associated mAbs and suitable toxic agents have been developed, but have had mixed success in the therapy of cancer, and virtually no application in other diseases ... »
US20160287722
[0052] «... Antibodies useful within the scope of the present invention include mAbs with properties as described above (and show distinguishing properties of different levels of internalization into cells and microorganisms) (...) Other useful antigens that may be targeted using these conjugates include HER-2 / neu, »US20160287722

Below is a comparison table between the characteristics granted by Resolution 12260 and the contributions made by US20160287722, US7,585,491, Wente et al., Rowinski, Inoue et al., Acchione et al .:

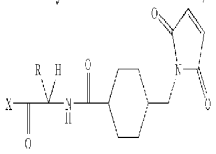
Exp. NC2016 / 0000187	US20160287722	US7585491	Wente	Rowinski	Inoue	Acchione
1. An antibody-drug conjugate characterized in that	YE S	YES			Y ES	Y ES
An antitumor compound represented by the following formula: Formula 1  (Exatecan)	SI6	SI7 (campothecin derivative)	Y ES	Y ES	Y ES	Y ES
a drug-linker structure selected from the following	YES 8	YES 9	YES	YES	YES	YES

6 22. A method of delivering a camptothecin to a target cell, comprising administering to an individual comprising the target cell in an immunoconjugate according to any of claims 1 to 21.

7 «The chemotherapeutic agent is selected from the group consisting of doxorubicin (DOX), epirubicin, morpholinodoxorubicin (morpholino-DOX), cyanomorpholino-Doxorubicin (cyanomorpholino-DOX), 2-pyrrolino-doxorubicin (2-PDOX), camptothecin (CPT), irinotecan (CPT-11), SN-38 (the active drug which is metabolically produced, in vivo, from the prodrug CPT- 11), topotecan, taxanes, geldanamycin, ansamycins, and epothilones. Targeting moiety is a monoclonal antibody (mAb). »
US7585491, Col. 5, ln. 34 to 41

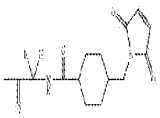
8 (c) a linker comprising a thiol-reactive functional group that binds to a thiol group on the antibody

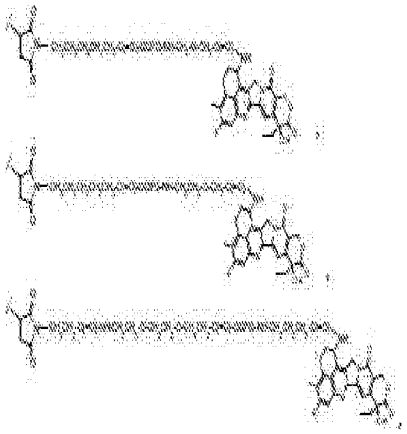
Wherein said chemotherapeutic moiety is attached to said linker via an intracellularly-cleavable moiety that is cleavable by intracellular esterases and comprises an ester formed from the α-carboxylic acid of an amino acid, and said linker contains an α-amino acid and is of the formula:



Wherein R is an amino acid side chain, said amino acid selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, proline, serine, threonine, cysteine, methionine, asparagine, glutamine, phenylalanine, tyrosine, tryptophan, lysine, arginine, histidine, aspartic acid and glutamic acid; and wherein X is a chemotherapeutic moiety.

9 A preferred linker has the formula:



Exp. NC2016 / 0000187	US20160287722	US7585491	Wente	Rowinski	Inoue	Acchione
Group  Wherein said drug-linker structure is connected to an antiHER2 antibody by a thioether bond which is formed in a disulfide bond portion present in the hinge part of the anti-HER2 antibody, - GGFG represents a peptide residue of -Gly-Gly- Phe-Gly-, and wherein the anti-HER2 antibody comprises a heavy chain and light chain Selected from the group:	Reveal The Group maleimido and a group carbon cyclic	Reveals The Group maleimido	Reveal T he conjugate DE-310	Reveal T he conjugate DE-310	Reveal Th e maleimido group and the group Of amino acids GGFG	Reveal T he maleimido group,
a heavy chain consisting of an amino acid sequence consisting of positions 1 to 449 of SEQ ID NO:1 and a light chain consisting of an amino acid sequence consisting of positions 1 to 214 of SEQ ID DO NOT:Two	Anti-HER-2	Anti-HER-2				
And a heavy chain consisting of the amino acid sequence of SEQ ID NO:1 and a light chain consisting of the amino acid sequence of SEQ ID NO:Two	Anti-HER-2	Anti-HER-2				

As it is observed, the Exatecan compound was already known as well as the anti-HER2 antibody trastuzumab, and in the case of the linker, Inoue et al., Reveals that the maleimido group can be accompanied by an amino acid residue of GGFG; consequently, the documents of the state of the art anticipated the matter granted in Resolution 12260, (the cytotoxic compound, the antibody trastuzumab, conjugates).

In view of the above, I ask you to analyze the arguments presented with the usual rigor and detachment, and that consequently the Office decrees, in addition to the denial of the request in question, the file of the file that contains it.

III. ANNEXES

I request that it be considered as such, without prejudice to those that the Office considers informally, the following:

- US Patent No. 7,585,491B2, entitled Immunoconjugates with an intracellularly-cleavable linkage, by Govindan S., published on September 23, 2004. Whole document.

- Acchione, M., Kwon, H., Jochheim, CM, & Atkins, WM (2012). Impact of linker and conjugation chemistry on antigen binding, Fc receptor binding and thermal stability of model antibody-drug conjugates. mAbs, 4 (3), 362-37. http://doi.org/10.4161/mabs19449

- Kazuhiro Inoue, Eiji Kumazawa, Hiroshi Kuga, Hiroshi Susaki, Noriko Masubuchi, Tetsuyo Kajimura. CM-Dextran-Polyalcohol-Camptothecin Conjugate DE-310 with A Novel Carrier System and Its Preclinical Data. Pg 145-146

- Rowinsky E., "Campothecins in cancer therapy", Chapter 14, Preclinical and Clinical Development of Exatecan (DX-8951f) A Hexacyclic Camptothecin Analog. Humana Press, New Jersey, 2005, pp. 317-318.

- Moritz N. Wente, Jörg Kleeff, Markus W. Buchler, Jantien Wanders, Peter Cheverton, Stephen Langman and Helmut Friess. DE-310, a macromolecular prodrug of the topoisomerase-I inhibitor exatecan (XX-8951), in patients with operable solid tumors. Investigational New Drugs 23: 339-347,2005. Pg 339-340.

Mr. Superintendent,

JUAN PABLO REYES VILLAMIZAR
CC 80.419.709 of Usaquén
TPA 92.189 of CSJ

SEÑOR
SUPERINTENDENTE DE INDUSTRIA Y COMERCIO
E. S. D.

REF:	**Expediente NC2016/0000187**
TÍTULO SOLICITADO:	**"CONJUGADO DE ANTICUERPO-FÁRMACO ANTI-RECEPTOR 2 DEL FACTOR DE CRECIMIENTO EPIDERMAL (HER2)"**
PUBLICACIÓN:	**Gaceta 780 de 30 de diciembre de 2016**
PRIORIDAD:	**JA 2014-017777 31/01/2014** **JA 2014-168944 22/08/2014** **JA 2014-227886 10/11/2014**
SOLICITANTE:	**DAIICHI SANKYO COMPANY,LIMITED**
APODERADO:	**CAROLINA MERCEDES DAZA MONTALVO**
TRÁMITE:	**Recurso de Reposición contra la Resolución 12260 de febrero 21 de 2018, por la cual se concede una patente de invención**

JUAN PABLO REYES VILLAMIZAR, mayor de edad, identificado como aparece al pie de mi firma, abogado en ejercicio, titular de la tarjeta profesional número 44655 del Consejo Superior de la Judicatura, obrando en calidad de apoderado de la sociedad **LABORATORIOS LEGRAND S.A.,** en el asunto de la referencia, de manera respetuosa y dentro de los términos de ley, presento **recurso de reposición** contra la Resolución **12260** de 21 de febrero de 2018 por la cual se concede a la solicitud **NC2016/0000187** el privilegio de patente de invención.

I. PETICIÓN

Sírvase, Señor Superintendente, reponer íntegramente la resolución **12260** de 21 de febrero de 2018, por la cual se declaró infundada la oposición presentada y se otorgó el privilegio de patente solicitado en el expediente de la referencia, y en su reemplazo emita otra que, con fundamento en los argumentos expuestos en el presente escrito, declare fundada dicha oposición y niegue el privilegio de patente mencionado, procediendo luego al definitivo archivo del expediente en cuestión.

En subsidio, se solicita ordenar la realización de un nuevo examen de patentabilidad a la solicitud por parte de la Dirección de Nuevas Creaciones, en el cual se consideren los argumentos y pruebas que planteo en el presente recurso.

II. FUNDAMENTOS DEL RECURSO Y RAZONES DE INCONFORMIDAD.

La resolución 12260 del 21 de febrero de 2018 emitida por esa Superintendencia presenta la concesión del beneficio de patente para la solicitud de la referencia, sin embargo, como se expone a continuación la materia reclamada carece de características que le otorguen nivel inventivo y aplicación industrial al Conjugado de anticuerpo-fármaco allí reclamado:

2.1. El Examinador técnico declaró infundada nuestra Oposición sin brindar argumentos técnicos sobre el documento US20160287722 –citado en la oposición–: i) El Examinador falla en demostrar que el documento US20160287722 no anticipaba las características técnicas reclamadas, ii) La Resolución 12260 no establece como el documento US20160287722 afectaba los requisitos de patentabilidad de la materia reclamada, iii) El Examinador no identificó documentos distintos a los aportados por nuestra oposición y, en consecuencia, no se identificó el documento más cercano a los inmunoconjugados reclamados, por lo tanto, el análisis de Patentabilidad no fue realizado correctamente.

En la Resolución 12260 se expone que las anterioridades presentadas en la Oposición no desvirtúan la materia reclamada por el solicitante debido a:

> *«Argumenta el opositor que la invención carece de nivel inventivo frente a las enseñanzas del documento US20160287722 porque divulgan anticuerpo anti HER2 conjugados con fármacos derivados de campotecinas.*
> *El solicitante no presentó respuesta a los argumentos del opositor.*
> *Este Despacho evaluó el documento aportado por el opositor y con el cual sustenta sus argumentos por falta de nivel inventivo y encontró que el documento fue publicado el 6 de octubre de 2016, después de la fecha de prioridad de la solicitud que corresponde al 31 de enero de 2014. Por lo anterior, y teniendo en cuenta que el opositor no aportó más elementos técnicos este Despacho considera infundada la oposición.»*

Precisando lo expuesto en la resolución, se tiene que las anterioridades citadas por la oposición no prosperaron porque:

a. El documento US20160287722 fue publicado en una fecha posterior a la fecha de prioridad.

Frente a este particular, manifestamos nuestro desacuerdo con la argumentación que declara infundada nuestra Oposición, debido a que el Examinador se limitó a analizar la fecha de publicación de la anterioridad y omitió realizar el debido análisis técnico del documento US20160287722. Al limitarse en el análisis técnico de las pruebas aportadas por la Oposición, el Examinador falló en considerar nuestros argumentos y en consecuencia no brindó su opinión técnica sobre la relevancia o no en el análisis del nivel inventivo de la materia reclamada.

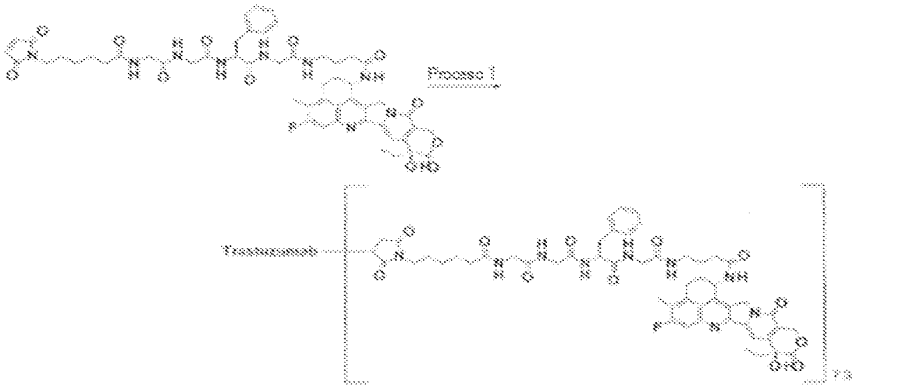
Otro hecho que deseo destacar, es que el Examinador no identificó otros documentos relevantes ni el documento del estado de la técnica más cercano a la materia reclamada, sino que simplemente concluyó en el Oficio 15105 que no existían anterioridades relacionadas con lo reclamado:

> «El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 31 de enero de 2014, y no encontraron documentos que anticipen el objeto de esta solicitud.» Resaltado fuera del texto original tomado del Oficio 15105, Exp. N° NC2016/0000187, numeral 6, p. 3 de 4.

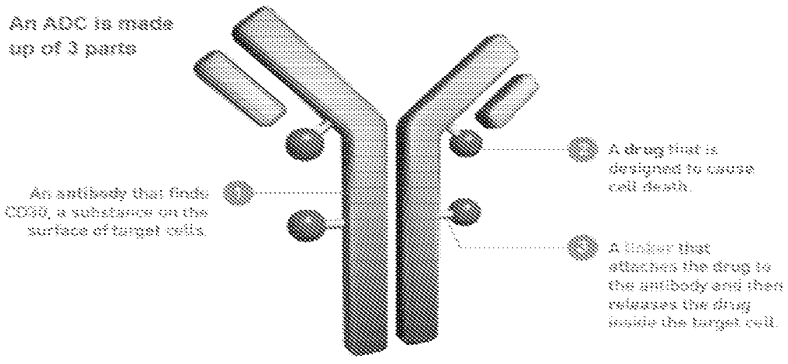
Teniendo en cuenta el resultado de la búsqueda del Examinador técnico –arriba citado–, respetuosamente presentamos nuestro desacuerdo puesto que la solicitud **NC2016/0000187** se refiere **a un conjugado de anticuerpo-fármaco –ADC–, en donde el fármaco tiene efecto antitumoral y está conjugado a un anticuerpo anti-HER2 mediante una estructura enlazadora,** (ver memoria descriptiva, p. 1, ln. 5 a 9), de forma específica el expediente **NC2016/0000187** se refiere a un inmunoconjugado que contiene trastuzumab, como se presenta a continuación:

Ejemplo 3 Conjugado anticuerpo-fármaco (3)

Fórmula 29

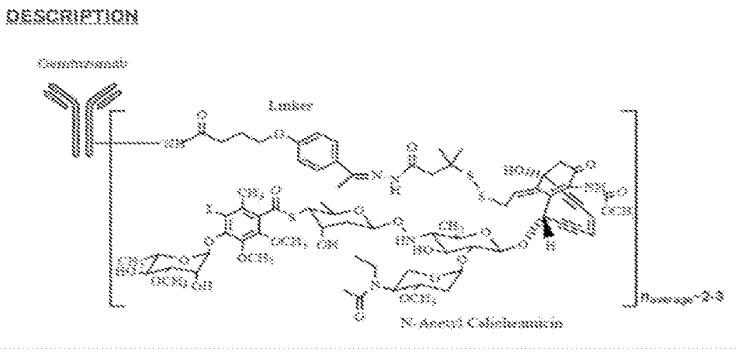


Ahora bien, en el campo técnico ya son conocidos los inmunoconjugados o ADC's –conjugados de anticuerpo-fármaco–que constan de un anticuerpo, un fármaco y un enlazador, del siguiente modo:



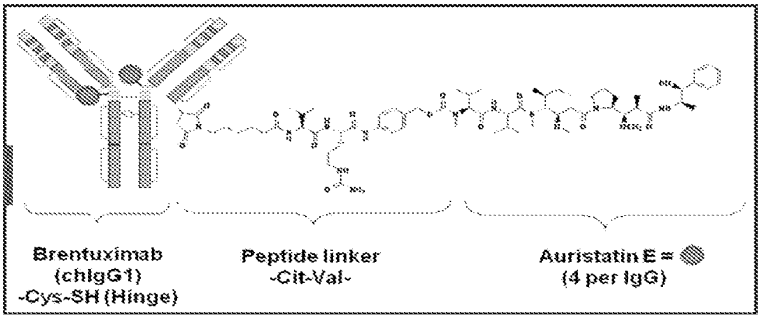
Tomado de https://adcetris.com/_in_cHL_Consolidation/MOA, marzo de 2018

A manera de ejemplo, se cita el inmunoconjugado Mylotarg®, el cual está compuesto por un anticuerpo monoclonal anti-CD33 unido mediante un enlazador a un agente citotóxico llamado N-acetil gamma calicheamicina. Veamos:



Tomado de https://www.rxlist.com/mylotarg-drug.htm, marzo 2018

Otro ejemplo, es Adcetris®[1], un inmunoconjugado que contiene el anticuerpo quimérico monoclonal llamado brentuximab unido a un agente antimitótico denominado Vedotin[2]:



Tomado de https://goo.gl/images/t1iSyR, marzo 2018

Teniendo en cuenta que el expediente **NC2016/0000187** está relacionado con inmunoconjugados que son bien conocidos en el campo técnico, no estamos de acuerdo con el Examinador técnico cuando sostiene que no hay documentos que anticipen las características técnicas del inmunoconjugado reclamado, más aún

[1] Tanto Adcetris como Mylotarg fueron inmunoconjugados mencionados en la misma memoria descriptiva.
[2] Tomado de https://newdrugapprovals.org/2013/02/03/health-canada-approves-adcetris-brentuximab-vedotin-for-the-treatment-of-relapsed-or-refractory-hodgkin-lymphoma-hl-and-systemic-anaplastic-large-cell-lymphoma-salcl/

cuando el mismo expediente menciona los ejemplos de Adcetris® y Mylotarg®[3] (ver página 1 de la memoria descriptiva), por lo tanto, es evidente que la solicitud si posee anterioridades válidas para analizar el nivel inventivo de la misma, contrario a lo argumentado por el Examinador que ni siquiera realiza el ejercicio de elección del documento más cercano a la materia reivindicada.

En este punto se concluye que el Examinador ignoró los argumentos técnicos de la oposición y omitió estudiar la materia técnica revelada por el documento **US20160287722** –aportado por la oposición–, así mismo, no identificó documentos diferentes a **US20160287722** relacionados con inmunoconjugados ni identificó un documento cercano al inmunoconjugado que contiene trastuzumab reclamado, en consecuencia, no se realizó un debido análisis de nivel inventivo.

2.2. El documento US20160287722 es relevante para el análisis de nivel inventivo de la solicitud en comento: i) US20160287722 revela inmunoconjugados que contienen un fármaco citotóxico, un enlazante y puede contener un anticuerpo anti-HER-2, como trastuzumab, ii) El documento US20160287722 es una divisional de la patente americana US7585491 publicada el 23 de septiembre de 2004, iii) Los documentos US20160287722 y US7585491, entre otros, anticipan las características técnicas reclamadas por el expediente NC2016/0000187.

En primer lugar, deseo mencionar que la portada del documento citado en la oposición, US20160287722, informa al lector que los datos de esta solicitud y están relacionados con otras solicitudes y patentes estadounidenses publicadas desde diciembre de 2003. Veamos:

> «*This application is a divisional of U.S. patent application Ser. No. 13/921,747, filed Jun. 19, 2013, which was a divisional of U.S. patent application Ser. No. 13/845,694 (now U.S. Pat. No. 9,133,268), filed Mar. 18, 2013, wich was a divisional of U.S. patent application Ser. No. 13/586,281 (now U.S. Pat. No. 8,425,912), filed Aug. 15, 2012, which was a divisional of U.S. patent application Ser. No. 12/512,526 (now U.S. Pat. No. 8,268,319), filed Jul. 30, 2009, which was a divisional of U.S. patent application Ser. No. 10/734,589, (now U.S. Pat. No. 7,585,491), filed Dec. 15, 2003, which claimed*

[3] Lo cual no debería admitirse dado que el Expediente se está refiriendo a marcas, lo que resta claridad a la solicitud.

priority to U.S. Provisional Application No. 60/433,017 filed Dec. 13, 2002, the contents of each of which are incorported herein by reference in their intirety.» (portada (62), y párrafo [0001])

Tales patentes publicadas de forma previa a la fecha prioridad del expediente **NC2016/0000187** –31 de enero de 2014–, se presentan a continuación:

- La patente estadounidense US9,133,268B2, **titulado Immunoconjugates with an intracellularly-cleavable linkage**, de Govindan S., publicada en agosto 22 de 2013
- La patente estadounidense US8,425,912, **titulado Immunoconjugates with an intracellularly-cleavable linkage**, de Govindan S., publicada en Diciembre 27 de 2012
- La patente estadounidense US8,268,319, titulado **Immunoconjugates with an intracellularly-cleavable linkage**, de Govindan S., publicada en Noviembre 26 de 2009
- La patente estadounidense US7,585,491B2, titulado **Immunoconjugates with an intracellularly-cleavable linkage**, de Govindan S., publicada en Septiembre 23 de 2004

Tomando como referencia en adelante la patente americana **US7,585,491**, procederé a demostrar que ésta anterioridad efectivamente anticipaba las características técnicas del expediente **NC2016/0000187**:

- La patente americana US7,585,491 enseña inmunoconjugados que contienen una parte marcadora y una parte terapéutica:

  «*The invention relates to therapeutic conjugates with improved ability to target various cancer cells containing a targeting moiety and a therapeutic moiety. The targeting and therapeutic moieties are linked via an acid cleavable linkage that increases therapeutic efficacy of the immunoconjugate.*» (US7,585,491, Abstract)

- También enseña que los anticuerpos monoclonales son usados para la entrega de fármacos altamente tóxicos:

  «*For many years it has been an aim of scientists in the field of specifically targeted drug therapy to use monoclonal antibodies (mAbs) for the specific delivery of toxic agents to human cancers. Conjugates of tumor-associated mAbs and suitable toxic*

agents have been developed, but have had mixed success in the therapy of cancer, and virtually no application in other diseases. The toxic agent is most commonly a chemotherapy drug, although particle-emitting radionuclides, or bacterial or plant toxins have also been conjugated to mAbs.
The advantages of using mAb-chemotherapy drug conjugates are that (a) the chemotherapy drug itself is structurally well defined; (b) the chemotherapy drug is linked to the mAb protein using very well defined conjugation chemistries, often at specific sites remote from the mAbs antigen binding regions; (c) mAb-chemotherapy drug conjugates can be made more reproducibly than chemical conjugates involving mAbs and bacterial or plant toxins, and as such are more amenable to commercial development and regulatory approval; and (d) the mAb-chemotherapy drug conjugates are orders of magnitude less toxic than radionuclide mAb conjugates.» (US7,585,491, Col. 1, ln. 15 a 35)

- También revela que los anticuerpos empleados en dichos conjugados pueden ser anti-HER-2:

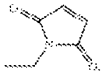
  «*In a preferred embodiment of the present invention, antibodies, such as mAbs, are used that recognize or bind to markers or tumor-associated antigens that are expressed at high levels on target cells and that are expressed predominantly or only on diseased cells versus normal tissues, and antibodies that internalize rapidly. (…)* ***Other useful antigens that may be targeted using these conjugates include HER-2/neu****, BrE3, (…)»* (US7,585,491, col 8, ln 47 a 54 y ln 65 a 68)

- En cuanto al agente terapéutico, la patente americana US7,585,491 menciona que puede ser Campotecina – como consta a continuación-:

-

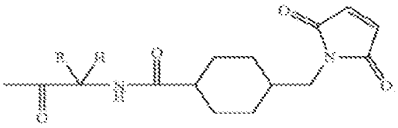
  «The chemotherapeutic moiety is preferably selected from the group consisting of doxorubicin (DOX), epirubicin, morpholinodoxorubicin (morpholino-DOX), cyanomorpholino-doxorubicin (cyanomorpholino-DOX), 2-pyrrolino-doxorubicin (2-PDOX), ***camptothecin (CPT),*** *irinotecan (CPT-11), SN-38 (the active drug which is metabolically produced, in vivo, from the prodrug CPT-11), topotecan, taxanes, geldanamycin, ansamycins, and epothilones. Targeting moiety is a monoclonal antibody (mAb).»* (US7,585,491, Col. 5, ln. 34 a 41)

- En cuanto al enlazante, menciona que contiene el grupo maleimido



, y que puede tener más de un aminoácido en la cadena interna del enlazante, así:

  «The targeting moiety preferably links to at least one chemotherapeutic moiety, more prerably it links to about 7 to 12 said chemotherapeutic moieties. A preferred linker has the formula:



The linker optionally comprises a functional group at the N-terminus, a water-solubilizing moiety at the C-terminus, and one or more internal basic amino acids with side chains available for attachment to said chemotherapeutic moiety. The water-solubilizing moiety is optionally DTPA, EDTA, TTHA, benzyl-DTPA, DOTA, benzyl-DOTA, NOTA, benzyl-NOTA, or N,N'-dialkyl substituted piperazine. The functional group is optionally a thiol-reactive or an amine-reactive group.»

En resumen, Govindan S, en su patente US7,585,491 (2004) revela las estructuras de los inmunoconjugados que opcionalmente pueden contener un anticuerpo monoclonal anti-HER-2, un agente citotóxico similar a la campotecina y un agente enlazante que contiene al grupo maleimido y mas de un aminoácido, tal como se pretende en la solicitud colombiana en mención.

2.3. Otros documentos enseñaban características técnicas que complementan las enseñanzas de Govindan S. (2004): i) Wente et al. (2005), y Rowinsky (2005) anticipaban que el compuesto exatecan presentaba una gran actividad antitumoral, ii) Acchione et al. (2012) anticipaban los factores a considerar para la elección de un enlazador que otorga estabilidad y buen perfil de unión con la diana terapéutica, iii) Inoue et al. (2012), enseña los factores que deben tenerse en cuenta para realizar la elección del agente enlazante para diferentes casos.

En este punto deseo manifestar que el compuesto Exatecan —el cual es mencionado en la reivindicación principal concedida—, ya era conocido en el estado de la técnica como un compuesto con una gran actividad antitumoral.

Es así como Wente et al. (2005), en el articulo titulado: «***DE-310, a macromolecular prodrug of the topoisomerase-I-inhibitor exatecan (DX-8951), in patients with operable solid tumors***», presenta el compuesto Exatecan -DX-8951, como un análogo sintético de la Campotecina con una actividad anti-tumoral amplia, al cual se le diseñó un sistema de entrega de fármacos denominado DE-310 –un conjugado– que aumentaba la acumulación de este citotóxico a nivel del tejido tumoral:

«*One of the recently introduced members of camptothecin analogs, exatecan mesylate (DX-8951), is a synthetic water-soluble hexacyclic camptothecin analogue with broad anti-tumor activity ... To utilize the potentially beneficial effects of DX-8951 over other camptothecins and to enhance accumulation of the drug in tumor tissues, DE-310 has been developed as a drug-delivery system. Each molecule of DX-8951 is linked to a carboxymethyldextran polyalcohol carrier via a glycyl-glycyl-phenylalanyl-glycyl (GGFG) peptidyl spacer (Figure 1). Upon enzymatic cleavage, DX-8951 and glycyl-DX-8951 (G-DX-8951) are released from DE- 310 [20]. The carboxymethyldextran polyalcohol carrier portion of DE-310 was designed to impart greater tumor specificity to the molecule. The slow release of DX-8951 from the carrier within the tumor provides for a sustained level of localized chemotherapeutic activity.*» p. 339 y 340

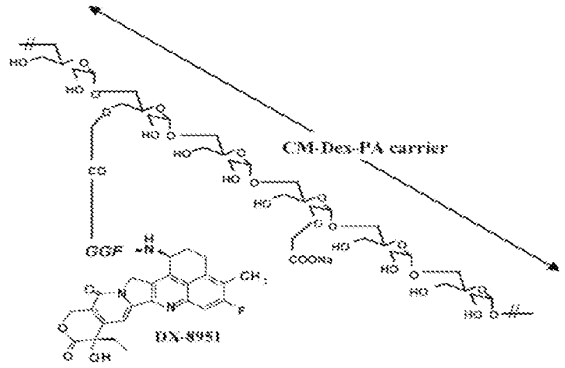


Figure 1. Chemical structure of DE-310.

Tomado de Wente el at., 2005

Así mismo, Rowinsky (2005), en el libro «*Campotecine in Cancer Therapy*», en su capítulo titulado: **«*Preclinical and Clinical Development of Exatecan (DX-8951f)*»,** publicado en 2005, divulga que el Exatecan desarrollado por Daiichi pharmaceutical Co., ya había pasado por fases clínicas y que se ha desarrollado un profármaco –DE-310– para el mismo:

«*The principal rationale for synthesizing exatecan (DX-8951f, Daiichi Pharmaceutical Co., Ltd, Japan) was to exploit physicochemical features of the camptothecins (CPTs) anticipated to yield an increased therapeutic advantage compared with currently available CPT analogs such as topotecan and irinotecan. The overall therapeutic profile sought in these efforts was greater and broader antitumor activity, decreased toxicity, and intrinsic activity without requiring metabolic activation, which may accentuate the fundamentally large interindividual variability in the pharmacologic behavior of the CPT analogs and consequently in their antitumor and toxicologic profiles. Exatecan has completed Phase I clinical development on a broad range of schedules and is currently undergoing more focused disease-directed Phase II and Phase III evaluations. Additionally, DE-310, a unique polymer prodrug of exatecan, which was developed to achieve protracted systemic exposure to the active drug after a single dose, is also undergoing early clinical development.*» p. 317 y 318

Por otro lado, Inoue K., Kumazawa E., Kuga H., Susaki H., Masubuchi N., Kajimura T. (2004), en el artículo titulado: **«*CM-Dextran-Polyalcohol-Camptothecin Conjugate.*»,** enseña el conjugado DE-310 obtenido mediante la optimización del conjugado CM-Dex-PA-GGFG-DX-8951[4] y que mostró una eficacia superior antitumoral:

> «*Thus for the application of a camptothecin analog DX- 8951f11, methanesulfonic acid salt of DX-8951, to the conjugate system, we utilized the CM-Dex-PA carrier with molecular sizes higher than renal filtration threshold to avoid a rapid renal excretion and thereby to maintain a high plasma level of the drug conjugate. In preclinical studies, DE-310, which was obtained by optimising the conjugate system CM-Dex-PA- GGFG-DX-8951, showed a long retention of high conjugate-levels in blood, a preferential tumor-accumulation and sustained releases of both DX-8951 and glycyl DX-8951 (G-DX-8951) in tumor tissue, and resulted in exhibition*
> *of a superior antitumor efficacy with a high therapeutic index by single iv dose. In addition, the carrier moiety of DE-310 was shown to be gradually depolymerized and excreted mainly into urine after iv dose of DE-310. Phase I trials of DE-310 have been going in US and Europe, while DX- 8951f is now in phase III.*» p. 145 y 146

Ahora bien, Acchione et al., (2012) en su artículo **«*Impact of linker and conjugation chemistry on antigen binding, Fc receptor binding and thermal stability of model antibody-drug conjugates*»,** enseña que para elegir el mejor enlazador de acuerdo a cada anticuerpo, se deben considerar algunos factores. Veamos:

> «*… General 'rules' about the advantages or disadvantages of specific linkers or conjugation methods are not well documented. Here we systematically evaluate the impact of linker and coupling chemistry on the properties of two model IgG1 antibodies (Fig. 1A). The results demonstrate some general trends concerning optimal linker strategies (…)*
> *Overall strategy. To explore the effects of IgG1 structure, conjugation chemistry and linker on ADC properties, we exploited two model IgG1 scaffolds, with multiple linkers of variable length and hydrophobicity. The linkers were used to attach the model 'drug' biotin to amines, reduced thiols, or oxidized carbohydrates on each IgG. In all cases, the form of biotin used for conjugation was biocytin, which allows for facile amine coupling of the biotin. Amine and carbohydrate linkers were purchased with this biocytin attached. For clarity, we refer to the group as biotin rather than biocytin in the remainder of this report. The structures of the linkers and biotin are shown in Figure 1B. For ADC identification, the subscripts 'N', 'T' and 'C' refer to coupling to amines, thiols or carbohydrates respectively…*
> *Of the three functional groups investigated, thiol conjugation resulted in the greatest decreases in observed Tm as monitored by DSC. Furthermore, the degree of change in Tm is correlated with the number of biotin molecules linked to the*

[4] CM-Dex-PA significa carboximetildextran polialcohol, GGFG es el espaciador peptidilo glicil-glicil-fenilalanil-glicil (GGFG) *y* DX-8951 es el análogo de campotecina.

antibody via thiols. The degree to which the Tm was independent of the length of the linker used or its hydrophobicity when the molecule being attached to the antibody is small and slightly hydrophobic. » p. 362

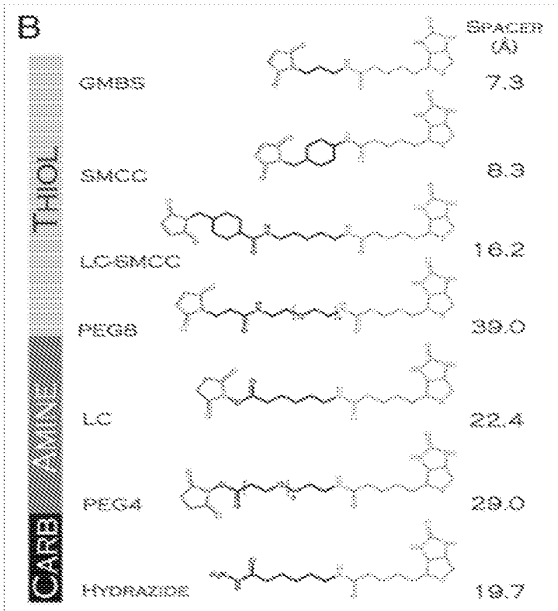


Figure 1. (A) Schematic representation of an IgG1 antibody showing sites of conjugation (thiol, amine and carbohydrate) used in generating the antibody-biotin conjugates. (B) Structure and length of linkers used in conjugating biotin (biocytin) to antibody. N-hydroxysuccinimide (blue), spacer arm (black), maleimide (violet), biotin (biocytin) (green).

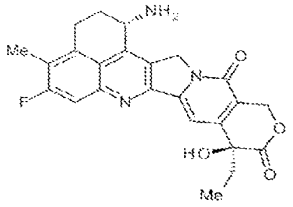
p. 363

2.4. La características técnicas de la invención habían sido divulgadas antes de la fecha de prioridad y por ende un técnico versado en la materia –con el ánimo de resolver el problema técnico planteado– habría podido tomar las sugerencias de las anterioridades aquí presentadas y llegar a los conjugados. De esta forma queda demostrado el incorrecto y superficial análisis de anterioridades realizado por el Examinador.

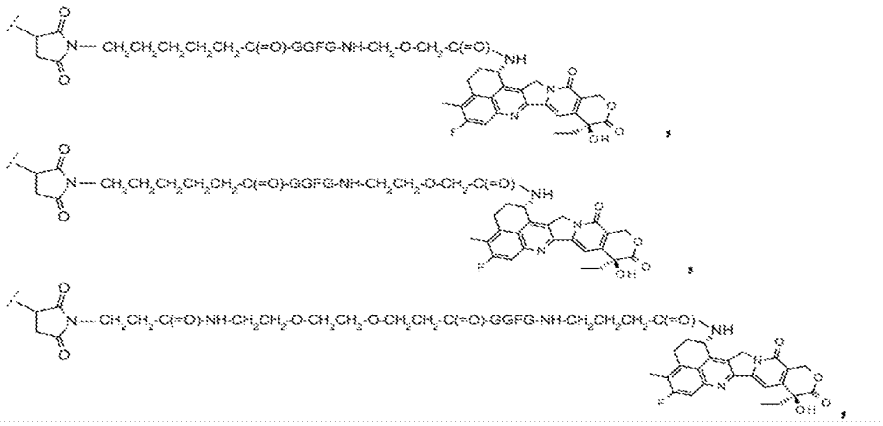
Según la Resolución 12260, la reivindicación principal concedida se refiere a un inmunoconjugado con las siguientes características:

«1. Un conjugado de anticuerpo-fármaco caracterizado porque un compuesto antitumoral representado por la siguiente fórmula:

Fórmula 1



está conjugado a un anticuerpo anti-HER2 como en forma de una estructura fármaco-enlazador seleccionada del siguiente grupo:



en donde

dicha estructura fármaco-enlazador está conectada a un anticuerpo anti- HER2 mediante un enlace tioéter el cual se forma en una porción de enlace disulfuro presente en la parte de articulación del anticuerpo anti-HER2, - GGFG- representa un residuo péptido de -Gly-Gly-Phe-Gly-,

y

en donde el anticuerpo anti-HER2[5] comprende una cadena pesada y cadena ligera seleccionada del grupo:

una cadena pesada que consiste en una secuencia de aminoácidos que consiste en las posiciones 1 a 449 de SEQ ID NO: 1 y una cadena ligera que consiste en

---

[5] Se pone de presente que el expediente **NC2016/0000187** se refiere a trastuzumab cuando reclama "anticuerpo anti-HER2", esto con base en las secuencias "reclamadas" y a lo revelado en la memoria descriptiva: «El término "HER2" como se usa en la presente, se usa con el mismo significado como proteína HER2. Ejemplos del anticuerpo anti-HER2 como se usa en la presente pueden incluir, pero no se limitan particularmente a, pertuzumab (Publicación de Patente Internacional No. WO 01/00245) y trastuzumab (Patente Estadounidense No. 5821337). Se prefiere trastuzumab. Sin embargo, el anticuerpo anti-HER2 de la presente invención está limitado a este en la medida en que es un anticuerpo anti-HER2 que se une específicamente a HER2, y más preferiblemente que tiene una actividad de internalización en células que expresan el HER2 por unión a HER2. El término "trastuzumab" como se usa en la presente es también llamado HERCEPTINA(R), huMAb4D5-8, o rhuMAb4D5-8 y es un anticuerpo humanizado que comprende una cadena pesada que consiste de una secuencia de aminoácido que consiste de residuos de aminoácidos 1 a 449 de la SEC ID NO: 1 (Figura 1) y una cadena ligera que consiste de una secuencia de aminoácido que consiste de residuos de aminoácidos 1 a 214 de la SEC ID NO: 2 (Figura 2)» Pág 45 y 46, memoria descriptiva.

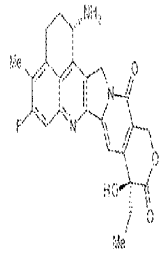
una secuencia de aminoácidos que consiste en las posiciones 1 a 214 de SEQ ID NO: 2 y
una cadena pesada que consiste en la secuencia de aminoácidos de SEQ ID NO: 1 y una cadena ligera que consiste en la secuencia de aminoácidos de SEQ ID NO: 2.»

Como se había mencionado, la patente americana US7,585,491 –y su divisional US20160287722– anticipa inmunoconjugados de un anticuerpo monoclonal para tratar el cáncer de mama, es decir un anticuerpo anti- HER-2, como trastuzumab y un compuesto citotóxico, como campotecina, y también divulga varias alternativas de enlazador para este conjugado. Veamos:

[0003] «*For many years it has been an aim of scientists in the field of* ***specifically targeted drug therapy to use monoclonal antibodies (mAbs) for the specific delivery of toxic agents to human cancers.*** *Conjugates of tumor-associated mAbs and suitable toxic agents have been developed, but have had mixed success in the therapy of cancer, and virtually no application in other diseases...*» US20160287722

[0052] «*... Antibodies useful within the scope of the present invention include mAbs with properties as described above (and show distinguishing properties of different levels of internalization into cells and microorganisms) (...) Other useful antigens that may be targeted using these conjugates include* ***HER-2****/neu,*» US20160287722

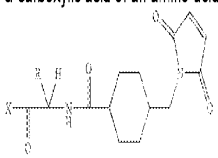
A continuación se presenta una tabla de comparación entre las características concedidas por la Resolución 12260 y los aportes hechos por US20160287722, US7,585,491, Wente et al., Rowinski, Inoue et al., Acchione et al.:

Exp. NC2016/0000187	US20160287722	US7585491	Wente	Rowinski	Inoue	Acchione
1. Un conjugado de anticuerpo-fármaco caracterizado porque	SI	SI			SI	SI
un compuesto antitumoral representado por la siguiente fórmula: Fórmula 1  (Exatecan)	SI[6]	SI[7] (derivado campotecina)	SI	SI	SI	SI
una estructura fármaco-enlazador seleccionada del siguiente	SI [8]	SI [9]	SI	SI	SI	SI

[6] **22**. A method of delivering a camptothecin to a target cell, comprising administering to an individual comprising the target cell an immunoconjugate according to any of claims 1 to 21.

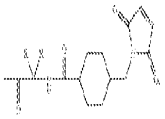
[7] «The chemotherapeutic moiety is preferably selected from the group consisting of doxorubicin (DOX), epirubicin, morpholinodoxorubicin (morpholino-DOX), cyanomorpholino-doxorubicin (cyanomorpholino-DOX), 2-pyrrolino-doxorubicin (2-PDOX), camptothecin (CPT), irinotecan (CPT-11), SN-38 (the active drug which is metabolically produced, in vivo, from the prodrug CPT-11), topotecan, taxanes, geldanamycin, ansamycins, and epothilones. Targeting moiety is a monoclonal antibody (mAb).»
US7585491, Col. 5, ln. 34 a 41

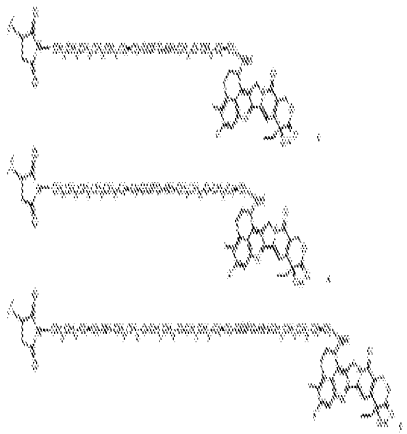
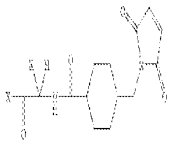
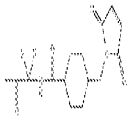
[8] (c) a linker comprising a thiol-reactive functional group that binds to a thiol group on the antibody
wherein said chemotherapeutic moiety is attached to said linker via an intracellularly-cleavable moiety that is cleavable by intracellular esterases and comprises an ester formed from the α-carboxylic acid of an amino acid, and said linker contains an α-amino acid and is of the formula:



wherein R is an amino acid side chain, said amino acid selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, proline, serine, threonine, cysteine, methionine, asparagine, glutamine, phenylalanine, tyrosine, tryptophan, lysine, arginine, histidine, aspartic acid and glutamic acid; and wherein X is a chemotherapeutic moiety.

[9] A preferred linker has the formula:



Exp. NC2016/0000187	US20160287722	US7585491	Wente	Rowinski	Inoue	Acchione
grupo  en donde dicha estructura fármaco-enlazador está conectada a un anticuerpo anti- HER2 mediante un enlace tioéter el cual se forma en una porción de enlace disulfuro presente en la parte de articulación del anticuerpo anti-HER2, - GGFG- representa un residuo péptido de -Gly-Gly-Phe-Gly-,y en donde el anticuerpo anti-HER2 comprende una cadena pesada y cadena ligera seleccionada del grupo:	Revela el grupo maleimido y un grupo cíclico de carbono 	Revela el grupo maleimido 	Revela el conjugado DE-310	Revela el conjugado DE-310	Revela el grupo maleimido y el grupo de aminoácidos GGFG	Revela el grupo maleimido,
una cadena pesada que consiste en una secuencia de aminoácidos que consiste en las posiciones 1 a 449 de SEQ ID NO: 1 y una cadena ligera que consiste en una secuencia de aminoácidos que consiste en las posiciones 1 a 214 de SEQ ID NO: 2	Anti-HER-2	Anti-HER-2				
y una cadena pesada que consiste en la secuencia de aminoácidos de SEQ ID NO: 1 y una cadena ligera que consiste en la secuencia de aminoácidos de SEQ ID NO: 2	Anti-HER-2	Anti-HER-2				

Como se observa, el compuesto Exatecan ya era conocido así como el anticuerpo anti-HER2 trastuzumab, y en el caso del enlazador, Inoue et al., revela que el grupo maleimido puede estar acompañado de un residuo de aminoácidos de GGFG; en consecuencia, los documentos del estado de la técnica anticipaban la materia concedida en la Resolución 12260, (el compuesto citotóxico, el anticuerpo trastuzumab, conjugados).

En vista de lo anterior, ruego se analicen los argumentos expuestos con el rigor y detenimiento habituales, y que en consecuencia el Despacho decrete, además de la negación de la solicitud en comento, el archivo del expediente que lo contiene.

III. ANEXOS

Ruego que se tenga como tal, sin perjuicio de aquellas que el Despacho considere oficiosamente, las siguientes:

- La patente americana US7,585,491B2, titulada **Immunoconjugates with an intracellularly-cleavable linkage**, de Govindan S., publicada en Septiembre 23 de 2004. Todo el documento.

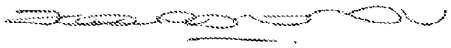
- Acchione, M., Kwon, H., Jochheim, C. M., & Atkins, W. M. (2012***). Impact of linker and conjugation chemistry on antigen binding, Fc receptor binding and thermal stability of model antibody-drug conjugates***. mAbs, 4(3), 362–372. http://doi.org/10.4161/mabs.19449

- Kazuhiro Inoue, Eiji Kumazawa, Hiroshi Kuga, Hiroshi Susaki, Noriko Masubuchi, Tetsuyo Kajimura. ***CM-Dextran-Polyalcohol-Camptothecin Conjugate DE-310 with A Novel Carrier System and Its Preclinical Data***. Pág 145-146

- Rowinsky E., "Campothecins in cancer therapy", Chapter 14, ***Preclinical and Clinical Development of Exatecan (DX-8951f) A Hexacyclic Camptothecin Analog.*** Humana Press, New Jersey, 2005, pág 317-318.

- Moritz N. Wente, Jörg Kleeff, Markus W. Buchler, Jantien Wanders, Peter Cheverton, Stephen Langman and Helmut Friess. ***DE-310, a macromolecular prodrug of the topoisomerase-I inhibitor exatecan (XX-8951), in patients with operable solid tumors.*** Investigational New Drugs 23: 339-347,2005. Pág 339-340.

Señor Superintendente,



JUAN PABLO REYES VILLAMIZAR
C.C. 80.419.709 de Usaquén
T.P.A. 92.189 de C.S.J.

3000 Ci/mM) (UTP) was then added, and the nuclear suspension was incubated at 30°C for 30 minutes, after which time 15 μl of DNase I (5 μg/ml) in 10 mM $CaCl_2$ (5 μg/ml) was added. After 5 minutes at 30°C, the reaction was made 1× SET (1 percent sodium dodecyl sulfate (SDS), 5 mM EDTA, 10 mM tris-HCl, pH 7.4), and proteinase K was added to a concentration of 200 μg/ml. After incubation at 37°C for 45 minutes, the solution was extracted with an equal volume of a mixture of phenol and chloroform, and the interphase was again extracted with 100 μl of 1× SET. Ammonium acetate (10M) was added to the combined aqueous phases (original plus reextraction) to a final concentration of 2.5M, an equal volume of isopropyl alcohol was added, and nucleic acid was precipitated (−70°C for 15 minutes). The precipitate was centrifuged in a microcentrifuge for 10 minutes, and the pellet was resuspended in 100 μl of TE (10 mM tris-HCl, 1 mM EDTA) and centrifuged through a G-50 (medium) spin column. The eluate was made 0.2M in NaOH and after 10 minutes on ice, HEPES was added to a concentration of 0.24M. Two and one-half volumes of ethanol were then added, and the solution containing the precipitate held overnight at −20°C. After centrifugation in a microcentrifuge for 5 minutes, the pellet was resuspended in hybridization buffer, which consisted of 10 mM TES, pH 7.4, 0.2 percent SDS, 10 mM EDTA, 0.3M NaCl, 1× Denhardt's, and *Escherichia coli* RNA (250 μg/ml). Nitrocellulose filters containing plasmid DNA's were prepared with a Schleicher & Schuell Slot Blot Apparatus under conditions suggested by S and S, except that wells were washed with 10× SSC (saline sodium citrate). These filters were first hybridized in the hybridization solution described above for a minimum of 2 hours at 63°C. After this preliminary hybridization, the filters were hybridized to the runoff products in hybridization solution for 36 hours. A typical reaction contained 2 ml of hybridization solution with 1×10^7 cpm/ml. After hybridization, filters were washed for 1 hour in 2× SSC at 65°C. The filters were then incubated at 37°C in 2× SSC with RNase A (10 mg/ml) for 30 minutes and were subsequently washed in 2× SSC at 37°C for 1 hour. Alternatively, after hybridization the filters were washed twice for 15 minutes in 0.1 percent SDS, 2× SSC at room temperature, and then washed at 60°C (0.1 percent SDS, 0.1× SSC) for 30 minutes. Either protocol for processing of the filters after hybridization yielded the same specificity in signal. Filters were then exposed to Kodak XAR film in cassettes containing Lightening-Plus screens at −70°C for various times.

45. C. Yanisch-Perron, J. Vierra, J. Messing, *Gene* **33**, 103 (1985).
46. S. L. McKnight, E. R. Gavis, R. Kingsbury, R. Axel, *Cell* **25**, 385 (1981).
47. M. Groudine and C. Casimir, *Nucleic Acids Res.* **12**, 1427 (1984).
48. We thank many of our colleagues for discussion and suggestions during the course of this work; Hal Weintraub, Paul Neiman, and Craig Thompson for comments on the manuscript; Craig Thompson for assistance in obtaining lymphocyte preparations; Bill Schubach for plasmid pBK25; and Kay Shiozaki for assistance with the manuscript. Supported by NIH grants CA 18282 (M.L.) and CA 28151 (M. L. and M.G.), and NSF grant PCM 82-04696 (M.G.), and a scholarship from the Leukemia Society of America (M.G.)

30 July 1985; accepted 15 October 1985

RESEARCH ARTICLE

Tyrosine Kinase Receptor with Extensive Homology to EGF Receptor Shares Chromosomal Location with *neu* Oncogene

Lisa Coussens, Teresa L. Yang-Feng, Yu-Cheng Liao
Ellson Chen, Alane Gray, John McGrath, Peter H. Seeburg
Towia A. Libermann, Joseph Schlessinger, Uta Francke
Arthur Levinson, Axel Ullrich

Growth factors and their receptors are involved in the regulation of cell proliferation, and several recent findings suggest that they also play a key role in oncogenesis (*1–4*). Of approximately 20 identified oncogenes, the three that have been correlated with known cellular proteins are each related to either a growth factor or a growth factor receptor. The B chain of platelet-derived growth factor (PDGF) is encoded by the proto-oncogene c-*sis* (*2*), the erb-B oncogene product gp68 is a truncated form of the epidermal growth factor (EGF) receptor (*3*), and the proto-oncogene c-*fms* may be related or identical to the receptor for macrophage colony-stimulating factor ($CSF\text{-}1^R$) (*4*).

The receptor-related oncogenes are members of a gene family in that each has tyrosine-specific protein kinase activity, and is associated with the plasma membrane (*5*). Such features are also shared by several other polypeptide hormone receptors, including those for insulin (*6*), PDGF (*7*), and insulin-like growth factor 1 (IGF-1) (*8*); hence more connections may be found between tyrosine kinase growth factor receptors and tyrosine kinase oncogene products.

Comparison of the complete primary structure of the human EGF receptor (*9*) with the sequence of the avian erythroblastosis virus (AEV) transforming gene, v-erbB (*10*), revealed close sequence similarity; in addition, there were amino and carboxyl terminal deletions that may reflect key structural changes in the generation of an oncogene from the gene for a normal growth factor receptor (*3, 9*). Another oncogene, termed *neu*, is also related to v-erbB and was originally identified by its activation in ethylnitrosourea-induced rat neuroblastomas (*11*). In contrast to v-erbB, which encodes a 68,000-dalton truncated EGF receptor, the *neu* oncogene product is a 185,000-dalton cell surface antigen that can be detected by cross-reaction with polyclonal antibodies against EGF receptor (*11*); *neu* may itself be a structurally altered cell surface receptor with homology to the EGF receptor and binding specificity for an unidentified ligand.

Using v-erbB as a screening probe, we isolated genomic and cDNA clones coding for an EGF receptor-related, but distinct, 138,000-dalton polypeptide having all the structural features of a cell surface receptor molecule. On the basis of its structural homology, this putative receptor is a new member of the tyrosine-specific protein kinase family. It is encoded by a 4.8-kb messenger RNA (mRNA) that is widely expressed in normal and malignant tissues. We have localized the gene for this protein to q21 of chromosome 17, which is distinct from the EGF receptor locus, but coincident with the *neu* oncogene mapping position (*12*). We therefore consider the possibility that we have isolated and characterized the normal human counterpart of the rat *neu* oncogene.

Tyrosine kinase–type receptor gene and complementary DNA. As part of our attempts to isolate and characterize the chromosomal gene coding for the human cellular homologue of the viral erbB gp68 polypeptide, AEV-ES4 erbB sequences (2.5-kb Pvu II fragment of pAEV) (*13*) were used as a ^{32}P-labeled hybridization probe for the screening of a human genomic DNA library at reduced stringency

Lisa Coussens, Yu-Cheng Liao, Ellson Chen, Alane Gray, Peter H. Seeburg, Arthur Levinson, and Axel Ullrich are in the Department of Molecular Biology, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080; John McGrath is currently with the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02142; Towia Libermann and Joseph Schlessinger are in the Department of Chemical Immunology at the Weizmann Institute of Science, Rehovot 76100, Israel; and Teresa L. Yang-Feng and Uta Francke are in the Department of Human Genetics at Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510.

(*14*). Clone λc-erbB/1 was isolated; it contained a hybridizing 1.8-kb Bam HI fragment, which was subjected to DNA sequence analysis. The 1838-bp sequence contains three complete and one partial erbB-homologous exons separated by short intervening sequences (Fig. 1). Comparison of this human gene sequence with our complete cDNA-derived human EGF receptor protein sequence (*9*) revealed 32 differences (18.7 percent) within the 171 amino acid stretch of combined exons, suggesting that this gene fragment was not derived from the human EGF receptor gene. Since this gene may code for an unknown tyrosine kinase–type receptor that is closely related to the human EGF receptor, we named it HER2.

Northern blot analysis (*15*) with the ^{32}P-labeled 1.8-kb HER2 fragment as a hybridization probe revealed a 4.8-kb mRNA in human term placenta poly(A)$^+$ RNA, distinct from the 5.8- and 10.5-kb EGF receptor mRNA's also present at high levels in this tissue (Fig. 2a, lane 1). Thus, we had isolated a portion of an EGF receptor–erbB-related but distinct gene. To obtain its complete primary structure, two single-stranded synthetic oligonucleotide probes (*16*) were prepared from HER2 exon sequence regions that differed sufficiently (less than 60 percent nucleotide sequence homology) from EGF receptor DNA sequences (Fig. 1, 1 and 2) and used to screen a term placenta complementary DNA (cDNA) library of 2×10^6 independent recombinant clones in λgt10 (*17*). Fifty-two clones were isolated; they hybridized strongly with both synthetic probes and weakly with an EGF receptor cDNA fragment (HER64-3) (*9*) containing the homologous region within the tyrosine kinase domain. One of these, λHER2-436, had the longest cDNA insert (4.5 kb), consisting of three Eco RI fragments (1.4, 1.5, and 1.6 kb).

The complete cDNA sequence of this clone is shown in Fig. 3. The longest open reading frame starting with a methionine codon codes for a 1255 amino acid polypeptide (137,828 daltons) and contains the 171 residues encoded by the four exons in the 1.8-kp HER2 gene Bam HI fragment (Fig. 1). This 3765-bp coding sequence is flanked by 150 bp of 5′ untranslated sequence and a TGA stop codon, followed by a 627-nucleotide 3′ untranslated sequence. No stop codon is found in the 5′ untranslated region. In support of our assignment, however, the initiation codon at position 151 is flanked by sequences that follow perfectly Kozak's rule (*18*) for translation initiation. The 3′ untranslated sequence contains a potential poly(A) addition signal sequence (AATATA) 12 nucleotides upstream from a stretch of 15 adenylate residues. We are not certain if this $(A)_{15}$ stretch is part of a poly(A) tail or represents an internal poly(A) stretch of a longer 3′ untranslated sequence.

those for EGF and insulin (*9*, *19*). Such features are apparent in the hydropathy profile (*20*) comparison (Fig. 4a). On the basis of this comparison, and on amino acid sequence alignment with the EGF receptor (Fig. 4b, region 1), we predict a 21 amino acid signal sequence (Fig. 4b,

Abstract. *A novel potential cell surface receptor of the tyrosine kinase gene family has been identified and characterized by molecular cloning. Its primary sequence is very similar to that of the human epidermal growth factor receptor and the v-erbB oncogene product; the chromosomal location of the gene for this protein is coincident with the* neu *oncogene, which suggests that the two genes may be identical.*

Comparison of EGF receptor and HER2 sequence. As already indicated by the v-erbB sequence homology used to isolate HER2, the putative HER2 protein is very similar in its overall domain organization and sequence to the EGF receptor. Nevertheless, there are differences that are likely to define a specific biological role for the HER2 polypeptide.

The predicted HER2 polypeptide contains each of the domain features found in hormone receptor precursors, such as

1), an amino terminal serine residue, and a 632 amino acid putative extracellular ligand-binding domain; a highly hydrophobic, 22–amino acid transmembrane anchor domain separates the extracellular domain from a 580-residue-long carboxyl-terminal cytoplasmic domain, which possesses the highest homology to v-erbB and other members of the tyrosine kinase family.

The 632–amino acid, putative HER2 ligand binding domain is about 40 percent homologous with the 621-residue

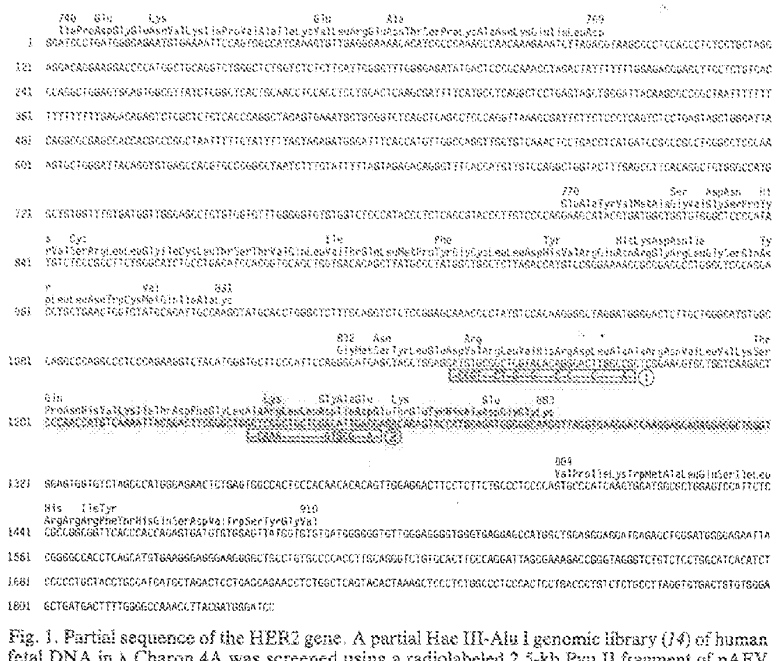


Fig. 1. Partial sequence of the HER2 gene. A partial Hae III-Alu I genomic library (*14*) of human fetal DNA in λ Charon 4A was screened using a radiolabeled 2.5-kb Pvu II fragment of pAEV (*13*) containing coding sequences for the tyrosine kinase domain. Hybridization was as described elsewhere (*31*), except that 30 percent formamide was used at 42°C. Three independent clones were isolated which shared a 1.8-kb hybridizing Bam HI fragment. This fragment and subsets thereof were isolated, subcloned into M13mp10 and M13mp11, and sequenced (*32*). The intron-exon organization was determined by comparison with v-erbB sequences (*10*). Amino acid numbering is based on the complete cDNA sequence shown in Fig. 3. Nucleotide sequence differences with the human EGF receptor sequence are shown in the regions that were used for the design of synthetic oligonucleotide probes 1 (30 nucleotides) and 2 (21 nucleotides). Amino acid sequence differences with the EGF receptor are shown above the HER2 sequence.

extracellular EGF binding domain of the EGF receptor. This homology includes two cysteine-rich subdomains of 26 and 21 regularly organized cysteine residues (Figs. 4a and 2c, subdomains 2 and 3), all of which are conserved in the EGF receptor. The cysteine residue spacing in this region is also homologous with the single cysteine-rich domain in the insulin receptor α subunit (*19*). In contrast, HER2 contains only eight potential *N*-linked glycosylation target sites (Asn-X-Thr or Ser) as compared to 12 in the corresponding region of the EGF receptor. Only five of these are conserved with respect to their relative position in each polypeptide.

The hydrophobic, putative membrane anchor sequence located between residues 653 and 676 (Fig. 4b, region 4) is flanked at its carboxyl terminus by a stretch of amino acids of predominantly basic character (KRRQQKIRKYTMRR) (*21*), as is found in the EGF receptor sequence (*9*) (Fig. 4b, region 5). This region of the EGF receptor contains Thr^{654}, which plays a key role in protein kinase C–mediated receptor modulation (*22*). A homologous threonine residue is embedded in a basic environment in the HER2 sequence at position 685 (Fig. 4, a and b).

The region of most extensive homology (78.4 percent) between EGF receptor and HER2 (beginning at residue 687) extends over 343 amino acids and includes sequences specifying the adenosine triphosphate (ATP) binding domain (*23*) and tyrosine kinase activity (Fig. 4b, region 6) (*5*). This region is also the most conserved between v-erbB and EGF receptor (95 percent) (*9*). The collinear homology between the EGF receptor-erbB and HER2 ceases at position 1032, but introduction of gaps into the EGF receptor or HER2 sequences reveals continued, although decreased, relatedness (Fig. 4b, region 7). This sequence alignment suggests that the two genes evolved by duplication of an ancestral receptor gene, and that subsequent nucleotide sequence divergence in this carboxyl terminal domain led to diverged biological roles for the encoded polypeptides.

The carboxyl terminal domain of HER2 is characterized by an unusually high proline content (18 percent) and predominant hydrophilicity (Fig. 4a). These general features are also found in the EGF receptor carboxyl terminal domain with a 10 percent proline content. The sequences in this region that are found to be conserved are almost exclusively centered around five tyrosine residues, which include the major (Tyr^{1173}) and two minor (Tyr^{1148}, Tyr^{1068}) in vitro autophosphorylation sites in the human EGF receptor (*24*) (Fig. 4, a and b). Three of these tyrosine residues of HER2 (positions 1139, 1196, 1248) are flanked by homologous sequences PQPEYV, ENPEYL, and ENPEYL (*21*), respectively (Fig. 4b, region 7).

HER2 chromosomal location. In situ hybridization of two ^{3}H-labeled HER2 probes (legend, Fig. 5a) to human chromosomes resulted in specific labeling at bands q12→q22 of chromosome 17 (Fig. 5a). Metaphase cells (100) were analyzed for each probe; 40 percent of cells scored for HER2 probe 1 (HER2-1) had silver grains over 17q12→q22 (Fig. 5b). Of the 209 grains observed, 42 (20 percent) were found at this specific region, with no other site labeled above background. For HER2 probe 2, 36 percent of cells had silver grains over the q12→q22 bands of chromosome 17. Of all silver grains, 17 percent (42/246) were localized to this chromosomal region. A secondary site of hybridization with 3.3 percent (8/246) of silver grains was detected at bands p13→q11.2 of chromosome 7.

To test whether this secondary site represented cross-hybridization with the EGF receptor gene, in situ hybridization was carried out with ^{3}H-labeled EGF receptor subclone 64-3. Of 100 cells examined, 30 had silver grains at bands p13→q11.2 of chromosome 7 and 3 percent (5/166) of total grains were found over q12→q22 of chromosome 17. With the other variant probe (HER2-1) no grain accumulation was observed at the EGF receptor site on chromosome 7.

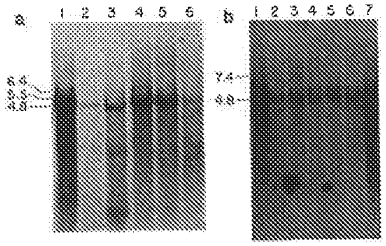


Fig. 2. Northern blot hybridization analysis of normal and malignant human tissues. (a) Fetal tissues; (lane 1) term placenta, (lane 2) 20-week placenta, (lane 3) 20-week liver, (lane 4) 20-week kidney, (lane 5) 20-week lung, (lane 6) 20-week brain. (b) Embryonic tumors; (lane 1) hepatoblastoma, (lanes 2 and 3) Ewing sarcoma, (lane 4) rhabdomyosarcoma, (lanes 5 and 6) neuroblastoma, (lane 7) Wilms' tumor. Total poly(A)$^+$ RNA was isolated as described (*33*); 4 μg per lane was analyzed on a 1 percent formaldehyde-agarose gel. ^{32}P-Labeled HER2-1 and HER-2 (legend to Fig. 5) were used as hybridization probes under high stringency conditions [50 percent formamide, 5× Denhardt's solution, 5× standard saline citrate (SSC), sonicated salmon sperm DNA (50 μg/ml), 50 μ*M* sodium phosphate buffer (pH 6.8), 1 m*M* sodium pyrophosphate, and 10 μ*M* ATP at 42°C for 16 hours; filters were washed three times for 15 minutes at 45°C with 0.2× SSC]. The filters were exposed at −60°C with a Cronex Lightning Plus intensifying screen (Dupont) for 7 days. Rat ribosomal RNA's were used as size standards (28*S*, 4.8 kb; 18*S*, 1.8 kb). RNA sizes are given in kilobases.

Southern blot analysis (*25*) of DNA extracted from nine somatic cell hybrids from human and rodent cells confirmed the localization of HER2 sequences to chromosome 17. ^{32}P-labeled HER2-1 and HER2-2 probes were hybridized to the same set of Eco RI–digested DNA samples. With HER2-1, a 13-kb hybridizing band was detected in human DNA (Fig. 5c, lane 1) and in DNA samples from hybrids containing human chromosome 17 (Fig. 5c, lanes 6, 8, 10, and 12). Likewise, hybridization of HER2-2 to a 6.6-kb DNA fragment was observed in human control DNA (Fig. 5c, lane 1) and in hybrids containing human chromosome 17 (Fig. 5c, lanes 6, 8, 10, and 12). Chromosome 17 was the only chromosome with perfect concordant segregation; all other chromosomes were excluded by two or more discordant hybrids.

Regional localization to chromosome 17 was also confirmed by Southern blot analysis. In a mouse-human hybrid containing a rearranged human chromosome 17 with region 17q21→qter, the human HER2 restriction fragments were detected (Fig. 5c, lane 4). The HER2 gene was therefore localized to region 17q21→qter, in agreement with the localization made by in situ hybridization.

Even though a low level of hybridization with probe HER2-2 was seen at the site of the EGF receptor gene on chromosome 7, we were able to show that this finding represented cross-hybridization. In a control experiment an EGF receptor probe cross-hybridized to the same extent with the HER2 site on 17q.

Taken together, the results of the in situ and Southern blot hybridizations permit the site of the HER2 sequences to be further narrowed down to bands 17q21-q22, with the major peak of silver grains at band 17q21.

HER2 expression in normal and malignant tissues. To obtain further clues regarding the function of this receptor both in normal cells and in neoplasms, Northern hybridization analyses (*15*) were carried out with several normal human tissues and randomly collected tumors. A hybridizing 4.8-kb mRNA was detected in all human fetal tissues analyzed, including term placenta, 20-week placenta, liver, kidney, lung, and brain obtained

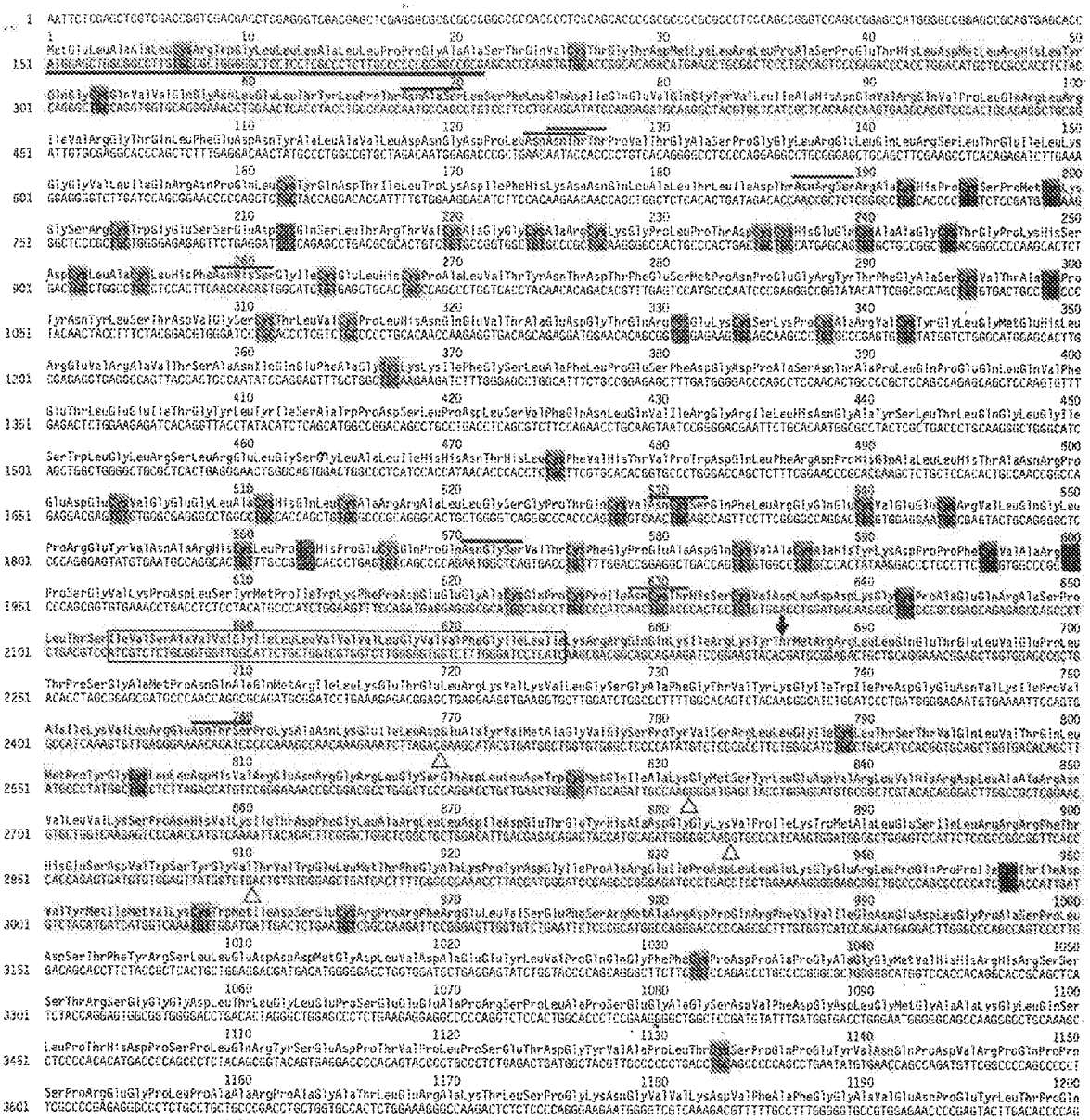
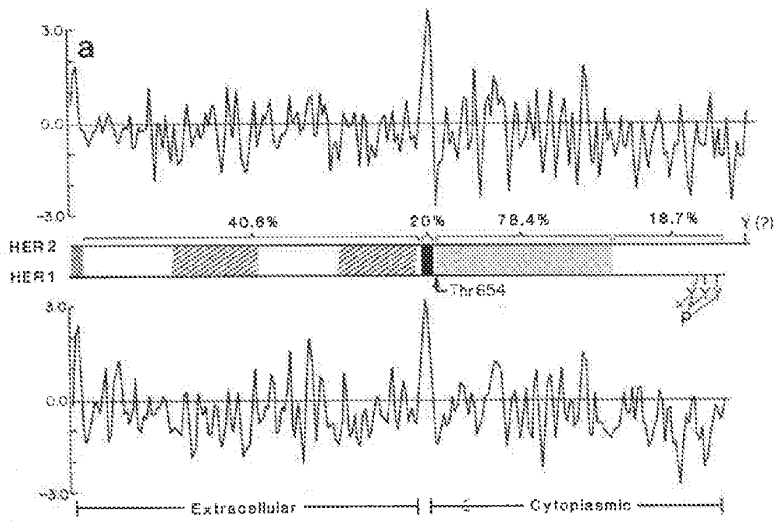


Fig. 3. Complete nucleotide and amino acid sequences of HER2 (clone λHER2-436). Synthetic probes 1 and 2 (Fig. 1) were used to screen 2×10^6 clones of a human placental cDNA library in λgt10 (*17*) as described (*9*). Fifty-two clones were isolated and characterized by Eco RI restriction digestion and Southern blot hybridization (*25*) with the radiolabeled synthetic oligonucleotides (*34*) and, on a duplicate blot, an EGF receptor Eco RI fragment (HER64-3) as probes. Labeling of oligonucleotides was achieved with 1 unit of T4 polynucleotide kinase (New England Biolabs), 10 pmol of oligonucleotide and 30 pmol of [γ-^{32}P]ATP (Amersham) as specified by the supplier. HER64-3 was labeled to high specific activity (>10^9 cpm/μg) as described (*34*). Clone λHER2-436 was used for nucleotide sequence analysis (*32*) of overlapping subclones. Amino acids are numbered starting with the initiation methionine (1). Underlining indicates the putative signal sequence (heavy) and the potential poly(A) addition signal (fine). Lines on top of the sequence indicate potential glycosylation sites, the black arrow demarcates the EGF receptor threonine 654 analogue (Thr 686), shading indicates cysteine residues, boxing shows the putative transmembrane region, large open triangles indicate some locations of introns in the HER2 gene, and the small triangle emphasizes a possible tyrosine autophosphorylation site by homology to Tyr 1173 in the EGF receptor sequence.

from a single fetus (Fig. 2a). Two mRNA's, of 5.4 and 6.4 kb, were also detected in term placenta. No cross-hybridization with the 5.8-kb and 10.5-kb EGF receptor mRNA's in term placenta mRNA was observed under these stringent hyridization conditions (legend, Fig. 2). Normal adult human tissues, including kidney, liver, skin, lung, jejunum, uterus, stomach, and colon, contained lower but significant amounts of the same 4.8-kb mRNA. Because of the magnitude of fetal expression, we also examined several embryonic tumors (Fig. 2b); each expressed large amounts of the 4.8-kb transcript, although not more than that detected in normal fetal tissue.

Thus, it appears that the HER2 gene is widely expressed, in both normal adult tissues and in several normal fetal tissues. While detected in most embryonic tumors, the HER2 gene was not present at higher levels than in fetal tissues; thus, the particular level may reflect the state of differentiation of a given tumor.

HER2 structurally characterized as cell surface receptor. Using the transforming gene of the avian erythroblastosis virus, v-erbB, as a hybridization probe, we isolated genomic and cDNA sequences of an uncharacterized human gene. The 1255 amino acid polypeptide sequence



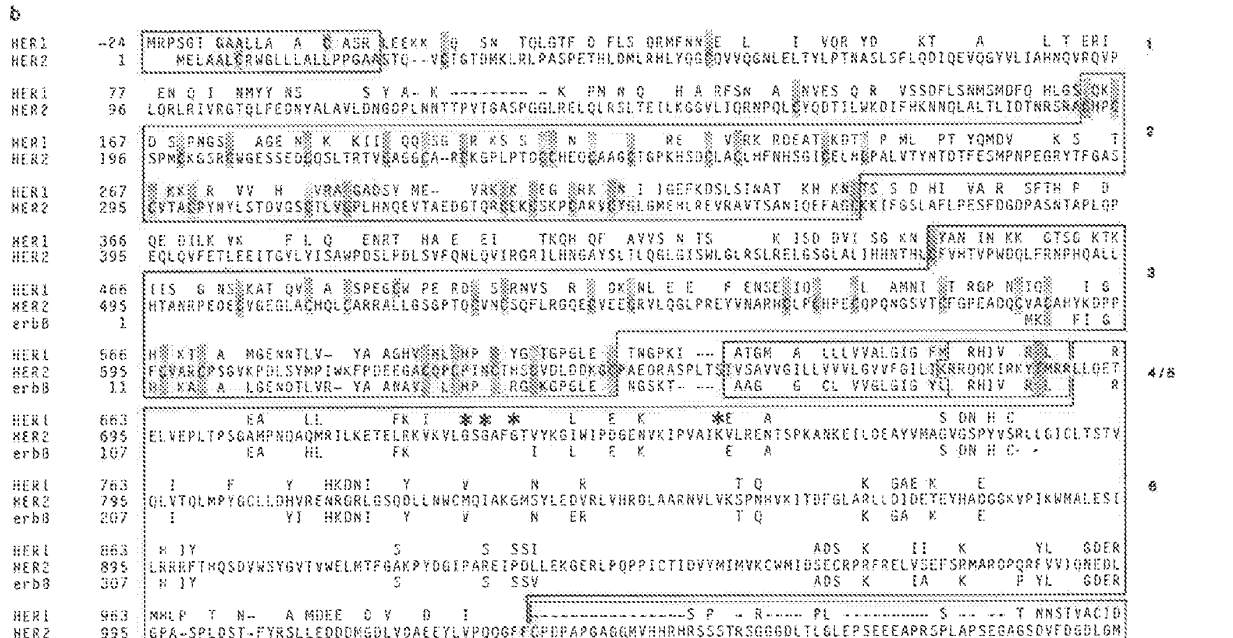


Fig. 4. (a) Hydropathy analysis (*20*) of HER2 (1255 amino acids) coding sequences and comparison with EGF receptor (HER1, 1210 amino acids). Different receptor domains and the extent of amino acid sequence homology are indicated. The autophosphorylation tyrosines in the EGF receptor sequence are shown, as is a potential analogue in HER2. The signal sequence is shown by fine shading, the cysteine-rich subdomains by hatching, the transmembrane region by a black bar, and the tyrosine kinase domain by coarse shading. (b) Comparison of HER2, EGF receptor (HER1), and v-erbB amino acid sequences. Identical residues are deleted, gaps are introduced to optimize alignment, cysteine residues are shaded, and carboxyl terminal tyrosines are in shaded boxes. The black and open triangles indicate the positions of the major and minor autophosphorylation sites in the EGF receptor, respectively. Asterisks indicate residues involved in ATP binding. Boxed regions include (i), signal sequence; (ii, iii), cysteine-rich domains; (iv), transmembrane domain; (v), protein kinase C modulation domain; (vi), tyrosine kinase domain; (vii), signal transfer domain.

derived from this cloned cDNA shows extensive homology to v-erbB and its cellular homologue, the human EGF receptor, and was therefore termed HER2. Its primary amino acid sequence displays all the structural features known to define a cell surface receptor for a polypeptide ligand. This finding represents the isolation and detailed structural characterization of a putative cell surface receptor before determination of its specific ligand, based solely on its structural homology to another receptor gene. In a similar case, the v-*fms* gene (*26*) was characterized because of its oncogenic activity, and was later determined to be identical or related to the receptor for CSF-1, a hematopoietic growth factor (*4*).

Examination of the HER2 primary sequence reveals extensive collinearity with the EGF receptor sequence. Detailed sequence comparison of these two closely related receptors, each having presumably different biological roles, provides important clues to the functional significance of the different regions of the proteins. A hydrophobic amino terminal sequence of HER2 presumably functions as a signal sequence (Fig. 4b, region 1) that, like the pre-EGF receptor, would be cleaved after alanine (*22*) to generate an amino terminal serine; however, cleavage after glycine (*19*) may occur, since signal peptidase frequently cleaves after small amino acid residues. Again, as in the case of the EGF receptor, the next 632 amino acids would represent the extracellular domain of the HER2 receptor that forms the binding pocket for a specific polypeptide ligand. Some structural features in this domain are highly conserved, while others have diverged extensively. Of the 51 cysteine residues of the EGF receptor ligand binding domain, 50 are conserved in HER2, of which 21 and 26, respectively, are clustered in subdomains 2 and 3 (Fig. 4b). Sequences between these conserved, regularly spaced cysteine residues, as well as the ones flanking subdomains 2 and 3 (Fig. 4b), have diverged to result in an overall homology of 40.6 percent (Fig. 4a).

Cysteine rich clusters (*27*) are found in receptors for EGF (*9*), low-density lipoprotein (*28*), and insulin (*19*), as well as in the EGF precursor (*29*), which is a potential receptor (Fig. 6). Since cysteine-rich domains are found in a family of cell surface receptors and potential receptors with very diverse functions, they are likely to form an essential structural backbone of the ligand binding domain, but not define ligand specificity. The ligand affinity of each of these receptors may be specified by sequences flanking these cysteine residue clusters.

The presence of regularly repeated, extracellular cysteine clusters (*27*) defines a gene family that overlaps with another gene family, the membrane-spanning proteins that use an intrinsic tyrosine-specific kinase activity in the signal transduction process (*5*) (Fig. 6). v-*fms*, the oncogenic homologue of the M-CSF-1 (colony-stimulating factor) receptor, contains a tyrosine kinase domain (*26*), but lacks cysteine-rich clusters in its extracellular domain, and thus is only a member of this second gene family.

Only 5 of the 12 potential *N*-linked glycosylation sites of the EGF receptor are conserved with respect to their approximate position in HER2, which also contains only eight such sites in this extracellular domain. The role of carbohydrate side chain differences in defining the biological function of these proteins requires further investigation.

The cytoplasmic domains of EGF receptor and HER2 also exhibit striking regional differences in sequence homology. These differences (Fig. 4, a and b) most likely distinguish regions that define receptor-specific, ligand-induced signal generation from support functions such as ATP binding and enzymatic tyrosine kinase activity.

Directly adjacent to the putative transmembrane domain, a cluster of 14 predominantly basic amino acids is found in both HER2 and EGF receptor sequences, which may interact with the membrane phospholipid head groups. The Thr^{654} residue, which is located within this region of the EGF receptor, is a phosphorylation substrate site for receptor activity down-modulation by protein kinase C (*22*). The HER2 sequence contains a threonine residue at an analogous position (686) (Figs. 3 and 4a and region 5, Fig. 4b) and may therefore also be subject to protein kinase C modulation.

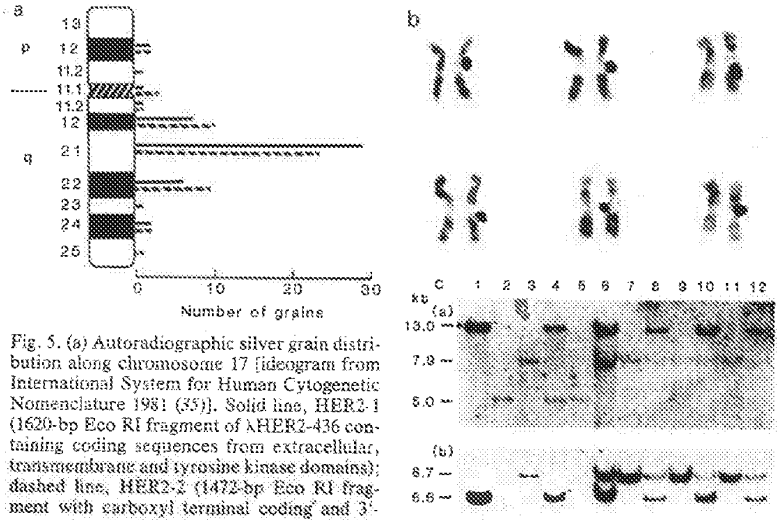


Fig. 5. (*a*) Autoradiographic silver grain distribution along chromosome 17 [ideogram from International System for Human Cytogenetic Nomenclature 1981 (*35*)]. Solid line, HER2-1 (1620-bp Eco RI fragment of λHER2-436 containing coding sequences from extracellular, transmembrane and tyrosine kinase domains); dashed line, HER2-2 (1472-bp Eco RI fragment with carboxyl terminal coding and 3′-untranslated sequences). Human metaphase and prometaphase chromosomes were prepared from methotrexate-synchronized peripheral lymphocyte cultures (*36*) of two normal individuals. The probes for in situ hybridization (*37*) were ^{3}H-labeled by nick translation with three labeled nucleotides to specific activities of 2.4×10^7 cpm/μg (HER2-1), 3.4×10^7 cpm/μg (HER2-2), and 2.5×10^7 cpm/μg (HER1 64-3); the probes (25 or 50 ng/ml) were hybridized to chromosome preparations (16 hours at 37°C). The emulsion-coated slides were exposed for 10 to 14 days at 4°C. Chromosomes were stained with Wright's stain for G-banding, and a second photograph was taken of the previously selected cells. G-banded chromosomes were analyzed for silver grain localizations. (*b*) Pairs of chromosome 17 from three cells, illustrating typical labeling at bands q12→q22 for HER2-1 (top row) and HER2-2 (bottom row). (*c*) Southern blot hybridization of HER2-1 (*a*) and HER2-2 (*b*) to Eco RI–digested DNA from somatic cell hybrids of humans and rodents. Somatic cell hybrids of four different series were used. Their origin and characterization has been reported (*38*). Samples (10 μg) of hybrid, mouse, Chinese hamster, and human DNA were cleaved with Eco RI, subjected to electrophoresis on 0.8 percent agarose gels, transferred to nitrocellulose filters, and hybridized to ^{32}P-labeled HER2 probes according to standard procedures (*26*, *39*). (Lane 1) Human lymphoblastoid control cell line; (lane 2) mouse 3T3 cells; (lane 3) Chinese hamster cell line; (lane 4) subclone of hybrid of mouse and human cells with a rearranged chromosome 17 containing only region q21→qter, retained by growth in hypoxanthine-aminopterin-thymidine (HAT) medium; (lane 5) same subclone as lane 4, except missing this rearranged chromosome after counterselection in bromodeoxyuridine medium; (lanes 6, 8, 10, and 12) clones of Chinese hamster and human hybrid cells containing human chromosome 17; (lanes 7, 9, and 11) Chinese hamster X human hybrid cell clones lacking human chromosome 17.

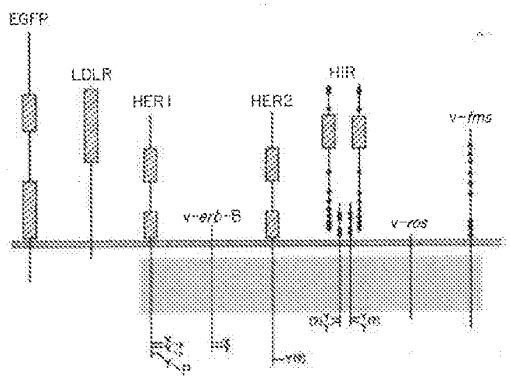


Fig. 6. Schematic comparison of HER2-related molecules. Hatched boxes indicate cysteine-rich regions within extracellular or putatively extracellular domains. The shaded region below the membrane bilayer indicates the location of the tyrosine kinase domain. Black circles within the human insulin receptor (HIR) and v-*fms* structures indicate cysteine residues that are not included in cysteine clusters and may be involved in heterotetramer (HIR) or ligand binding pocket formation (v-*fms*). EGFP, EGF precursor; LDLR, LDL receptor; HER1, for a growth or differentiation factor and is consistent with a more general role in fetal development. No clues were obtained from tumor RNA screening to suggest that increased expression was involved in tumor generation or growth. In some tissues, such as term placenta, fetal kidney, and embryonic tumors, we found additional larger (5.5, 6.4, and 7.4 kb) mRNA's hybridizing with our probe, suggesting transcriptional diversity or the presence of homologous mRNA's more closely related to HER2 than to the EGF receptor. Further experiments should establish the biological role of the HER2 gene, and its role in oncogenesis.

The subsequent 341 residues are not only highly homologous to the EGF receptor and the v-erbB oncogene, but also to all the other members of the *src* family of tyrosine kinase oncogenes (*22*), as well as the β subunit of the insulin receptor (*19*). This sequence similarity suggests that this HER2 region encompasses at least a portion of the kinase domain that may be activated by extracellular ligand binding. A Gly X Gly XX Gly sequence at HER2 position 727 and a lysine at position 753 are known from studies on other members of this gene family to be involved in ATP binding (*23*). Overall, this HER2 region (6 in Fig. 4b) is the most homologous to v-erbB and EGF receptor (78.4 percent; Fig. 4a), which explains why the initially isolated genomic clone (Fig. 1) contained exons coding for part of this tyrosine kinase domain (intron positions are shown in Fig. 3).

The region within the sequence of a tyrosine kinase receptor most likely to be responsible for translation of the activation signals intitiated by specific, extracellular ligand binding into physiological action is localized at the carboxyl terminus of the receptor polypeptide chain. This region contains the 32-amino acid sequence deletion that plays a crucial role in the generation of the AEV transforming gene v-erbB from the EGF receptor proto-oncogene (Fig. 6) (*30*), as well as the tyrosine residues that are major (1173) and minor (1068 and 1148) sites of ligand-induced autophosphorylation in the EGF receptor (*24*) (Fig. 4, a and b). This region contains the most extensive differences in length and primary structure between HER2 and EGF receptor/erbB. However, five of seven tyrosines are conserved in HER2, including short surrounding sequences. This suggests that, like the conserved cysteine residues in the ligand binding domain, these tyrosine residues may serve an auxiliary activity-control function in the generation of a cytoplasmic signal. Other sequences in this signal-control and transfer domain are likely to define specificity, which may include recognition of possible substrates and/or cofactors. Future work will be needed to determine if tyrosine 1248 of HER2 plays a role analogous to tyrosine 1173, the major autophosphorylation site of the EGF receptor, and if carboxyl terminal truncation may lead to the generation of a HER2-derived oncogenic polypeptide.

The oncogenic potential of the HER2 gene is further suggested by its chromosomal location. Results of in situ and Southern blot hybridizations enabled us to assign the HER2 gene to chromosome 17 (17q21-q22), precisely coinciding with our previous assignment of the *neu* oncogene (*12*), a transforming sequence isolated from rat neuroblastoma (*11*). As documented elsewhere (*12*), our results with the rat *neu* probe were virtually identical to those obtained with the two HER2 probes described here, except for the lack of cross-hybridization of rat *neu* genomic sequence with the EGF receptor locus on chromosome 7. Our mapping results support the notion that the proto-oncogene *neu* and the HER2 gene, although both are very likely not distinct from each other. Further detailed analysis will be necessary to establish whether they actually represent the same gene.

Can we obtain clues to the cellular role of this putative receptor by examination of its gene expression? Northern blot hybridization analysis of poly(A)$^+$ RNA's from a variety of normal and malignant tissues implicates a role for the HER2 receptor-ligand system in many cell types in both fetal and adult life. Highest levels of the 4.8-kb mRNA were found in several fetal tissues, which rules out a cell lineage–specific receptor

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20 September 1985; accepted 30 October 1985

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Clinical Cancer Research

Development of an Fc-Enhanced Anti–B7-H3 Monoclonal Antibody with Potent Antitumor Activity

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Clinical Cancer Research

Cancer Therapy: Preclinical

Development of an Fc-Enhanced Anti–B7-H3 Monoclonal Antibody with Potent Antitumor Activity

Deryk Loo[1], Ralph F. Alderson[2], Francine Z. Chen[1], Ling Huang[2], Wenjun Zhang[2], Sergey Gorlatov[2], Steve Burke[2], Valentina Ciccarone[2], Hua Li[2], Yinhua Yang[2], Tom Son[1], Yan Chen[1], Ann N. Easton[1], Jonathan C. Li[1], Jill R. Rillema[1], Monica Licea[1], Claudia Fieger[1], Tony W. Liang[1], Jennie P. Mather[1], Scott Koenig[2], Stanford J. Stewart[1], Syd Johnson[2], Ezio Bonvini[2], and Paul A. Moore[2]

Abstract

Purpose: The goal of this research was to harness a monoclonal antibody (mAb) discovery platform to identify cell-surface antigens highly expressed on cancer and develop, through Fc optimization, potent mAb therapies toward these tumor-specific antigens.

Experimental Design: Fifty independent mAbs targeting the cell-surface immunoregulatory B7-H3 protein were obtained through independent intact cell-based immunizations using human tissue progenitor cells, cancer cell lines, or cell lines displaying cancer stem cell properties. Binding studies revealed this natively reactive B7-H3 mAb panel to bind a range of independent B7-H3 epitopes. Immunohistochemical analyses showed that a subset displayed strong reactivity to a broad range of human cancers while exhibiting limited binding to normal human tissues. A B7-H3 mAb displaying exquisite tumor/normal differential binding was selected for humanization and incorporation of an Fc domain modified to enhance effector-mediated antitumor function via increased affinity for the activating receptor CD16A and decreased binding to the inhibitory receptor CD32B.

Results: MGA271, the resulting engineered anti–B7-H3 mAb, mediates potent antibody-dependent cellular cytotoxicity against a broad range of tumor cell types. Furthermore, in human CD16A-bearing transgenic mice, MGA271 exhibited potent antitumor activity in B7-H3–expressing xenograft models of renal cell and bladder carcinoma. Toxicology studies carried out in cynomolgus monkeys revealed no significant test article-related safety findings.

Conclusions: This data supports evaluation of MGA271 clinical utility in B7-H3–expressing cancer, while validating a combination of a nontarget biased approach of intact cell immunizations and immunohistochemistry to identify novel cancer antigens with Fc-based mAb engineering to enable potent antitumor activity. *Clin Cancer Res; 18(14); 3834–45. ©2012 AACR.*

Introduction

Antigens that are tumor specific or overexpressed on cancer cells represent opportunities for development of target-specific antibody-based therapeutics with a range of possible therapeutic modalities. For example, unmodified IgG1 monoclonal antibodies (mAb) directed to the EGF receptor (EGFR) family, neutralize tumor-promoting activities of such molecules through antiproliferative and tumoricidal mechanisms. Although such targeted therapies have shown clinical benefit, the responses are rarely sustained and are often limited to subsets of patients expressing the antigen (e.g., amplified Her 3+ metastatic breast cancer patients treated with trastuzumab; refs. 1–3). More recently, modifications to mAbs have been employed including drug conjugation, radionuclide labeling, and glycosylation or amino acid substitutions in Fc domains that have shown particular therapeutic advantages in preclinical and clinical studies. In the case of the latter, these Fc modifications can enhance variable region–dependent signaling properties of the mAb and Fc and immune cell–dependent effector functions such as antibody-dependent cellular cytotoxicity (ADCC; refs. 4, 5).

The rationale for improving effector cell function has been supported independently by the correlation of clinical outcomes of mAb therapies with the natural polymorphisms of Fcγ receptors (FcγRs) in patients who participated in these trials. The allelic FcγR variants show different affinities for the Fc domain of IgG1 and more favorable clinical

Authors' Affiliation: [1]MacroGenics, Inc., South San Francisco, California and [2]MacroGenics, Inc., Rockville, Maryland

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Authors: Deryk Loo, MacroGenics, Inc., One Corporate Drive, South San Francisco, CA 94080. Phone: 650-624-2636; Fax: 650-624-2693; E-mail: lood@Macrogenics.com and Paul A. Moore, MacroGenics, Inc., 9640 Medical Center Drive, Rockville, MD 20850. Phone: 301-354-2692; Fax: 301-251-5321; E-mail: moorep@Macrogenics.com

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Translational Relevance

Using a monoclonal antibody (mAb)–driven process to identify cell-surface antigens highly expressed in cancer, we discovered a panel of mAbs against B7-H3, a member of the B7 family of immunomodulatory molecules. To translate this discovery for clinical evaluation, we developed MGA271, a humanized B7-H3 mAb displaying broad tumor reactivity but limited normal tissue binding that incorporates Fc-domain modifications designed to enhance antitumor effector-mediated function. The rationale that potent mAb mediated effector functions will be clinically beneficial is supported by comparison of clinical outcomes of mAb therapies with natural polymorphisms of FcγRs, which revealed more favorable outcome for patients homozygous for the higher affinity alleles of the activating receptors CD16A and CD32A. We hypothesize that the enhanced antitumor effects of MGA271 in preclinical studies, combined with its favorable safety profile in non-human primates, as shown in this article, will translate into potent antitumor activity toward B7-H3–positive cancers in the clinic.

responses were observed in patients who were homozygous for the higher affinity alleles of FcγR IIA (CD32A) and, in particular, FcγR IIIA (CD16A). These therapeutic mAbs include rituximab for treatment of follicular lymphoma (4, 5), trastuzumab for treatment of metastatic breast cancer (6), and, albeit controversial, cetuximab for treatment of metastatic colorectal cancer (mCRC; ref. 7). These results support the notion that potent immune-mediated effector functions can be clinically beneficial, and mAbs with enhanced Fc-mediated activity may favor not only patients with lower affinity allelic variants for FcγRs but also those individuals who are homozygous for the higher binding alleles. This strategy can be exploited for cell-surface cancer targets irrespective of their causality in the initiation or progression of the tumor.

To identify cell-surface cancer antigens suitable for mAb-based targeting, we have characterized mAbs generated in a target-unbiased fashion from intact cell immunizations of serum-free adapted tissue progenitor cells or cancer cell lines, including those exhibiting cancer stem cell properties (8, 9). Immunohistochemical profiling of these mAbs reveals those displaying differential expression on human cancer tissues compared with normal tissue, whereas biochemical analyses identify the cell-surface cancer antigen recognized (10). Here we describe characterization of a panel of 50 independent mAbs identified from this approach that recognize the cell-surface protein B7-H3 (CD276), a member of the B7 family of immune regulators (11). The limited normal tissue binding and broad tumor reactivity exhibited by specific B7-H3 mAbs prompted development of MGA271, a fully humanized anti-B7H3 mAb bearing an engineered Fc domain optimized to enhance antitumor effector–mediated function through combination of enhanced binding to the activating receptor CD16A and reduced binding to the inhibitory receptor CD32B. To determine the clinical potential of MGA271, antitumor activity was evaluated in a series of murine xenograft models and its toxicology profile evaluated in non-human primates.

Materials and Methods

Cell lines

Renal (A498, 786-0, and ACHN), prostate (LnCap), lung (SK-MES-1), breast (MDA-MB-468), bladder (SW780 and HT-1197), melanoma (UACC-62) cancer cell lines, and Raji B-cell lymphoma were obtained from American Type Culture Collection and cultured according to recommended protocol for fewer than 20 passages.

Immunohistochemistry

OCT-embedded, frozen tissues, and positive control B7-H3–expressing Caki2 and Hs700T cells were sectioned at 7 μm. Following drying, sections were incubated in 4°C acetone, air dried, then processed in a Dako Autostainer. Formalin-fixed paraffin-embedded (FFPE) tissue microarray sections were deparaffinized, rehydrated, then processed in a Dako Autostainer. Endogenous peroxidase activity was quenched with 3% H_2O_2, then nonspecific binding sites were blocked with 5% normal goat serum. Primary mAbs (BRCA84D and BRCA69D) were detected using EnVision horseradish peroxidase (HRP) anti-mouse polymer (Dako) in conjunction with 3,3′-Diaminobenzidine (Sigma-Aldrich). Slides were counterstained with hematoxylin.

Protein engineering

chBRCA84D was generated by fusing the BRCA84D VL and VH coding sequences to human c-Kappa or human gamma 1 constant region cDNA, respectively. To construct hBRCA84D, humanized BRCA84D VL (hBRCA84D VL) and BRCA84D VH (hBRCA84D VH) amino acid sequences were designed using the CDR sequences from the mouse mAb BRCA84D and framework sequences from human germline V-kappa or VH segment, respectively. The hBRCA84D VL and hBRCA84D VH coding sequences were synthesized *de novo*, fused to the human C-Kappa or human gamma 1 constant region cDNA, respectively. Single mutations, L46A in VL and A93G in VH, were introduced by site-directed mutagenesis to optimize the binding affinity for B7-H3.

MGA271 was generated from hBRCA84D by exchanging its Fc domain for MGFc0264 (L235V, F243L, R292P, Y300L, and P396L). hBRCA84D-aglycosyl was generated from hBRCA84D by mutagenesis at the N-glycosylation site of the WT Fc domain. Antibodies were produced in stably transfected Chinese hamster ovary cells.

ADCC

Peripheral blood mononuclear cells (PBMC) were isolated from healthy human donor blood (Ficoll-Paque Plus; GE Healthcare). Target cells, effector cells, and antibody

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were incubated overnight in Dulbecco's modified Eagle's medium/F-12 containing 5% FBS. PBMC effector cells were added at ratios of 25:1 to 30:1. LDH release (Promega Corp.) was measured after overnight incubation. Cytotoxicity (%) = (experimental cell lysis − antibody-independent cell cytolysis)/(maximum target lysis − spontaneous target lysis) × 100. Fcγ receptor genotypes were determined by sequencing PCR-amplified DNA. To evaluate the ability of murine B7-H3 mAbs to support ADCC, FITCylated anti–B7-H3 murine mAbs were mixed with a bispecific DART (Dual Affinity ReTargeting; CD16×FITC) comprising specificity of human CD16 and fluorescein isothiocyanate (FITC) and incubated with cancer target cells together with resting human PBMC effector cells (E:T = 30:1).

Mass spectrometry

GB8 antigen was immunoprecipitated from A498 cell membranes using biotinylated antibody and streptavidin-coated resin (Pierce). After washing, antigens were eluted with low pH buffer and concentrated using Strataclean beads (Agilent Technologies, Inc.), treated with reducing sample buffer, and subjected to SDS-PAGE. Antigen gel bands were excised, subjected to enzymatic digestion, followed by liquid chromatography tandem mass spectrometry analysis.

Capture ELISA

A MaxiSorp ELISA plate (Nalge Nunc Intl.) was coated with soluble human B7-H3-4Ig-His (0.3 μg/mL) in BupH bicarbonate buffer (Thermo Fisher Scientific) overnight at 4°C. The plate was blocked with PBS containing 0.5% bovine serum albumin (BSA) and 0.1% Tween-20 (PBST/BSA) for 30 minutes. Antibodies were diluted in PBST/BSA and applied to the ELISA plate for 1 hour. Following wash with PBST, HRP-conjugated goat anti-mouse IgG (H+L; dilution 1:10,000 in PBST/BSA; Jackson ImmunoResearch) was added for 1 hour, the plate washed and developed with 80 μL/well of TMB peroxidase substrate and terminated with 40 μL/well 1% H_2SO_4. Absorbance at 450 nm (A_{450}) was determined and data analyzed using GraphPad Prism 5 software.

SPR analysis of human B7-H3 binding to selected mAbs

Binding of the B7-H3 mAb panel to human B7-H3 was analyzed by surface plasmon resonance (SPR) in a BIAcore 3000 biosensor (Biacore AB) as previously described (12, 13). mAbs were captured on goat anti-mouse F(ab')$_2$ fragment (Jackson ImmunoResearch) coated CM-5 sensor chips and binding curves obtained following injection of human B7-H3(4Ig)-His (R&D Systems). Experimental binding curves were also generated following injection of BRCA84D and its humanized forms to both human and cynomolgus monkey B7-H3 on the CM-5 sensor chip. Data were analyzed using BIAevaluation 4.0 software. Kinetic constants, k_{a1} and k_{d1} describing the binding of first arm of antibody to immobilized antigen were estimated by global fitting analysis of the association/dissociation curves to the bivalent analyte interaction model. The equilibrium dissociation constant (K_D) was calculated as $K_D = k_{d1}/k_{a1}$.

In vivo efficacy

All mouse experiments were carried out under protocols approved by the MacroGenics Institutional Animal Care and Use Committee (IACUC). mCD16$^{-/-}$ hCD16A^{+} RAG2$^{-/-}$ mice (mCD16 knockout mice expressing the hCD16A-158F transgene consistent with the distribution of CD16A in human tissues) were bred at MacroGenics. Tumor cells (5×10^6 per mouse) in PBS + Matrigel were implanted subcutaneously and antibodies administered intravenously weekly, beginning approximately 1 week following tumor implantation or after tumors of approximately 200 to 300 mm^3 had been allowed to form. Tumor sizes were monitored twice weekly by orthogonal measurements with electronic calipers. Statistical differences in tumor sizes were assessed by 2-way ANOVAs and Bonferroni posttest analyses (GraphPad Prism 5.02).

Pharmacokinetics/toxicology studies in cynomolgus monkeys

Cynomolgus monkey experiments were conducted at SNBL USA, which adheres to the regulations outlined in the USDA Animal Welfare Act and the conditions specified in the Guide for the Care and Use of Laboratory Animals. The study protocols were approved by the Testing Facility IACUC. A single-dose study was conducted with 24 cynomolgus monkeys randomized into 4 groups (3/gender/group) receiving vehicle control or MGA271 at 1, 30, or 150 mg/kg by 60-minute intravenous infusion. Terminal group animals (2/gender/group) were necropsied 7 days following dose administration. Recovery group animals (1/gender/group) were euthanized 40 days following dose administration for necropsies. A repeat-dose study was conducted with 52 cynomolgus monkeys randomized into 5 groups receiving vehicle control (6/gender/group) or MGA271 (5/gender/group) weekly for 4 weeks at 1, 10, 30, or 150 mg/kg by 60-minute intravenous infusion. Terminal group animals (4/gender/group for vehicle control; 3/gender/group for MGA271) were necropsied 7 days following the final dose administration. Recovery group animals (2/gender/group) were necropsied 70 days following the final dose.

Results

Identification of a panel of B7-H3 mAbs

Through a series of intact cell immunizations, we have obtained more than 1,500 mAbs reactive with antigens expressed on cancer cells (8). Antibodies discovered include those recognizing common cancer antigens such as EGFR, HER2, and CEACAM5 and those binding unique cancer antigens such as RAAG12 (10). A subset of antibodies that displayed strong normal/tumor differential by immunohistochemistry (Fig. 1A) exhibited a distinct binding fingerprint across a panel of cancer cell lines, suggesting reactivity to an alternate cancer-associated

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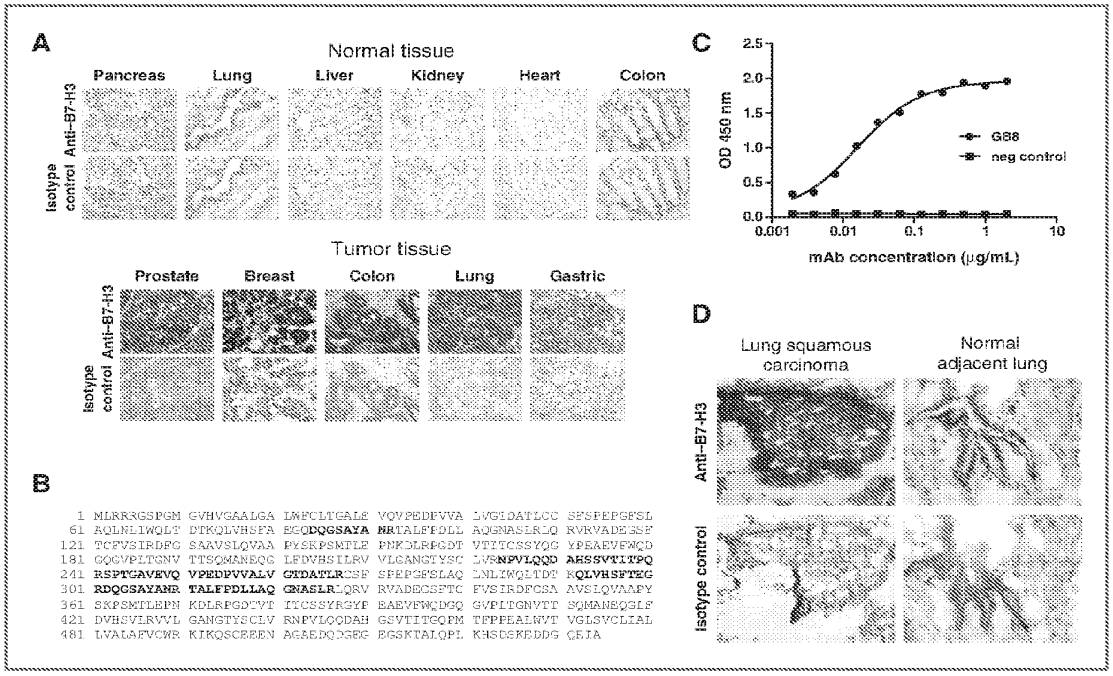


Figure 1. Tumor-specific panel of mAbs are reactive with human B7-H3. A, anti–B7-H3 mAb TES7 exhibits strong differential reactivity to multiple solid tumor tissues compared with normal tissues. No positive staining was noted in human normal pancreas, lung, liver, kidney, and heart. The very weak staining in the epithelium of the crypt of the colon was consistent with a nonspecific staining pattern. B, tandem mass spectrometry analysis of protein immunoprecipitated by GB8 mAb from A498 membrane lysate identifies B7-H3 as the potential antigen. TOF peptide matches are highlighted in bold. C, confirmation of reactivity of GB8 for human B7-H3 by ELISA using recombinant human B7-H3. D, anti–B7-H3 mAb BRCA84D exhibits strong differential reactivity to lung squamous carcinoma compared with normal adjacent lung tissue from the same patient specimen.

antigen. Tandem mass spectrometry of protein immunoprecipitated from A498 renal cell carcinoma cell membranes by one mAb in the set, GB8, yielded several peptides corresponding to B7-H3 (Fig. 1B). A recombinant B7-H3–based ELISA assay confirmed that GB8 (Fig. 1C), as well as the other mAbs in the subset (data not shown), were reactive with B7-H3. The tumor-specific reactivity of this set of mAbs prompted screening of the larger panel for additional mAbs and led to the identification of 50 B7-H3–reactive mAbs. A subset of the B7-H3–reactive mAbs, which recognized nonoverlapping epitopes on B7-H3 and displayed a range of binding affinities, is shown in Table 1.

To determine the ability of the B7-H3 mAb panel to support immune effector cell-based interventions, mAbs were tested for ADCC activity. This was assessed in a cell-based cytotoxicity assay using a FITCylated panel of B7-H3 mAbs, together with a bispecific DART molecule recognizing FITC and CD16A (CD16AxFITC DART; ref. 14). The CD16AxFITC DART molecule engages the targeting mAb via the FITC specificity and engages immune effector cells through the anti-CD16 arm, thus bringing together target and effector cells. This strategy obviates the lack of effector function intrinsic in the majority of the murine B7-H3 mAbs. All but one of the B7-H3 mAbs tested efficiently redirected immune-mediated killing of B7-H3–positive A498 renal cell carcinoma cells (Table 1).

Selection, humanization, and Fc-optimization of BRCA84D as a clinical candidate for B7-H3–directed interventions

Among the panel of B7-H3 mAbs initially screened on frozen tissue specimens, BRCA84D exhibited the least reactivity to normal tissues, while retaining strong reactivity with prostate, breast, colon, lung, gastric, and renal cancers (Supplementary Fig. S1A). As a further test of the differential reactivity of BRCA84D, analysis of reactivity toward lung squamous carcinoma compared with normal adjacent lung tissue from the same patient specimen confirmed that BRCA84D exhibited strong reactivity toward the lung squamous carcinoma, although exhibiting minimal or no reactivity toward the normal adjacent lung tissue (Fig. 1D). On the basis of the immunohistochemical profile, binding properties, biologic activity, and non-human primate

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Table 1. Select panel of tumor-reactive mAbs recognizing nonoverlapping epitopes on human B7-H3

Antibody	Isotype	Input cell line	Epitope group	Affinity (KD, nmol/L)	*In Vitro* redirected killing[a] (% cytotoxicity)
BRCA69D	IgG1	MCF-7	A	2.4	82
BRCA84D	IgG1	MCF-7	B	28.3	72
GB8	IgG1	Fetal gall bladder progenitor	C	22.7	38
OVCA64	IgG1	Ovarian CSCs	D	0.8	72
PRCA157	IgG1	H460	E	15.0	59
SG27	IgG2b	Fetal sweat gland progenitor	F	263.2	15
TES7	IgG1	Fetal testes progenitor	G	45.3	83

[a]Redirected killing of A498 renal cell carcinoma tumor cells using FITCylated murine anti–B7-H3 mAbs together with bispecific (CD16×FITC) DART in the presence of resting human PBMC effector cells (E:T = 30:1).

cross-reactivity, BRCA84D was chosen for further development. To generate MGA271, the coding sequences of the murine IgG1 mAb variable light and heavy chain genes were humanized and then fused in-frame with MGFc0264, an optimized human IgG1 Fc domain containing 5 amino acid changes that impart increased affinity for both alleles of the human activating FcγR, CD16A, and decreased affinity for the inhibitory FcγR, CD32B (12). Control humanized BRCA84D mAbs containing either the wild-type human IgG1 Fc domain (RES240) or a mutated human IgG1 Fc domain that lacks FcγR binding (RES240-aglycosyl) were also constructed. The recombinant mAbs retained the binding affinity of the parental BRCA84D mAb for both human and cynomolgus monkey B7-H3 (Supplementary Table S1). Importantly, a survey analysis confirmed the restricted reactivity of MGA271 with a broad set of normal tissues. Membrane staining was limited to basal squamous epithelium of the skin (1–2—, occasional) when tested at the optimal staining concentration. Additional membranous staining (1+, rare) of squamous epithelium of the esophagus was observed when stained at 5 times the optimal concentration. Cytoplasmic staining (1+, rare to occasional) was observed in various epithelium and stroma at the optimal concentration and was modestly increased in intensity and frequency at 5 times the staining concentration (Supplementary Table S2). Because of the possible immune regulatory capacity of B7-H3, reactivity of MGA271 in lymphatic tissues was also examined. No reactivity of MGA271 with lymph node and spleen tissues was observed (Supplementary Fig. S1B).

B7-H3 is highly expressed in multiple cancers with high penetrance

An expanded panel of FFPE tumor tissues was screened to confirm the expression level and penetrance of B7-H3 in cancer. Because BRCA84D does not perform adequately on paraffin-embedded tissues, we employed BRCA69D, another B7-H3 mAb from our panel that performs appropriately

Table 2. Summary of immunohistochemical staining of FFPE tumor specimens with anti–B7-H3 mAb BRCA69D to evaluate B7-H3 expression across a broad range of cancer types

		Positive staining (any grade)		Moderate to high staining (2+ or greater)	
Tissue	Type	Positive/total	%Positive	Positive/Total	%Positive
Melanoma	Primary	48/51	94	25/51	49
	Metastatic	18/19	95	7/19	37
	Total	66/70	94	32/70	46
Kidney cancer	Primary	77/78	99	75/78	96
Prostate cancer	Primary	88/99	89	51/99	52
Pancreatic cancer	Primary	69/78	88	45/78	58
Gastric cancer	Primary	100/115	87	100/115	87
Breast cancer	Primary	76/90	84	74/90	82
Ovarian cancer	Primary	39/52	75	19/52	37
	Metastatic	4/8	50	2/8	25
	Total	43/60	72	21/60	35
Small cell lung cancer	Primary	12/75	16	6/75	8

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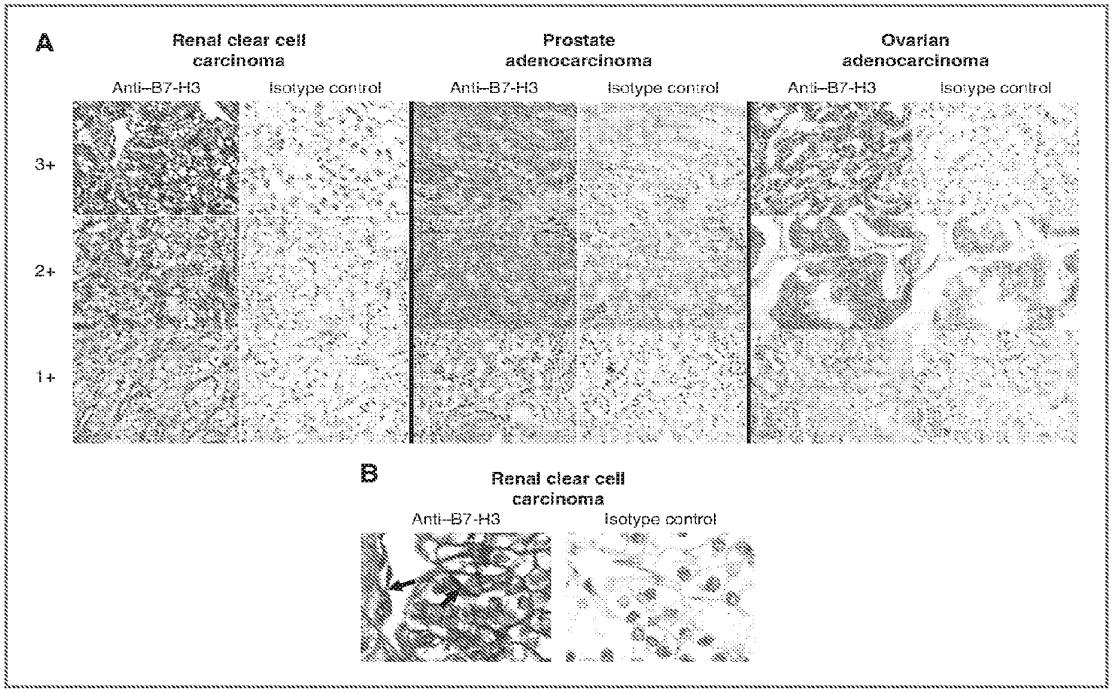


Figure 2. Expression of B7-H3 across a broad range of cancer types. A, representative examples of 1+, 2+, and 3+ staining on renal clear cell carcinoma, prostate adenocarcinoma, and ovarian adenocarcinoma. B, higher magnification image showing vascular endothelium staining in renal cell carcinoma (arrows).

on paraffin-embedded tissues. The tumor reactivity against a panel of solid primary and metastatic tumor tissue samples is summarized in Table 2. Representative examples of low (1−), moderate (2+), and high (3+) B7-H3 expression levels in cancer tissues, as detected by BRCA69D, are shown in Fig. 2A. In kidney cancer, substantial expression was observed not only in the epithelial component of the tumor but also in the surrounding tumor vasculature (Fig. 2B).

Humanized/Fc-optimized BRCA84D (MGA271) mediates potent ADCC *in vitro*

To confirm retention of ADCC activity and determine the effect of the MGFc0264, MGA271 was compared with chimeric BRCA84D (chBRCA84D, WT Fc domain), humanized BRCA84D (RES240, WT Fc domain), and aglycosylated humanized BRCA84D (RES240-aglycosyl, inactivated Fc domain) using human PBMC donors representing the 3 CD16A genotypes (low-affinity 158F homozygous, high-affinity 158V homozygous, and 158F/V heterozygous). As shown in Fig. 3A and B, RES240 mediates ADCC *in vitro* against B7-H3–expressing A498 renal cell carcinoma cells with a potency equivalent to chimeric BRCA84D, indicating that humanization did not alter ADCC function. Importantly, MGA271 exhibited enhanced potency in the ADCC assay relative to RES240. The enhanced ADCC potency of MGA271 was evident with PBMCs from all 3 CD16A donor genotypes (Fig. 3A–C), with the greatest enhancement observed for the homozygous low-binding 158F allele of CD16A, consistent with the enhanced FcγR-binding characteristic of MGA271 Fc domain for this CD16A allele. As expected, RES240-aglycosyl did not mediate ADCC (Fig. 3C), indicating that the cytotoxic activity observed with MGA271, RES240, and chBRCA84D is a function of Fc/FcγR interactions. The MGFc0264 Fc of MGA271 retained binding to cynomolgus monkey FcγR (13) and translated into functional engagement, as shown by the ability of MGA271 to mediate ADCC against A498 cells with cynomolgus monkey PBMCs (Fig. 3D).

MGA271-mediated ADCC activity was determined for a panel of tumor cell lines displaying a range of B7-H3 cell-surface expression and representing multiple cancer types, including kidney, prostate, lung, breast, and bladder carcinoma as well as melanoma (Supplementary Fig. S2). All cancer cell lines that displayed detectable levels of B7-H3 expression exhibited sensitivity to MGA271-mediated ADCC (Fig. 4). Consistent with the enhanced ADCC observed with MGA271 against A498 cells, MGA271 exhibited enhanced ADCC potency relative to RES240 against all

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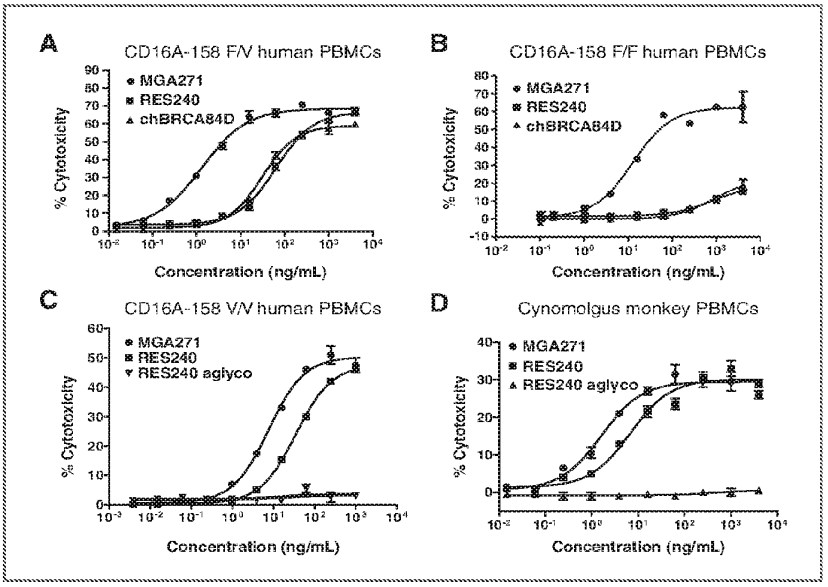


Figure 3. MGA271 mediates *in vitro* ADCC on A498 renal cell carcinoma cells with human PBMC effector cells representing all 3 CD16A-158 genotypes and with cynomolgus monkey PBMC effector cells. Evaluation of the ability of MGA271 to mediate ADCC on the A498 renal cell carcinoma cell line using resting PBMCs representative of each CD16A-158 genotype—A, F/V; B, F/F; or C, V/V and compared with the indicated control molecules: humanized BRCA84D (RES240), chimeric BRCA84D (chBRCA84D), and aglycosylated humanized BRCA84D (RES240-aglycosyl). D, evaluation of the ability of cynomolgus monkey PBMCs to support MGA271-mediated ADCC on the A498 renal cell carcinoma cell line.

B7-H3–expressing tumor cell lines examined (data not shown). In contrast, MGA271 did not mediate ADCC against the B7-H3–negative Raji B-cell lymphoma.

MGA271 exhibits potent *in vivo* efficacy against renal cell carcinoma xenografts

To evaluate the antitumor activity of MGA271 *in vivo*, xenograft studies were carried out in mice that have the murine *CD16* gene knocked out and are transgenic for human CD16A-158F, the low-affinity binding allele of this FcγR (mCD16$^{-/-}$ hCD16A^{+}). This model was developed to overcome the discrepancy in binding of the MGFc0264 Fc domain to murine CD16, compared with the human CD16A counterpart (13), and provides the greatest sensitivity for evaluating the potential enhanced Fc-dependent activity mediated by mAbs with engineered Fc domains. The pharmacokinetics of MGA271 was evaluated at a dose of 5 mg/kg in mCD16$^{-/-}$ hCD16A FOXN1 nu/nu mice. The estimated half-life of MGA271 was 249 hours with a C_{max} of 43 μg/mL, supporting once weekly administration of MGA271 in xenograft efficacy models.

Treatment with MGA271 once weekly for 5 weeks at doses ranging from 0.1 to 10 mg/kg, initiated 7 days following tumor cell implantation, resulted in sustained inhibition of the growth of A498 tumor xenografts over the course of the 50-day study (Fig. 5A). A cytostatic response was achieved over the course of the study at doses of 0.5 mg/kg or greater, with a near cytostatic response achieved at the 0.1 mg/kg dose.

Efficacy studies were also carried out against 786-0 renal cell carcinoma and HT-1197 bladder carcinoma tumor xenografts. Treatment with MGA271 once weekly for 5 weeks at doses of 1, 2.5, and 5 mg/kg initiated 7 days following tumor cell implantation resulted in sustained inhibition of the growth of 786-0 tumor xenografts over the course of the study (Fig. 5B). Tumor growth inhibition was also observed with RES240 in this study; however, growth inhibition by MGA271 was significantly greater than that observed with the RES240, indicating that the MGFc0264 Fc mediated enhanced antitumor activity *in vivo*. The tumor growth inhibition observed was Fc mediated, as treatment with RES240-aglycosyl did not inhibit tumor growth (Fig. 5C). Potent antitumor activity was also observed against HT-1197 tumor xenografts. Treatment with MGA271 once weekly for 5 weeks at doses of 1, 5, and 10 mg/kg, initiated 7 days following tumor implantation, led to sustained inhibition of the growth of HT-1197 tumor xenografts over the course of the study (Fig. 5D). The antitumor activity of MGA271 was also evaluated in a late-treatment mode, wherein the 786-0 tumor xenografts were allowed to grow to an average volume of approximately 260 mm^3 before treatment. As shown in Fig. 5E, once weekly treatment with MGA271 at doses of 1 mg/kg or greater resulted in a significant inhibition of tumor growth. At 5 or 10 mg/kg, a cytostatic response was achieved until day 52, after which the average tumor volume of the 5 mg/kg treatment group remained near predose administration levels, whereas the 10 mg/kg group exhibited a nonsignificant trend toward relapse.

Toxicokinetic and toxicology assessment in cynomolgus monkeys

The toxicokinetic and toxicologic profile of MGA271 was assessed in cynomolgus monkeys following a single

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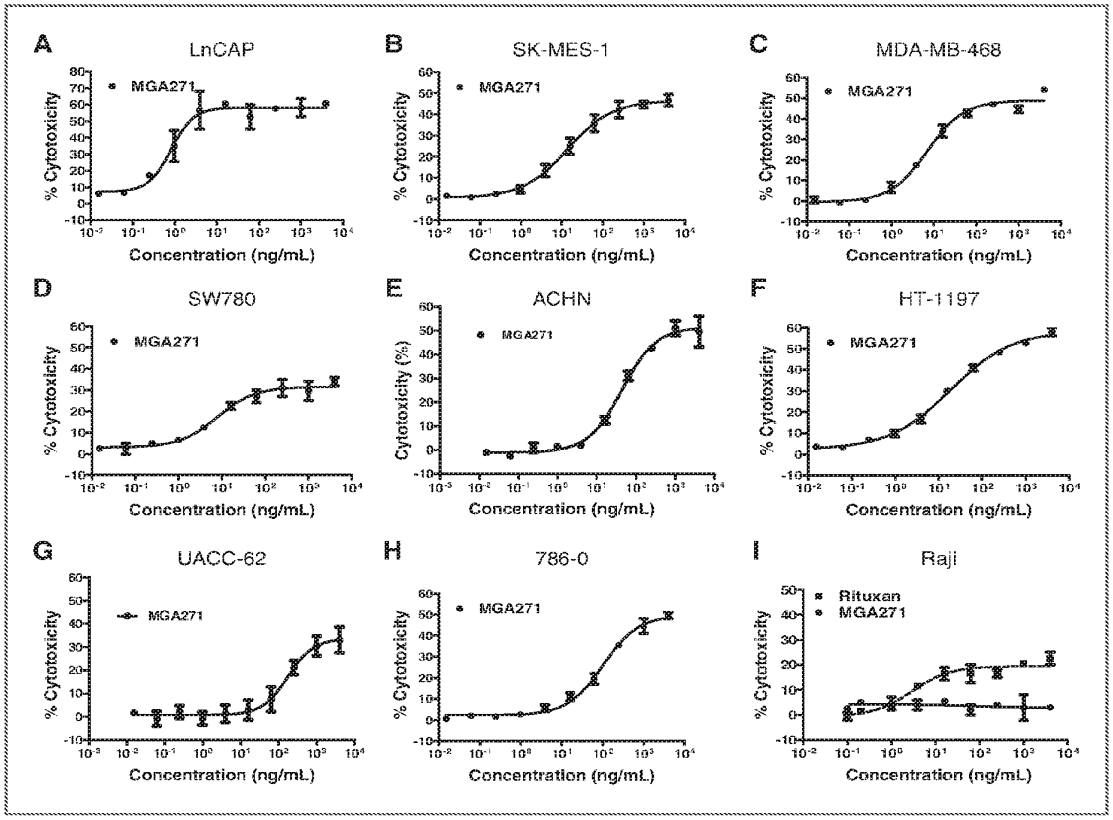


Figure 4. MGA271 mediates *in vitro* ADCC across a panel of ATCC cancer cell lines exhibiting a range of B7-H3 expression. The ability of MGA271 to mediate ADCC was evaluated on B7-H3–positive cancer cell lines with resting PBMCs. A, LnCAP prostate adenocarcinoma; B, SK-MES-1 lung carcinoma; C, MDA-MB-468 breast adenocarcinoma; D, SW780 colon adenocarcinoma; E, ACHN renal cell carcinoma; F, HT-1197 bladder carcinoma; G, UACC-62 melanoma; H, 786-0 renal cell carcinoma; I, B7-H3–negative Raji B cell lymphoma.

administration and 4 once weekly administrations of MGA271. Cynomolgus monkeys exhibit MGA271 reactivity comparable with that observed with human tissues. In addition, cynomolgus monkey FcγRs bind to the optimized Fc domain of MGA271 (13), and as previously shown (Fig. 3D), cynomolgus monkey PBMCs were capable of mediating MGA271-dependent ADCC of a magnitude similar to that mediated by human PBMCs. No significant adverse MGA271-mediated changes were observed following a single administration and 4 once weekly administrations of MGA271 at dose levels up to 150 mg/kg, the highest dose examined. Minor transient MGA271-related elevations in the serum level of interleukin 5 (IL-5), IL-6, and TNF-α were observed following administration of MGA271 (Supplementary Fig. S3), which were not accompanied by clinical symptoms. A time- and dose-dependent recoverable reduction in circulating natural killer cells was observed following administration of MGA271 (Supplementary Fig. S4) that, owing to the transient nature of the response, was not considered adverse. On the basis of these results, the no-observed-adverse-effect level (NOAEL) in cynomolgus monkeys for MGA271 was established at 150 mg/kg. The mean terminal half-life ranged from 8 to 12 days.

Discussion

We previously outlined an approach to generate mAbs directed against cell-surface antigens overexpressed in cancer, based on whole-cell immunization and selection for favorable tumor:normal tissue expression (8). These mAbs recognize a spectrum of potential targets with some linked to a functional role in tumor formation and/or progression (e.g., adhesion molecules, growth factor receptors, receptor tyrosine kinases, metabolic targets, and metalloproteases) and others with an undiscovered role in tumorigenesis. The selection of highly tumor-specific targets enables the latter

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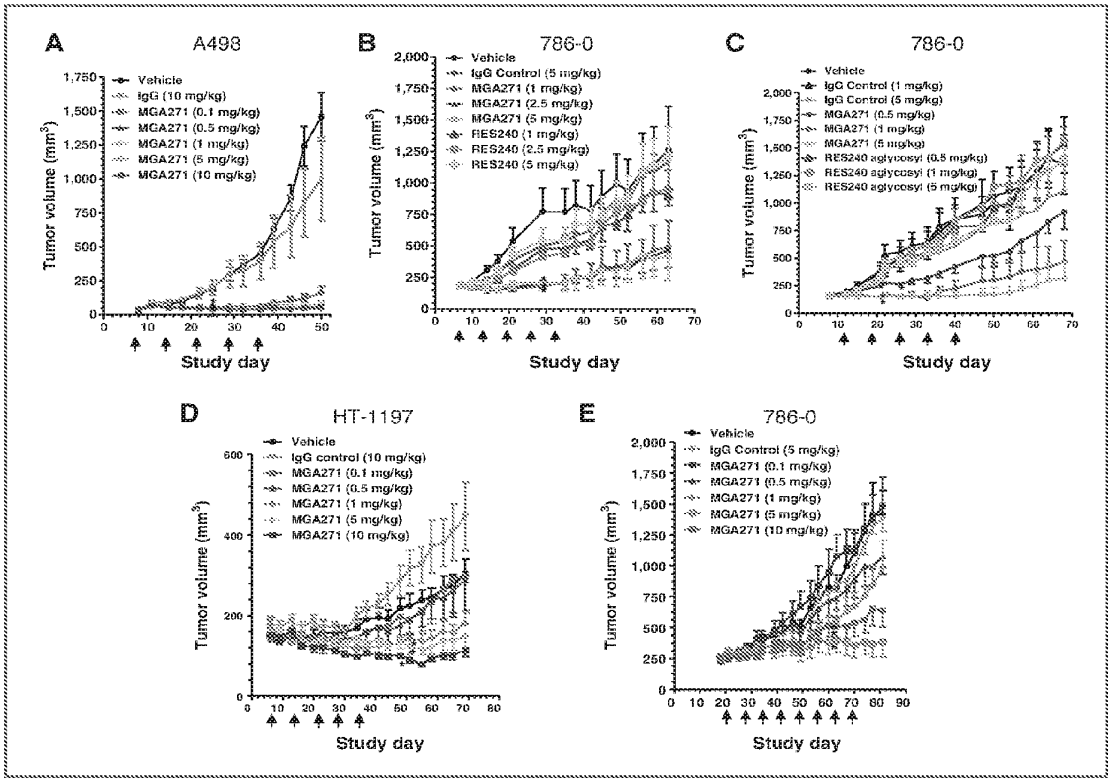


Figure 8. MGA271 exhibits potent *in vivo* antitumor activity toward tumor cell carcinoma xenografts. Xenografts were established by implanting A498 renal cell carcinoma, 786-0 renal cell carcinoma, or HT-1197 bladder carcinoma cells subcutaneously into the mCD16$^{-/-}$ hCD16A^{+} mice. MGA271, the indicated control mAbs, or PBS vehicle were administered by intravenous injection once weekly (arrows) at the indicated dose levels beginning 6 to 12 days (early treatment mode; A–D) or 21 days (late treatment mode, average tumor volume 260 mm^3; E) following tumor cell implantation. Asterisks indicate time point at which the tumor volume for the treatment group reached statistical significance ($P < 0.05$) compared with IgG treatment group.

category of antigens to be candidates for Fc-optimization to enhance the ability of the mAb to bind to activating Fcγ receptors and mediate enhanced Fc-dependent antitumor activities.

In this article, we have described identification and characterization of a panel of mAbs with specificity for B7-H3, a protein overexpressed on many cancers, including prostate (15–17), renal (18), pancreatic (19, 20), colorectal (21), non–small cell lung (NSCLC; ref. 22), ovarian (23), bladder (24), melanoma (25), and neuroectodermal (25) cancers and postulated to mediate immunomodulatory activity (26). Our immunohistochemical analysis confirmed and extended published data indicating B7-H3 is highly expressed across a variety of solid cancers. We observed high levels of B7-H3 expression in kidney, prostate, pancreatic, breast, gastric, and ovarian cancer, as well as melanoma, but limited expression in normal human tissues. B7-H3 expression was observed in the epithelial compartment of the tumor as well as the tumor vasculature. It is worth noting that genetic expression screens would not have identified B7-H3 as a tumor-specific target. B7-H3 mRNA has been reported to be broadly expressed across normal organ and immune tissues (27), whereas B7-H3 protein expression is more limited in normal tissues. Consistent with our study, 2 independent reports describe differential B7-H3 protein expression on tumor tissues compared with normal tissues. A membrane-bound tumor-associated antigen defined by the mAb 376 (28) and subsequently identified as B7-H3 (29) was reported to be expressed on melanoma, glioma, neuroblastoma, sarcoma, and breast cancer cells and tissues, but minimally or undetectably expressed on adult normal tissues (28, 30). Modak and colleagues (25) reported that the 8H9 mAb, whose binding antigen was ultimately determined to be B7-H3 (31), exhibited undetectable binding in most normal tissues and only limited cytoplasmic staining in normal pancreas, stomach, liver, and adrenal cortex.

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Evidence suggests that posttranslational regulation by the miRNA miR-29 may contribute to the observed lack of correspondence between B7-H3 mRNA and protein expression levels (31).

The functional role of B7-H3 remains largely undissected and controversial. Reports suggesting a costimulatory role in immune modulation for B7-H3 (27) have been countered by a preponderance of evidence suggesting a tumor-supportive role in terms of immunologic escape (11, 32–34) and increased resistance to treatment (35). Overexpression of B7-H3 has been correlated with disease severity and poor outcome in a growing number of cancer types, including pathologic indicators of aggressiveness and negative clinical outcome in prostate cancer (15–17), increased grade and decreased T-lymphocyte infiltration in tumor nests and stroma in colorectal cancer (21), and a reduction in T-lymphocyte infiltrates in NSCLC (22). B7-H3 expression on tumor-associated vascular endothelia suggests additional roles for B7-H3 that favor tumor growth and progression, albeit by mechanism(s) not yet uncovered. Consistent with this notion, the presence of B7-H3 on the epithelial or vasculature component of renal cell carcinoma was associated with multiple adverse clinical and pathologic features and an increased risk of death (18). In addition, in ovarian cancer, B7-H3 expression in tumor vasculature was associated with significantly shorter patient survival and higher incidence of disease recurrence (23). Tumor-specific effects of B7-H3 have also been described. Two studies have shown that siRNA knockdown of B7-H3 expression reduced cell adhesion, migration, and invasion of melanoma and breast cancer cells (29) and prostate cancer cells (36) *in vitro*, suggesting B7-H3 may play a role in tumor progression and metastasis. Silencing of B7-H3 expression has also been reported to regulate intracellular signaling of breast cancer cells and modulate chemotherapy resistance (34). The derivation of a B7-H3 mAb from an ovarian cancer line displaying cancer stem cell (CSC) properties (Table 1; ref. 37) also warrants exploration of the expression and potential biologic role for B7-H3 on CSCs and whether B7-H3 can serve as a therapeutic target for CSCs in addition to the broader tumor cell population.

Because the role of B7-H3 in tumorigenesis and immune escape is not fully understood, and given the exquisite tumor-specific expression of the target, the clinical candidate mAb BRCA84D was engineered with an Fc region that imparts increased affinity for the human activating Fcγ receptor CD16A and decreased affinity for the human inhibitory Fcγ receptor CD32B (12, 13). This choice reflects the notion that enhancing the ability of an mAb to mediate Fc-dependent activity may translate to improved clinical efficacy—a concept supported by clinical data that correlate clinical outcome with Fcγ receptor polymorphisms. Specifically, patients homozygous for the higher affinity variant of CD16A or CD32A have more favorable clinical outcomes following treatment with rituximab for follicular lymphoma (4, 5), trastuzumab for metastatic breast cancer (6), or cetuximab for mCRC under certain settings (7). In the latter case, *KRAS* mutations were associated with lower response rates to cetuximab; however the high-affinity polymorphism was associated with more positive outcomes irrespective of KRAS status. Thus, cetuximab mediates a positive clinical response in mutated *KRAS* mCRC under conditions in which the EGFR pathway is constitutively active and insensitive to the direct cytostatic mechanism of the antibody, supporting the importance of the role of Fc-mediated mechanisms in the efficacy of cetuximab.

Consistent with the enhanced binding of MGA271 to CD16A, MGA271 exhibited enhanced ADCC activity against all B7-H3–positive cancer cell lines evaluated when compared with forms of the mAb with a wild-type Fc domain. Although strong correlations between B7-H3 expression and sensitivity to MGA271-mediated ADCC cannot be made based on the tumor cell lines tested in this study, overall the cell lines expressing the greatest level of cell-surface B7-H3 exhibited the greatest sensitivity. The cytotoxic activity observed *in vitro* was reflected in the potent antitumor activity in tumor xenograft models *in vivo*. Significant inhibition of tumor growth and cytostasis with xenografts representing renal cell carcinoma and bladder carcinoma was observed following weekly administration of MGA271. The antitumor activity was dependent on the Fc region of the mAb and was enhanced in comparison with a wild-type Fc version. Significant antitumor activity was also observed against a variety of additional xenografts representing tumors of gastric, lung and colon cancer, and melanoma (data not shown). Consistent with the limited expression of B7-H3 in normal tissues, MGA271 was well tolerated in cynomolgus monkeys, and the NOAEL was determined to be 150 mg/kg.

The focus of our approach to developing MGA271 as an anti–B7-H3 mAb therapy has been on exploiting the favorable tumor/normal expression profile of B7-H3 to provide a therapeutic window for enhanced tumor cell killing through Fc-engineering. However, it is important to note that MGA271 retains the potential to target additional mechanisms of action, including directly modulating tumor cell functions, mediating antitumor vasculature activities, and modulating immune suppressive activity. These areas of potential therapeutic intervention remain to be explored and will be greatly facilitated by identification of the B7-H3 receptor.

In summary, we have developed MGA271, a B7-H3–reactive, Fc-engineered mAb that mediates potent antitumor activity *in vitro* as well as in tumor xenograft studies; these data, together with its favorable safety profile in cynomolgus monkey toxicology studies, support the exploration of MGA271 in the treatment of B7-H3–positive cancers. A phase I/IIa clinical study of MGA271 in patients with B7-H3–positive metastatic or recurrent adenocarcinoma has been initiated.

Disclosure of Potential Conflicts of Interest

D. Loo, R.F. Alderson, F.Z. Chen, L. Huang, S. Gorlatov, S. Burke, V. Ciccarone, H. Li, Y. Yang, T. Son, Y. Chen, A.N. Easton, J.C. Li, J.R. Rillema, M. Licea, C. Fieger, J.P. Mather, S.J. Stewart, S. Johnson, E. Bonvini, and P.A. Moore have ownership interest (including patents) in MacroGenics, Inc. S.

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Koenig has held the title of president and CEO of MacroGenics Inc. The other authors disclosed no potential conflicts of interest.

Authors' Contributions

Conception and design: D. Loo, R.F. Alderson, L. Huang, T.W. Liang, J.P. Mather, S. Koenig, S. Johnson, E. Bonvini, P.A. Moore
Development of methodology: D. Loo, R.F. Alderson, F.Z. Chen, L. Huang, W. Zhang, S. Burke, H. Li, Y. Chen, J.C. Li, T.W. Liang, J.P. Mather
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Loo, R.F. Alderson, W. Zhang, S. Gorlatov, V. Ciccarone, H. Li, Y. Yang, Y. Chen, J.C. Li, J. Rillema, M. Licea, C. Fieger, T.W. Liang, P.A. Moore
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Loo, R.F. Alderson, F.Z. Chen, W. Zhang, Y. Yang, T. Son, J.C. Li, J. Rillema, M. Licea, T.W. Liang, J.P. Mather, S. Koenig, S.J. Stewart, S. Johnson, E. Bonvini, P.A. Moore
Writing, review, and/or revision of the manuscript: D. Loo, R.F. Alderson, F.Z. Chen, L. Huang, V. Ciccarone, A.N. Easton, J.C. Li, T.W. Liang, S. Koenig, S.J. Stewart, S. Johnson, E. Bonvini, P.A. Moore
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Loo, T. Son
Study supervision: D. Loo, R.F. Alderson, L. Huang, W. Zhang, S. Koenig, S. Johnson, E. Bonvini, P.A. Moore

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Review

Expert Opinion

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Vaccines & Antibodies

Tumour-targeted chemotherapy with immunoconjugates of calicheamicin

Nitin K Damle
Oncology Discovery Research, Wyeth Pharmaceuticals, 200/4604, 401 North Middletown Road, Pearl River, NY 10965, USA

Antibody-targeted chemotherapy is a therapeutic strategy in cancer therapy that involves a monoclonal antibody specific for a tumour-associated antigen, covalently linked via a suitable linker to a potent cytotoxic agent. Tumour-targeted delivery of a cytotoxic agent in the form of an immunoconjugate is expected to improve its antitumour activity and safety. Calicheamicin is a cytotoxic natural product isolated from *Micromonospora echinospora* that is at least 1000-fold more potent than conventional cytotoxic chemotherapeutics. Calicheamicin binds DNA in the minor groove and causes double-strand DNA breaks, leading to cell death. Immunoconjugates of calicheamicin targeted against tumour-associated antigens exhibit tumour-specific cytotoxic effects and cause regression of established human tumour xenografts in nude mice. Gemtuzumab ozogamicin is the first clinically validated cytotoxic immunoconjugate in which a humanised anti-CD33 antibody is linked to a derivative of calicheamicin. Gemtuzumab ozogamicin is indicated for the treatment of elderly patients with relapsed acute myeloid leukaemia. A similar conjugate, inotuzumab ozogamicin, is being evaluated at present in Phase I clinical trials in patients with non-Hodgkin's lymphoma. A number of tumour-targeted immunoconjugates of calicheamicin are being explored preclinically at present for their therapeutic applications.

Keywords: calicheamicin, cancer, chemotherapy, monoclonal antibody, tumour-targeted

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1. Introduction

Treatment of metastatic cancer with cytotoxic agents (chemotherapy) is a common therapeutic strategy that has been practised for the last several decades. The cytotoxic activity of existing chemotherapeutic agents is primarily targeted directly to either cellular DNA or microtubules, or to the associated cellular enzymatic machinery. It is not uncommon to use various chemotherapeutic agents in combination in order to maximise their individual therapeutic potential. A vast majority of the chemotherapeutic agents are administered as intravenous infusions. Although such systemic delivery of potent cytotoxic agents can confer transient therapeutic benefit, tumours often develop resistance to the cytotoxic agents, resulting in disease relapse. Moreover, systemic delivery of cytotoxic chemotherapeutic agents cannot discriminate between cancerous tissues and normal tissues, often resulting in multiorgan toxicity. Thus, such agents can only be administered to cancer patients for a limited number of cycles. The inadequate antitumour therapeutic effects and indiscriminate toxicity toward normal tissues are the two major limitations of conventional chemotherapy and have provided impetus to the development of various tumour-targeted therapeutic strategies.

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Antibody-targeted chemotherapy (ATC) is one such strategy that relies on the use of monoclonal antibodies (mAbs) specific for tumour-associated antigens (TAAs) as tumour-targeting agents [1,2]. The use of mAbs in cancer therapy is no longer a novel approach; at least five humanised antibody therapeutics have been licensed and a number of others are in various phases of clinical evaluations and regulatory review. TAA-specific mAbs have also been used in the form of immunotoxins to deliver potent toxins derived from plants or microbes [3], or radioimmunotherapy to deliver radioactive isotopes [4]. Two such radioimmunotherapeutic agents have been licensed for therapeutic use in non-Hodgkin's lymphoma [101,102]. In addition, although not an antibody, a fusion protein between a truncated form of IL-2 and diphtheria toxin has been approved for the treatment of IL-2 receptor-positive cutaneous T cell lymphoma [103].

ATC involves the use of a potent cytotoxic agent that is chemically attached to the TAA-specific mAb via a suitable linker to create a cytotoxic immunoconjugate [1,2]. When introduced in the systemic circulation, the covalent linkage between the cytotoxic agent and the targeting mAb in the conjugate should not readily allow uptake of the cytotoxic agent into the intracellular compartments of normal tissues, thus resulting in reduced toxicity. In contrast, the specificity of the targeting mAb should allow the cytotoxic immunoconjugate to seek out TAA^+ tumour cells in the body and deliver the attached cytotoxic agent to the tumour cells. Therefore, the basic promise of ATC is the focused intratumoural delivery, retention and accumulation of the potent cytotoxic agent into the TAA^+ tumour cells that helps maximise its antitumour efficacy and spare the TAA^- normal tissues of the body from the toxic effects of the same agent, resulting in a significantly improved therapeutic index.

The concept of ATC has been studied extensively in a number of preclinical and clinical explorations. Although various preclinical studies with immunoconjugates of some of the cytotoxic chemotherapeutic agents used at present [1,2] demonstrated promise, no significant clinical benefit was derived from their clinical evaluation until gemtuzumab ozogamicin (Mylotarg®, Wyeth Research), which provided the first clinical validation of the ATC strategy. Gemtuzumab ozogamicin was approved in May 2000 by the US FDA for its therapeutic use in patients with acute myeloid leukaemia (AML). Gemtuzumab ozogamicin is a CD33-targeted immunoconjugate of a derivative of the natural product calicheamicin.

Calicheamicins are a family of potent cytotoxic antibiotics of the enediyne (war-head) class, synthesised by the soil microbe *Micromonospora echinospora* ssp. *calichensis*, which were first discovered in the mid-1980s and, subsequently, structurally elucidated at Lederle Laboratories (now a division of Wyeth Pharmaceuticals) [5-8]. Gamma calicheamicin is the dominant type of calicheamicins and is used in all studies discussed below. Gamma calicheamicin binds DNA in the minor groove and, in a relatively sequence-specific manner, causes cellular thiol-dependent structural rearrangement in its enediyne moiety, resulting in the generation of a diradical that then abstracts hydrogens from the phosphodiester backbone of the DNA, creating double-strand breaks [8-10]. Such double-strand DNA breaks are difficult to repair in cycling cells and often result in apoptosis and cell death. Although gamma calicheamicin may not be the most potent cytotoxic agent, it is at least 1000-fold more potent than conventional cytotoxic chemotherapeutic agents [8,9]. Due to its narrow therapeutic index and late-emerging toxicities, therapeutic applications of gamma calicheamicin have not been pursued. In order to improve its structural stability and therapeutic effect, gamma calicheamicin, a trisulfide, has been chemically modified to create a disulfide, *N*-acetyl gamma calicheamicin dimethyl hydrazide (CalichDMH). CalichDMH is 10- to 50-fold less potent than gamma calicheamicin, but is still 10 – 100 times more potent than conventional chemotherapeutic agents [9-12]. CalichDMH can be successfully conjugated to any mAb of desired specificity to create a tumour-targeted chemotherapeutic agent. The following account reviews progress made so far with antibody-targeted calicheamicin therapeutic agents.

ATC using immunoconjugates of CalichDMH has been studied extensively in preclinical model systems. Tumour-targeted immunoconjugates of CalichDMH have shown strong antitumour activity, both *in vitro* and *in vivo*, against histologically diverse human tumours grown as xenografts in immunocompromised mice [9-15]. Based on the author's own preclinical studies and also those of others, the following generalisations can be made about ATC. The success of the ATC strategy is influenced by a combination of at least five independent components:

- TAA to be targeted
- TAA-targeting antibody
- cytotoxic agent to be targeted
- the linker used to conjugate the cytotoxic agent to the targeting antibody
- sensitivity of the tumour cells to the targeted cytotoxic agent

The following are the ideal features for each of these components:

TAA:

- high levels of homogenous surface expression on tumour cells with no expression on normal cells
- if the TAA is expressed on normal cells, its expression should be confined to lineages whose loss is not life-threatening
- antibody bound to TAA should be internalised for efficient intracellular delivery of the conjugated cytotoxic agent
- TAA should not be present in soluble form in circulation in quantities that may interfere with tumour-targeting of the conjugate

Targeting antibody:

- affinity of the targeting mAb for TAA should be sufficiently high (e.g., K_D: 100 pM – 1 nM); targeting antibodies with

ultrahigh affinity (K_D: < 20 pM) were not able to distribute evenly throughout the tumour mass and were concentrated primarily on the perivascular regions within the tumour [16]
- the targeting mAb should be either humanised or fully human to avoid immunogenicity in humans; chimeric mAbs can be used if there is sufficient evidence of their lack of immunogenicity in humans; other alternatives, such as the use of 'de-immunised' antibodies, can also be considered
- if antibody fragments are used, such as Fab', $F(ab')_2$, scFv, diabody or minibody, they should be imparted sufficient hydrodynamic volume to prevent their rapid clearance via filtration through the kidneys [17]

Cytotoxic agent:

- cytotoxic agent to be targeted should be more potent than conventional chemotherapeutic agents being used in a non-targeted manner at present; antibody targeting may allow a limited number of molecules to be delivered to the tumour and, thus, the antibody-mediated tumour-targeted delivery of the conjugated cytotoxic drugs with greater potency may generate greater antitumour activity than that of non-targeted conventional cytotoxics
- cytotoxic agent should be able to reach its intended intracellular molecular target (predominantly nuclear DNA or cytoplasmic microtubules)
- cytotoxic agent is preferably not a substrate for drug efflux pumps that may influence its intracellular accumulation and targeting
- cytotoxic agent should not profoundly alter pharmacokinetic and functional properties of the targeting antibody

Linker:

- the linker used to conjugate the cytotoxic agent to the targeting mAb should be stable in the extracellular compartment of the peripheral blood
- the linkage between the cytotoxic agent and the targeting mAb should be conditionally stable, allowing the release of the drug in the intracellular compartments, such as lysosomes, to which the internalised vesicles often traffic

The following account focuses on three distinct immunoconjugates of calicheamicin that have been advanced to their clinical evaluation.

2. Gemtuzumab ozogamicin

Gemtuzumab ozogamicin, also known as Mylotarg or CMA-676, is the first cytotoxic immunoconjugate to be approved by the US FDA for clinical use [18,19]. It is an immunoconjugate of calicheamicin in which a humanised IgG4 anti-CD33 antibody, hP67.6, is conjugated to CalichDMH via a hybrid 4-(4′-acetylphenoxy)butanoic acid (AcBut) linker. The linkage between calicheamicin and the targeting antibody involves an acid-labile hydrazone functionality that facilitates rapid release of CalichDMH from its conjugated state under the acidic conditions such as those in the lysosomal vesicles. The target of gemtuzumab ozogamicin is CD33, a myeloid lineage-specific molecule. CD33 (also known as Siglec-3) is a 67 kDa adhesion molecule, a member of the Siglec family of sialic acid-binding lectins and also a member of Ig superfamily, which binds specifically α2,3-linked sialyl residues displayed on various glycoproteins. It is expressed on the surface of cells of all maturational stages of the myeloid lineage, but not on $CD34^+$ hematopoietic stem cells [20]. Its increased expression on malignant cells of myeloid lineages has made it an attractive target for the treatment of myeloid leukaemias. Gemtuzumab ozogamicin binds CD33 with high affinity and is rapidly internalised into lysosomal vesicles wherein the acidic pH facilitates the hydrolysis of the hydrazone functionality, releasing calicheamicin, which attacks cellular DNA, leading to cell death [11,12]. Gemtuzumab ozogamicin was selectively cytotoxic *in vitro* against $CD33^+$ leukaemic cells relative to those lacking the expression of CD33, and also caused regression of $CD33^+$ HL-60 xenografts in nude mice [11,12].

Gemtuzumab ozogamicin is indicated for the treatment of patients with $CD33^+$ AML in first relapse who are ≥ 60 years old and who are not considered candidates for other cytotoxic chemotherapy [18,19,21-24]. The clinically recommended dose of gemtuzumab ozogamicin is 9 mg/m^2 administered as a 2-h intravenous infusion for a total of two infusions administered two weeks apart. The efficacy and safety of gemtuzumab ozogamicin as a single agent have been evaluated in 277 patients in three single-arm, open-label studies in patients with $CD33^+$ AML in first relapse. These studies included 84, 95 and 98 patients, respectively. In the first two studies, patients were ≥ 18 years of age with a first remission duration of ≥ 6 months. In the third study, only patients ≥ 60 were enrolled and their first remission had to have lasted for ≥ 3 months. Patients with secondary leukaemia or white blood cell (WBC) counts ≥ 30,000/μl were excluded. Some patients were leukoreduced with hydroxyurea or leukapheresis to lower WBC counts below 30,000/μl in order to minimise the risk of tumour lysis syndrome. The treatment course included two doses of gemtuzumab ozogamicin at 9 mg/m^2 separated by 14 days and a 28-day follow-up after the last dose. Although smaller doses had elicited responses in earlier studies, the 9 mg/m^2 was chosen because it would be expected to saturate all CD33 sites, regardless of leukaemic burden. A total of 157 patients were ≥ 60 old. The primary end point of the three clinical studies was the rate of complete remission (CR), which was defined as:

- leukaemic blasts absent from the peripheral blood
- ≤ 5% blasts in the bone marrow, as measured by morphology studies
- haemoglobin ≥ 9 g/dl, platelets ≥ 100,000/μl, absolute neutrophil count ≥ 1500/μl
- red cell and platelet-transfusion independence (no red cell transfusions for 2 weeks; no platelet transfusions for 1 week)

In addition to CR, a second response category, CRp, was defined as patients satisfying the definition of CR, including platelet transfusion independence, with the exception of platelet recovery of ≥ 100,000/μl. This category was added because the treatment with gemtuzumab ozogamicin appears to delay platelet recovery in some patients. Clinical equivalence between CR and CRp responses has not yet been established. All patients were premedicated with acetaminophen 650 – 1000 mg and diphenhydramine 50 mg to decrease acute infusion-related symptoms. Growth factors and cytokines were not permitted. Use of prophylactic antibiotics was not specified. The median relapse-free survival of the 42 patients who attained remission was 6.8 months. The overall remission rate of gemtuzumab ozogamicin is not greatly dissimilar in the elderly (26% in ≥ 60 years of age) and younger (34% in < 60 years of age) patients with AML in first relapse. For elderly patients with AML in first relapse who are not candidates for other cytotoxic chemotherapy, gemtuzumab ozogamicin remains a meaningful therapeutic option. Studies conducted postregistration of gemtuzumab ozogamicin suggest that the overall response rate observed in Phase II clinical trials of gemtuzumab ozogamicin has been maintained in the postapproval phase [23,24].

Although gemtuzumab ozogamicin caused complete remission in ~ 26 – 30% of the patients with relapsed AML, the remaining 70% of the treated patients demonstrated a variable response in the Phase II trials [18,19,21-24]. The inability of gemtuzumab ozogamicin to clear marrow blasts and induce remission in these patients correlated with the surface expression of MDR1/Pgp-1 and MRP1 and increased dye efflux activity by leukaemic blasts, but not the surface expression of CD33 or its internalisation [25-28]. Calicheamicin is a substrate for the MDR1/Pgp-1 pump and the expression of molecules responsible for exhibiting multi-drug resistance on the surface of leukaemic blasts is consistent with the clinically observed resistance to gemtuzumab ozogamicin therapy. Even though there is a correlation between the clinical resistance of myeloid leukaemic blasts to gemtuzumab ozogamicin and their expression of drug efflux pumps, there may be additional, yet unclearly defined mechanisms underlying the clinically observed resistance to gemtuzumab ozogamicin. In some *in vitro* studies, the observed resistance to gemtuzumab ozogamicin was partially reduced by multi-drug resistance-reducing agents, such as cyclosporin A, PSC833 and MS209 [27,28]. Whether such increased sensitivity to conjugated calicheamicin can be translated into clinical advantage remains to be determined.

The safety profile of gemtuzumab ozogamicin observed in the three Phase II studies in relapsed AML patients was distinct from that of standard induction chemotherapy. The treatment with gemtuzumab ozogamicin was generally well-tolerated. Infusion-related adverse events reported for the 142 patients in the Phase II studies included chills, fever and hypotension. These infusion-related symptoms occurred in most patients receiving gemtuzumab ozogamicin, despite prophylactic treatment with antihistamines and acetaminophen. The incidence of infusion-related events was less common during the second infusion of gemtuzumab ozogamicin. In addition, National Cancer Institute (NCI) grade 3 or 4 haematological toxicity with neutropenia and thrombocytopenia was commonly observed with gemtuzumab ozogamicin, which is consistent with the myeloid lineage-targeted cytotoxicity of gemtuzumab ozogamicin [18,19]. Various other toxicities that are often seen with traditional combination chemotherapy, such as alopecia, mucositis, cardiotoxicity or cerebellar toxicity, were absent in patients treated with gemtuzumab ozogamicin. Approximately a quarter of the patients treated with gemtuzumab ozogamicin exhibited NCI grade 3 or 4 elevation of serum bilirubin and transaminases, indicative of liver toxicity that was transient and reversible [18,19]. The clinically observed haematological and liver toxicities of gemtuzumab ozogamicin are consistent with those observed during preclinical safety evaluation in rats and cynomolgus monkeys (Wyeth Research, unpublished). None of the patients in the Phase II trials exhibited an antibody response to the humanised antibody or the calicheamicin-linker complex.

The most striking manifestation of the hepatic toxicity of gemtuzumab ozogamicin is the development in ≤ 10% of the treated patients of often fatal hepatic veno-occlusive disease (hVOD), also known as hepatic sinusoidal obstruction syndrome, characterised by hepatomegaly, hepatic portal hypertension, hepatic necrosis, subendothelial oedema, sinusoidal fibrosis and, occasionally, fibrous venular occlusion [29-32]. The hVOD was more pronounced in those patients who received hematopoietic stem cell transplant after the gemtuzumab ozogamicin therapy. The incidence of hVOD in patients not receiving hematopoietic stem cell transplant was lower than that of patients receiving hematopoietic stem cell transplant after gemtuzumab ozogamicin therapy. The development of hVOD is not uncommon in patients receiving hematopoietic stem cell transplant after standard combination chemotherapy [33,34]. Both the preclinical and clinical studies conducted with gemtuzumab ozogamicin indicate its potential for causing hepatic toxicity characterised by hepatic necrosis, increased serum transaminases and bilirubin. Although reversible hepatic necrosis was seen in rats and cynomolgus monkeys during preclinical safety evaluation of gemtuzumab ozogamicin, hVOD was never seen in these studies (Wyeth Research, unpublished). Whether or not the observed hVOD in gemtuzumab ozogamicin-treated patients is a CD33-targeted event has not yet been established [31]. The incidence of hVOD was not correlated with the remission induction in the AML patients treated with gemtuzumab ozogamicin. It has been speculated that gemtuzumab ozogamicin triggers a series of cellular events that leads to sinusoidal obstruction, rather than directly causing hepatic necrosis [31]. Irrespective of the molecular mechanism(s) underlying the development of gemtuzumab ozogamicin-associated hVOD, exposure to gemtuzumab ozogamicin prior to myeloablative allogeneic stem cell transplant

has been identified to increase the risk of developing hVOD in this AML patient population [35,36].

More recently, gemtuzumab ozogamicin has been evaluated in combination with cytarabine and daunorubicin in the treatment of untreated AML patients with promising results. This combination therapy was well-tolerated in Phase II clinical studies, with lower incidence of hematopoietic and hepatotoxicity and greatly improved remission induction (> 80%) [35-38]. More importantly, there was a low incidence of hVOD in these Phase II studies. The ability of gemtuzumab ozogamicin to cause remission has also been documented in patients with acute promyelocytic leukaemia [39,40]. At present, additional studies are evaluating gemtuzumab ozogamicin in combination with, or as an alternative to, other chemotherapeutic agents used in AML [41,42]. Despite its potential for treatment-related toxicity, gemtuzumab ozogamicin still remains a meaningful option for older patients with relapsed AML. In recognition of this feature, gemtuzumab ozogamicin has been added to the National Comprehensive Cancer Network practice guidelines to oncologists regarding the treatment of AML. Ongoing and future studies with gemtuzumab ozogamicin, in combination with conventional chemotherapy in *de novo* first-line patients with AML, will indicate how widely it could be used in the treatment of this and other malignancies of the myeloid lineage.

3. CMB-401: a MUC1-targeted calicheamicin therapeutic

The first opportunity to evaluate antitumour activity of conjugated calicheamicin in solid tumours was presented with CMB-401. This conjugate targets the MUC1 molecule (polyepithelial mucin or CD227), which is aberrantly glycosylated and overexpressed on the surface of various epithelial malignancies including breast, cervical, lung, oesophageal, gastric, ovarian, pancreatic and renal carcinomas [43]. The targeting antibody used in CMB-401 is a humanised IgG4 antibody (hCTMO1) that recognises the peptide core epitope of MUC1 and is rapidly internalised when bound to the cell-surface MUC1 on the surface of MUC1$^+$ carcinoma cells [44-46]. This antibody targeted to human MUC1 does not crossreact with MUC1 homologues in experimental animals, including rodents and primates. Imaging studies conducted in cancer patients with radiolabelled hCTMO1 indicated the superiority of hCTMO1 over other clinically tested anti-MUC1 antibodies in its tumour uptake and accumulation [44-46]. CMB-401 is comprised of hCTMO1 covalently linked to *N*-acetyl gamma calicheamicin dimethyl acid via the acid-stable 'Amide' linker. One important feature in which CMB-401 differs from gemtuzumab ozogamicin is the absence of acid-labile hydrazone functionality in the linkage between the targeting antibody and the drug in CMB-401. Thus, conjugates such as CMB-401 are believed to require much more extensive intracellular processing in order to release the drug from its conjugated state. As a result, in order to be effective, CMB-401 requires internalisation by the target cells.

In preclinical tumour models, CMB-401 exhibited strong antitumour activity against MUC1$^+$ breast, lung, epidermoid and ovarian carcinoma cell lines and caused regression of established MUC1$^+$ breast, lung and epidermoid carcinoma xenografts in nude mice (Hamann *et al.*, manuscript in preparation). Preclinical safety profile of CMB-401 was similar to that of gemtuzumab ozogamicin. Strong antitumour activity of CMB-401 against established carcinoma xenografts supported its evaluation in cancer patients with MUC1$^+$ carcinomas.

CMB-401 was initially evaluated in a two-centre Phase I trial in 32 patients with progressive epithelial ovarian carcinoma not amenable to platinum-based standard therapy [46]. These patients, who had previously received an average of 3.2 previous chemotherapeutic regimens, received up to four cycles of dual infusion of 35 mg/m^2 of unconjugated anti-MUC1 mAb (hCTMO1) as a predose, followed by doses of CMB-401 starting from 2 mg/m^2 and progressing up to 16 mg/m^2. The predosing with unconjugated hCTMO1 prior to the administration of CMB-401 was deemed essential to saturate the MUC1 sites in normal tissues and soluble MUC1 antigen in circulation [45-47] in order to enhance the delivery of CMB-401 to the tumour cells. CMB-401 was generally well-tolerated. However, transient fever, emesis and dose-dependent increase in malaise in patients occurred, necessitating routine prophylaxis. World Health Organization grade 3 – 4 toxicities, irrespective of causality, included predominantly nausea/vomiting (76%) and anaemia (21%), and in < 10% of the patients, granulocytopenia, thrombocytopenia, liver transaminases, sepsis and haemorrhage. The maximum tolerated dose was reached at 16 mg/m^2. During this study, 12% of the patients had > 50% reduction in circulating CA125 antigen, and 9% of these patients had radiological evidence of reduction in tumour bulk. Overall, CMB-401 seemed to have an acceptable safety profile.

Based on its acceptable safety profile, CMB-401 was evaluated in a Phase II clinical trial as monotherapy for the treatment of 21 patients with recurrent platinum-sensitive epithelial ovarian carcinoma [48]. Tumour response and serum CA125 levels were assessed before and after active treatment. After an initial predosing with unconjugated hCTM01, CMB-40 was administered at 16 mg/m^2 over 60 min for up to seven cycles, with 4 weeks between cycles. Nineteen patients were evaluable. Measurable changes observed following administration of CMB-401 did not meet the criteria for partial remission (PR). CMB-401 was not effective as monotherapy for patients with epithelial ovarian carcinoma. Adverse events experienced by patients in this study included nausea (95%), asthenia (90%), abdominal pain (62%), headache (57%), anorexia (57%) and diarrhoea (57%), mostly at a toxicity grade level of 1 or 2. Due to the lack of clinically acceptable efficacy of CMB-401, further development of CMB-401 was discontinued.

Although the reasons underlying the lack of clinical efficacy of CMB-401 remain unexplored, the lack of therapeutically meaningful tumour-targeting of the conjugate, in part due to the high levels of circulating soluble MUC1 in patients and the presence of acid-stable linker in CMB-401, may have contributed to the clinically observed lack of its antitumour efficacy. Whether or not replacing the acid-stable linker in CMB-401 with the acid-labile AcBut linker would have provided better antitumour activity in patients remains unexplored. However, the high levels of circulating MUC1 antigen in the appropriate patient population may neutralise any advantage conferred by this acid-labile linker.

4. CMC-544: a CD22-targeted calicheamicin therapeutic

Antibody-targeted calicheamicin therapy has been applied further to B lymphoid malignancies using CD22 as a molecular target [13,14]. CD22, a member of Ig superfamily, is a sialic acid-binding lectin (Siglec-2), which binds α2,6-linked sialic acid residues, is a marker of mature B lymphoid lineage and is expressed preferentially on both normal and malignant B cells [49-51]. Antibodies bound to CD22 are internalised rapidly and, thus, CD22 can be used for targeted delivery of cytotoxic agents in order to obtain a therapeutic advantage. CMC-544 (inotuzumab ozogamicin, Wyeth Research) is a CD22-targeted calicheamicin therapeutic agent in which calicheamicin is conjugated to a humanised IgG4 anti-CD22 mAb, g5/44, via the AcBut linker containing an acid-labile hydrazone functional group [13,14]. In this regard, CMC-544 is similar to gemtuzumab ozogamicin (CMA-676), except that these two conjugates recognise distinct molecular targets. CMC-544 binds human CD22 with subnanomolar affinity and conjugation to calicheamicin does not significantly affect its binding to CD22 [13,14]. The CD22-mediated targeted intracellular delivery of calicheamicin brings about cellular arrest in G2/M phase, followed by cell death.

CMC-544 has been studied extensively in preclinical models of human B cell lymphoma. It exerted a potent and selective cytotoxic effect against $CD22^+$ cells and, in various models of human B cell lymphomas, caused dose-dependent regression of both small and large established B lymphoma xenografts, producing cures with therapeutic indices (maximum tolerated non-lethal dose/minimum efficacious dose) of > 10 [13,14]. Cures are defined by tumour-free survival for ≥ 100 days after the initiation of therapy of established xenografts. CMC-544 also caused long-term survival of severe combined immunodeficient mice with systemically disseminated human B lymphoma [52]. The targeting mAb, G544, had no effect on the growth of B lymphomas in nude mice over a wide range of doses, consistent with its effector function-deficient IgG4 isotype. The preclinical antitumour efficacy observed with CMC-544 strongly supports its evaluation as a targeted chemotherapeutic in patients with B cell malignancies. Preclinical safety of CMC-544 has been evaluated in rats and cynomolgus monkeys. CD22 from neither of these species is recognised by g5/44, the targeting agent in CMC-544. Therefore, the observed safety features of CMC-544 are reflective of antigen-nonspecific effects of immunoconjugates of calicheamicin. In this regard, preclinical profiles of both gemtuzumab ozogamicin and CMC-544 are similar. CMC-544 is being evaluated at present in Phase I clinical trials in patients with non-Hodgkin's B lymphoma. Therapeutic applications of CMC-544 in other malignancies of B lymphoid lineage are also envisaged. CMC-544 may prove to be an effective chemotherapeutic option for the treatment of B lymphoid malignancies.

5. Expert opinion and conclusions

Antibody-targeted chemotherapy using immunoconjugates of calicheamicin can be an effective therapeutic strategy for human cancers. Calicheamicin immunoconjugates targeted to antigens overexpressed on cancer cells represent an important therapeutic advancement whose focused application may result in the availability of meaningful therapeutic options in the clinical management of malignant diseases.

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Affiliation

Nitin K Damle PhD
Oncology Discovery Research, Wyeth Pharmaceuticals, 200/4604, 401 North Middletown Road, Pearl River, NY 10965, USA
Tel: +1 845 602 3984; Fax: +1 845 602 5557;
E-mail: damlen@wyeth.com

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Abstract

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DX-8951f: summary of phase I clinical trials.

De Jager R[1], Cheverton P, Tamanoi K, Coyle J, Ducharme M, Sakamoto N, Satomi M, Suzuki M; DX-8931f Investigators.

Author information

Abstract

Exatecan mesylate (**DX-8951f**) is a new hexacyclic camptothecin analogue with favorable attributes compared to topotecan and CPT-11, including watersolubility, greater potency against topoisomerase I, lack of esterase-dependent activation, broad antitumor activity, and low cross-resistance against MDR-1 overexpressing tumors. In preclinical studies, the compound demonstrated a favorable toxicology profile with hematologic dose-limiting toxicity and moderate gastrointestinal toxicity, linear pharmacokinetics, P450 hepatic metabolism (CYP3A4 and CYP1A2), and predominately fecal excretion. The results of six U.S. and European **phase I clinical trials** as well as two Japanese studies are presented including total DX-8951 and lactone DX-8951 pharmacokinetics. The toxicity profile was similar for all schedules of administration. Hematologic toxicity was dose-dependent and reversible. Neutropenia was dose-limiting in minimally pretreated patients, whereas neutropenia and thrombocytopenia were dose-limiting in heavily pretreated patients. Non-hematologic toxicity included moderate gastrointestinal toxicity (nausea, vomiting > diarrhea), transient elevation of hepatic transaminases, asthenia, and alopecia. Two cases of acute pancreatitis not predicted by preclinical toxicology were also observed. Antineoplastic activity was detected in several solid tumor types: non-small cell lung cancer, extrapulmonary small cell cancer, colorectal cancer, hepatocellular cancer, and sarcoma. Antitumor activity was seen in CPT-11 and topotecan-resistant tumors. Pharmacokinetics were linear within the dose range tested. A pharmacokinetic/pharmacodynamic model predictive of **DX-8951f**-induced neutropenia in individual patients was developed. The daily x5, every 3-week schedule with the drug administered as a 30-minute intravenous infusion was selected for future **phase II clinical trials** based on its superior antitumor activity.

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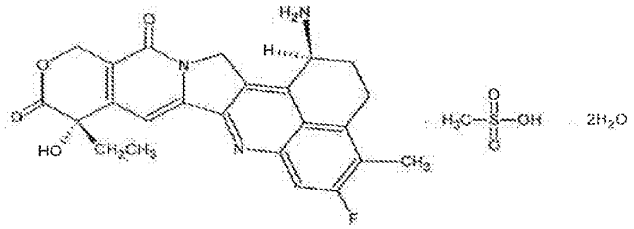
R. DE JAGER, P. CHEVERTON, K. TAMANOI, J. COYLE, M. DUCHARME, N. SAKAMOTO, M. SATOMI, M. SUZUKI, AND THE DX-8951f INVESTIGATORS

Daiichi Pharmaceutical Corporation (USA); Daiichi Pharmaceutical Limited (UK); Daiichi Pharmaceutical Co., Ltd. (Japan); Phoenix International Life Sciences Inc. (Canada)

ABSTRACT: Exatecan mesylate (DX-8951f) is a new hexacyclic camptothecin analogue with favorable attributes compared to topotecan and CPT-11, including watersolubility, greater potency against topoisomerase I, lack of esterase-dependent activation, broad antitumor activity, and low cross-resistance against MDR-1 overexpressing tumors. In preclinical studies, the compound demonstrated a favorable toxicology profile with hematologic dose-limiting toxicity and moderate gastrointestinal toxicity, linear pharmacokinetics, P450 hepatic metabolism (CYP3A4 and CYP1A2), and predominately fecal excretion. The results of six U.S. and European phase I clinical trials as well as two Japanese studies are presented including total DX-8951 and lactone DX-8951 pharmacokinetics. The toxicity profile was similar for all schedules of administration. Hematologic toxicity was dose-dependent and reversible. Neutropenia was dose-limiting in minimally pretreated patients, whereas neutropenia and thrombocytopenia were dose-limiting in heavily pretreated patients. Non-hematologic toxicity included moderate gastrointestinal toxicity (nausea, vomiting>diarrhea), transient elevation of hepatic transaminases, asthenia, and alopecia. Two cases of acute pancreatitis not predicted by preclinical toxicology were also observed. Antineoplastic activity was detected in several solid tumor types: non-small cell lung cancer, extrapulmonary small cell cancer, colorectal cancer, hepatocellular cancer, and sarcoma. Antitumor activity was seen in CPT-11 and topotecan-resistant tumors. Pharmacokinetics were linear within the dose range tested. A pharmacokinetic/pharmacodynamic model predictive of DX-8951f–induced neutropenia in individual patients was developed. The daily ×5, every 3-week schedule with the drug administered as a 30-minute intravenous infusion was selected for future phase II clinical trials based on its superior antitumor activity.

INTRODUCTION

Exatecan mesylate (DX-8951f, Daiichi Pharmaceutical Co., Ltd., Japan) was synthesized to impart greater antitumor efficacy and less toxicity than those of existing camptothecin analogues such as CPT-11 and topotecan. DX-8951f: (1,5,9,5)-1-amino-9-ethyl-5-fluoro-1,2,3,9,12,15-hexahydro-9-hydroxy-4-methyl-10H-benzo(de) pyrano (3,4: 6,7) indolizino (1,2-b) quinoline-10,13-dione monomethanesulfonate (salt), dihydrate is a water-soluble, fluorinated, hexacyclic camptothecin derivative that, unlike CPT-11, does not require metabolic activation. The anhydrous



DX-8951f: Monomethanesulfonate dihydrate
DX-8951: Anhydrous free base

Molecular Weight: 567.59 (DX-8951f, monomethanesulfonate dihydrate)
435.45 (DX-8951, anhydrous free base

FIGURE 1. Chemical structure of DX-8951f.

free-base form of the drug is referred to as DX-8951. The chemical structure is shown in FIGURE 1. Similar to other camptothecin derivatives, the lactone form of DX-8951 is hydrolyzed into an open-ring hydroxy-acid form. The two species coexist in solution according to a reversible pH-dependent equilibrium. DX-8951f was approximately 3 times more potent than SN-38, the active metabolite of CPT-11, 10 times more potent than topotecan, and 20 times more potent than camptothecin as an inhibitor of topoisomerase I activity *in vitro*, and 5 times more potent than SN-38 as an inhibitor of DNA synthesis. The inhibition of human topoisomerase I by the lactone form was about 300-fold more potent than that by the hydroxy-acid form.

In cell-based cytotoxicity assays, DX-8951f was 7–30 times more active than SN-38 or topotecan against a wide range of breast, lung, gastrointestinal, prostate, brain, and pediatric tumor cell lines. DX-8951f was also effective in inhibiting the growth of clonogenic cells from human head and neck, liver, non-small cell lung, breast, colon, and ovarian and prostate tumors *in vitro*. DX-8951f has shown activity *in vivo* against a wide range of human tumor xenografts in nude mice, including gastric, pancreatic, colon, breast, ovary, and lung tumors.[1–5] In addition, DX-8951f exhibited potent antitumor activity in an intracranial xenograft of human RH 30 rhabdomyosarcoma, murine lung and liver metastasis models, and early and late disease models of human acute myelogenous leukemia.[6,7] Several *in vitro* and *in vivo* experiments using human tumor cell lines and drug-resistant variants have demonstrated that DX8951f, unlike SN-38 or topotecan, is not affected by mechanisms of drug resistance based on the overexpression of P glycoprotein or the decrease in levels of topoisomerase I mRNA or protein.[8] DX-8951f is not a substrate for the P glycoprotein multidrug carrier associated with the MDR phenotype, whereas topotecan and SN-38 are weak substrates. Studies in the *in vivo* homologous Meth A mouse fibrosarcoma model demonstrated that a cyclical dosing pattern gave superior antitumor activity, at lower doses of DX-8951f, than was seen with single dose administration.

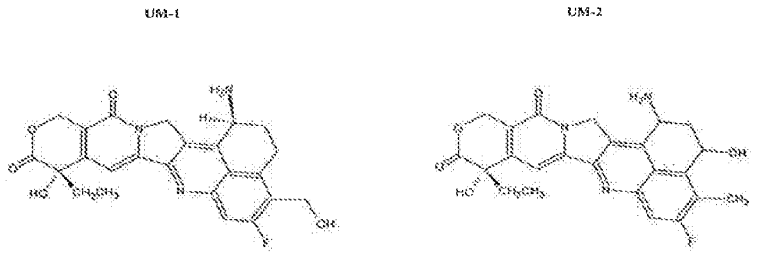


FIGURE 2. DX-8951f metabolites.

Preclinical pharmacologic and toxicologic studies were performed in rodents and dogs. ^{14}C-DX-8951f and high-performance liquid chromotography were used for differential quantification of total drug and lactone. Pharmacokinetic studies in mice and dogs showed that the t½ for lactone and total DX-8951 was similar, that the area under the concentration time curve (AUC) for the lactone was approximately 50% of the total drug AUC, and that the AUC was linearly related to the dose. The distribution of DX-8951 into tissues was reported in all species tested. Very low levels were detected in brain tissue. Therefore, DX-8951 does not appear to cross the intact blood brain barrier. DX-8951 is highly bound to plasma proteins.

In vitro, the observed plasma protein binding values were approximately 93% in the rat, 86% in the dog, 96% in the monkey, and 97% in man. Metabolic *in vitro* studies using human liver microsomes indicate that CYP3A4 and CYP1A2 of the P450 superfamily of enzymes play key roles in the metabolism of DX-8951, resulting in the formation of two hydroxylated metabolites, UM-1 and UM-2 (FIG. 2).[9,10]

Antitumor activity of both metabolites is much less potent than that of DX-8951f. The principal route of excretion was the feces (70–90%), whereas 10–25% of the drug was recovered in the urine. The toxicity profile was consistent across animal species: hematologic toxicity (anemia, neutropenia, lymphopenia, and thrombocytopenia), gastrointestinal toxicity (vomiting, diarrhea), and alopecia. Hematologic toxicity was schedule- and dose-dependent. Neutropenia was the dose-limiting toxicity. The dog was the most sensitive species. The human equivalent of the 1/3 dog toxic dose low was used to determine the starting dose for each corresponding schedule of administration in human clinical trials.

GLOBAL PHASE I CLINICAL DEVELOPMENT

DX-8951f was tested according to a parallel and integrated phase I clinical development plan in the United States, Europe, and Japan with the goal of selecting a single best dosing regimen for future phase II studies. To achieve this goal, a core protocol was established to standardize clinical data evaluation including: toxicity and efficacy criteria; definitions of dose-limiting toxicity (DLT) and maximal tolerated dose (MTD); inclusion and exclusion criteria; and supportive therapy. Data were ex-

TABLE 1. Parallel development in 3 regions

PRT 001, 30 min, every 3 weeks	
	Institute Gustave Roussy (France)
	National Cancer Center East (Japan)
PRT 002, 30 min, daily 5 days, every 3 weeks	
	Institute for Drug Development (USA)
	National Cancer Center Hospital (Japan)
PRT 003, 24 hr, every 3 weeks	
	MD Anderson Cancer Center (USA)
PRT 004, 24 hr, weekly	
	Memorial Sloan-Kettering Cancer Center (USA)
PRT 005, Cont., 5 days – 21 days	
	Institute for Drug Development (USA)
PRT 006, 30 min, weekly	
	The Churchill, Oxford Radcliffe Hospital (UK)
	Vrije Universiteit Amsterdam (The Netherlands)

changed in a timely manner among the three regions so that all investigators had access to the data necessary for decision-making as dose escalation was in progress. Data collection and processing were done in compliance with the ICH guidelines.[11]

Six phase I studies were conducted outside of Japan according to three schedules of administration and two durations of intravenous infusion (TABLE 1). Two studies were duplicated in Japan. However, these Japanese studies included the pharmacokinetics of DX-8951 lactone as well as total DX-8951, whereas the other studies were limited to the measurement of total DX-8951 due to logistical considerations.

STUDY OBJECTIVES

For each dosage regimen and duration of infusion of DX-8951f, the objectives of the studies were to determine the MTD, DLT, toxicity profile, pharmacokinetics, and pharmacodynamics; to detect evidence of antitumor activity; and to define a dose for phase II studies. The selection of a single dosage regimen for phase II studies was to be based on a comparison of the results of the six individual phase I clinical trials.

STARTING DOSE AND DOSE ESCALATION

For each study, the starting dose was chosen as 1/3 of the toxic dose low for the corresponding schedule of administration in the dog, which was the most sensitive species. There was no standardization of the method of dose escalation. A modified Fibonacci method was used in studies PRT 001, 006, and the two Japanese studies, whereas a modified continual reassessment method was used in studies PRT 002, 003, 004, and 005.[12,13] No significant differences were noted between the two methods in terms of number of dose escalation steps. MTDs were determined separately

for minimally (MP) and heavily pretreated patients (HP). HP patients were defined as those who had received more than six courses of alkylating-agent–containing chemotherapy (or more than four courses of carboplatin), radiation therapy to more than 25% of hematopoietic reserves, and two or more courses of mitomycin C or nitrosurea. All toxic effects were graded according to the National Cancer Institute Common Toxicity Criteria. DLT was defined as: febrile (≥38.5°C) or 5-day grade 4 neutropenia; other grade 4 hematologic toxicity; grade 4 vomiting with maximum supportive care; any grade 3 nonhematologic toxicity (excluding nausea and vomiting); any grade 3 or higher adverse event requiring intensive care treatment; or the inability to start a second course after a 2-week delay due to toxicity. The MTD was defined as the dose at which 20% of patients experienced DLT during the first course.

PHARMACOKINETIC AND PHARMACODYNAMIC ANALYSIS

The pharmacokinetics of DX-8951 were determined in all phase I studies (PRT001 to PRT006) using standard noncompartmental and compartmental pharmacokinetic methodologies.[14–17] The latter methodology was used in addition to noncompartmental analysis in order to obtain additional protein kinase (PK) parameters, a better understanding of the pharmacokinetics of the drug, as well as more information on the relationship between the pharmacokinetics of the drug and its pharmacological effects as it relates to toxicity.

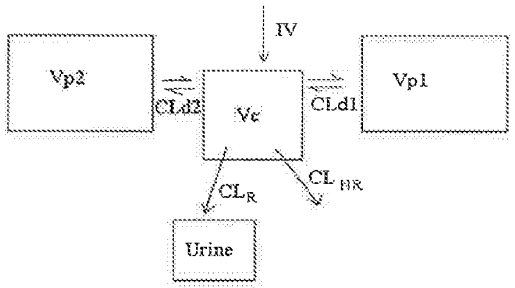
Compartmental PK/PD analyses were performed with a population methodology (IT2S) in order to obtain more robust estimations of the individual PK and PK/PD parameters.[18] No evidence of nonlinear pharmacokinetics (e.g., "hockey-stick" profiles, change in elimination of the drug between different study days) could be seen when looking at each individual patient plots of plasma concentrations versus time following administration of the drug by different regimens. Discrimination between different pharmacokinetic models was performed for each study by minimizing the Akaike information criterion test and optimizing both the fitted versus the observed concentrations versus time and the weighted residuals versus the observed values relationship. The most appropriate pharmacokinetic model to describe DX8951 plasma concentrations and excreted urinary amounts for each study is presented in FIGURE 3.

When the drug was given by a 24-hour infusion and concentrations available for 24 hours after a dose (PRT003), the quality of the fitting was as good using a two- or three-compartment model. Therefore, the first model was chosen because it is simpler. When DX-8951f was given as a 24-hour infusion and concentrations available for only 6 hours after a dose, a single compartment model fitted the data as well as the two- or three-compartment one. These observations are not surprising, as a 24-hour–hour constant infusion may mask a distribution phase (hence, a two-compartment appears as good as a three-compartment), and the lack of plasma observations after 6 hours prevents one from capturing the terminal elimination phase of the drug (hence, a one-compartment appears as good as a three-compartment). The relationship between the pharmacokinetics of DX8951 and its pharmacological effects on platelets and neutrophils was investigated using regression analysis. These clearly indicated that DX8951 exposure (i.e., the area under the plasma concentration time

TABLE 2. Distribution of Patients ($n = 165$)

Schedule		30-min infusion	24-h infusion
d 1 q3wk	(E)	12	22
	(J)	15	
d 1-5 q3wk	(US)	36	25[a]
	(J)	28	
wk ×3 q4wk	(E)	35	27

[a]5–21 day CIVI.



IV: Intravenous administration rate (mg/h); Vc: Central volume of distribution (L),
CLd1: distributional clearance between Vp1 and Vc (L/h);
Vp1: First peripheral volume of distribution (L);
CLd2: distributional clearance between Vp2 and Vc (L/h),
Vp2: Second peripheral volume of distribution (L);
CL_R: Renal clearance (L/h); CL_{NR}: non-renal clearance (L/h).

FIGURE 3. Structural pharmacokinetic model for DX-8951.

curve [AUC]) was related to the decrease in the neutrophil and platelet counts in every subject. All neutrophil and platelet counts were therefore modeled in each subject using the indirect response PK/PD model.[14]

PATIENT DISTRIBUTION

A total of 201 patients were enrolled in phase I studies: 127 patients received DX-8951f as a 30-minute infusion and 74 patients were treated by intermittent 24-hour or continuous 5–21-day infusions. The distribution by study is shown in TABLE 2. The 5–21 day continuous infusion study was still ongoing at the time of this writing.

TOXICITY

The toxicity profile was similar for all schedules of administration. Hematologic toxicity was dose-dependent and predictable. Neutropenia was dose-limiting in MP

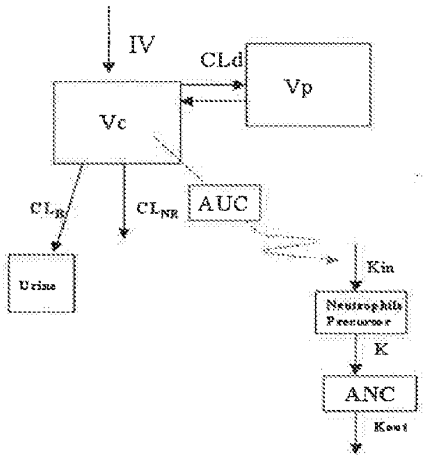
TABLE 3. Comparison of PK models for 30-minute and 24-hour continuous infusions

Study	Administration regimen of DX8951	Sampling strategy	Most appropriate PK model to describe plasma concentrations of DX8951
PRT002	30-minute constant infusion daily for 5 consecutive days	Multiple plasma samples on day 1 and day 5 (until 24 hours after each dose), Cmin on days 2, 3, and 4	3-compartment linear PK model
PRT003	24-hour continuous infusion every 3 weeks	Multiple plasma samples during infusion and for 24 hours following first infusion	2-compartment linear PK model
PRT004	24-hour continuous infusions every week for 3 weeks	Multiple plasma samples during infusion and 6 hours after the infusion on weeks 1 and 3.	1-compartment linear PK model

TABLE 4. DX-8951f: phase I studies MTDs

Schedule	MTD (mg/m^2/day)	>MTD (mg/m^2/day)	Prior treatment
d1, q 3wk	5.33	7.1	MP/HP
(30-min)			
d1-5, q 3wk	0.5	0.6	MP
(30-min)	0.3	0.4	HP
wk ×1, q 3wk	2.4	3.0	HP
(24-hr)	<2.4	3.6	HP
wk ×3, q 4wk	1.0	1.2	MP
(24-hr)	<0.8	0.8	HP
5-21d CIVI q 4wk	0.23	0.30	MP
(24-hr)	0.23	ND	HP
wk ×3, q 4wk	2.75	3.13	MP
(30-min)	2.06	2.35	HP

patients, whereas neutropenia and thrombocytopenia were dose-limiting in HP patients. Neutrophil and platelet count nadirs occurred between days 10 and 15, with recovery by day 22 in most patients receiving DX-8951f in 3-week courses. In studies in which DX-8951f was given according to a 4-week course, nonhematologic toxicity consisted mostly of mild to moderate (Grade 1 or 2) gastrointestinal toxicity (nausea/vomiting, diarrhea, stomatitis, asthenia, fever, and alopecia). Nausea and vomiting were controlled by standard antiemetic drugs (compazine, ondansetron). Transient and reversible elevation of serum transaminases were also observed. In addition, there were two cases (1%) of acute pancreatitis. Nonhematologic toxicities for the daily ×5, every 3 weeks (30-minute infusion) schedule are shown in TABLE 3.



IV: Intravenous administration rate (mg/h); Vc: Central volume of distribution (L);
CLd: distributional clearance between Vp and Vc (L/h);
Vp: peripheral volume of distribution (L);
CL_R: Renal clearance (L/h), CL_{NR}: non-renal clearance (L/h);
AUC: Predicted total exposure of the drug following an administered dose (mcg.h/L)
Kin: Zero-order formation rate constant; K: first-order formation rate constant
ANC: Absolute neutrophil counts; Kout: First-order elimination rate constant of neutrophils

FIGURE 4. Structural pharmacokinetics pharmacodynamics model for DX-8951.

Values for the MTDs and stopping doses (>MTD) are listed in TABLE 4. The MTDs for MP patients were greater than the MTDs for HP patients except for the single-dose, every 3 weeks (30-minute) regimen for which a single MTD was determined for MP and HP patients. This study involved only 12 patients, and the MTD was close to the SD. The number of dose escalation steps in each study reflected the MTD/SD ratios and did not correlate with the dose escalation method. It was not possible to evaluate the impact of the modified continual reassessment method in these studies because of the small number of patients and because the opinion of investigators prevailed over the modified continual reassessment method-assigned doses at toxic dose levels.

ANTINEOPLASTIC ACTIVITY

Antitumor activity was detected in several solid tumor types including partial responses in non-small cell lung cancer, extrapulmonary small cell cancer, colorectal cancer, hepatocellular cancer, and sarcoma. Disease stabilization, including minor responses, was seen in colorectal cancer, hepatocellular cancer, prostate cancer, and peritoneal cancer. In particular, antitumor activity was seen in CPT-11–resistant and topotecan-resistant cancers.[19–31] The daily ×5 (30-minute), weekly ×3 and the 5–21

TABLE 5. Phase I studies: average PK parameters

Parameter	Pharmacokinetic methodology	
	Noncompartmental	Population compartmental
CL (L/h per m^2)	1.87 (67%)	1.63 (64%)
Fe (%)	N/C	9.9 (60%)
Vc (L per m^2)	N/C	6.4 (28.7%)
Vss (L per m^2)	17 (39%)	17.65 (28.9%)
$T_{1/2}$ (h)	9.1 (54%)	12.3 (60%)

TABLE 6. PK parameters: single dose, q3 weeks (n = 15)

Dose (mg/m^2)	Plasma		Urinary excretion	
	$\frac{AUC_{lactone}}{AUC_{total}}$	$\frac{AUC_{UM-1}}{AUC_{DX-8951}}$	DX-8951 (%)	UM-1 (%)
3	0.29 ± 0.12	0.04 ± 0.01	6.3 ± 1.6	10.5 ± 1.2
5	0.33 ± 0.05	0.05 ± 0.01	7.6 ± 3.0	15.6 ± 2.1
6.65	0.28 ± 0.09	0.04 ± 0.01	11.0 ± 5.3	19.2 ± 4.6
Total	0.30 ± 0.08	0.04 ± 0.01	8.7 ± 4.2	16.0 ± 4.5

H. Minami (ASCO, 1999.)

day (continuous infusion) regimens appeared to be more active than the single-dose regimens or the 24-hour intermittent regimens. There was no correlation between the number of patients showing antitumor effects and the dose intensity of the regimen used in individual studies. The daily ×5 (30-minute) regimen, which appeared to be the best in terms of antineoplastic activity, was selected for phase II trials, whereas the corresponding MTDs for MP and HP patients were selected as phase II doses.[32]

PHARMACOKINETICS AND PHARMACODYNAMICS

A summary of the average PK parameters along with their interindividual variability for all phase I studies can be found in TABLE 5. The pharmacokinetics of DX-8951 were linear within the range of doses tested. Plasma C_{max} and AUC were linearly related to dose. There was no accumulation of the drug in the plasma over 5 days with the daily ×5 regimen in most patients. Clearance and Vss were dose-independent. Both total DX-8951 and its lactone form were measured only in the single-dose, every 3 weeks (30-minute) study conducted in Japan (TABLE 6). The lactone AUC was 30% of the AUC of the total DX-8951. The plasma AUC of the UM-1 metabolite was 4% of the total DX-8951 AUC. In the urine, 25% of the drug was recovered as DX-8951 with a 2:1 ratio between the UM-1 metabolite and DX-8951. Pharmacokinetic parameters were not different whether calculated by a noncompartmental or a compartmental method. The interindividual variability was within expected range. The PK model

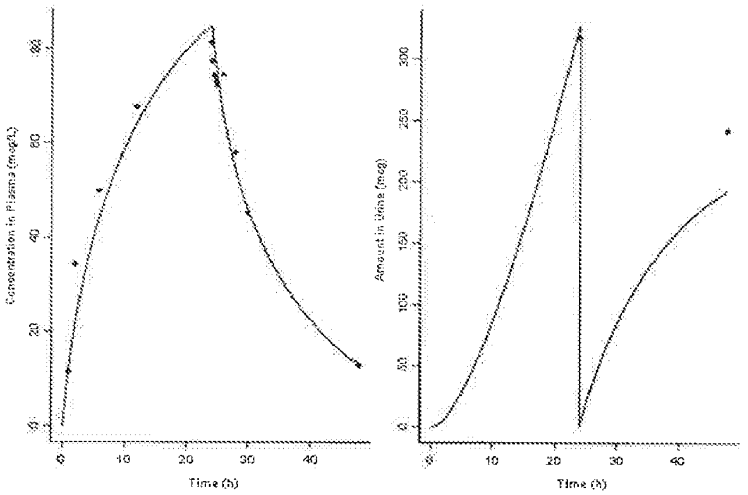


FIGURE 5. Comparison of predicted and observed plasma and urine concentations in a representative patient (PRT 003).

used in every study was explaining the observed plasma concentrations and excreted urinary amounts very well, as demonstrated by a representative patient from PRT003 (24-hour infusion) and PRT002 (5 days of daily administration). The pharmacokinetic/pharmacodynamic (PK/PD) correlation has been analyzed so far for the daily ×5 (30-minute) and single-dose (24-hour continuous infusion) studies. Using a population PK/PD method (IT2S), results were best described by a sigmoid E-max method or an E-max method. For PRT003, the relationship between the pharmacokinetics of the drug and the evolution in absolute neutrophil counts could be described by the PD parameters in TABLE 6. The neutrophil counts were associated with a normal elimination half-life of 15.2 hours (calculated as 0.693/Kout). The observed decrease in neutrophil counts started to occur (Tlag) on average 34 and 54 hours after the beginning of the first and second administration of DX8951, respectively. The duration of the inhibition in neutrophil production (Trel) by DX8951 appeared to be on average 352 and 342 hours for the first and second administration of the drug, respectively. The evolution in the absolute neutrophil counts in each subject was well explained by the PK/PD model, as demonstrated by the graph (FIG. 6) of the observed and fitted neutrophil counts versus time, coming from a representative patient. This PK/PD model may be very useful clinically and for future studies, as it may be able to predict in a given patient what dosing regimen of DX8951 would minimize the decrease in the absolute neutrophil counts.

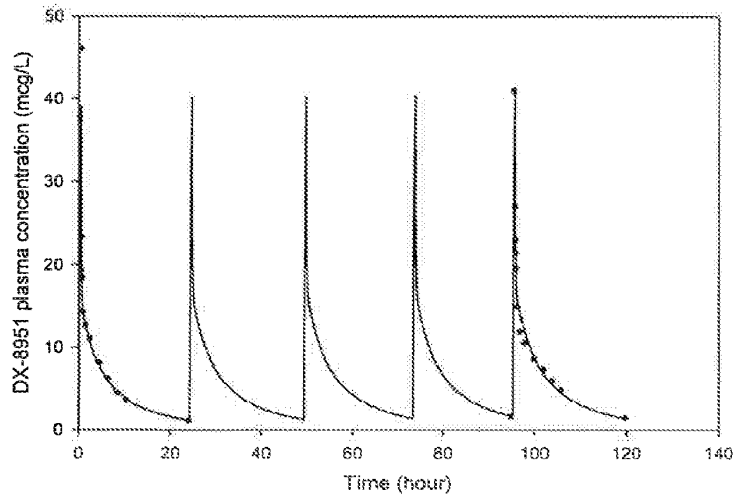


FIGURE 6. Comparison of predicted and observed plasma concentrations in a representative patient (PRT 002).

CONCLUSION

DX-8951f is a new camptothecin derivative that was selected for phase I clinical trials based on favorable characteristics identified during preclinical development, including broad spectrum *in vitro* and *in vivo* antitumor activity, the relative absence of cross-resistance with CPT-11 and topotecan, and a better safety profile than that of CPT-11. These preclinical observations were predictive of the results of the human phase I clinical studies. The safety profile, which is the same for all schedules tested, has been established. Neutropenia is the principal DLT. Thrombocytopenia may be associated with neutropenia in HP patients at the MTD. Gastrointestinal toxicity was moderate, consisting of nausea and vomiting controlled by standard anti-emetics In contrast to CPT11, severe diarrhea was not observed with DX-8951f. Other non–dose-limiting toxicities include asthenia, transient elevation of transaminases, moderate alopecia, fever, and mucositis. In addition to the favorable safety profile of DX-8951, the drug exhibited linear pharmacokinetics within the dose range listed, and antitumor activity was detected including that against CPT-11 and topotecan-resistant tumors. Based on the favorable results obtained in phase I clinical development, disease-oriented phase II studies of DX-8951f were initiated. The daily ×5, every 3 weeks schedule, with the drug administered as an (30-minute) intravenous infusion showed the most antitumor activity and was selected for phase II clinical trials.

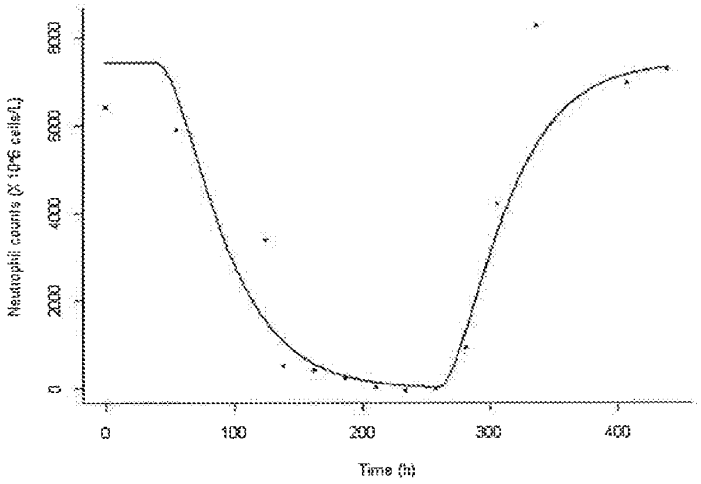


FIGURE 7. Comparison of predicted and observed values of ANC versus time.

TABLE 7. Correlation between the pharmacokinetic parameters and neutrophil counts

PD parameter	Patient average	Interindividual variability
Tlag 1	34.2 h	66%
Tlag 2	54.2 h	77%
Trel 1	352 h	20%
Trel 2	342 h	19%
Kout	0.046 h^{-1}	15
Emax	80%	N/C
AUC50	550 mcg.h/L	64%
K	38.6 h^{-1}	412%

APPENDIX: INVESTIGATORS

JEAN-PIERRE ARMAND, MD, ANDRE RAYMOND, MD, ERIC ROWINSKY, MD, and THOMAS JOHNSON, MD, *Institut Gustave Roussy, Villejuif, France*

CHARLES GEYER, MD and DANIEL VON HOFF, MD, *Cancer Treatment & Research Center, San Antonio, Texas*

MELANIE ROYCE, MD, PAULO HOFF, MD, and RICHARD PAZDUR, MD, *MD Anderson Cancer Center, Houston Texas*

LEONARD SALTZ, MD and SUNIL SHARMA, MD, *Memorial Sloan Kettering Cancer Center, New York*

DENNIS TALBOT, MD, *Oxford Radcliffe Hospital, Oxford, England*
EPIE BOVEN, MD, *Vrije Universiteit Amsterdam, Amsterdam, Netherlands*
YASUTSUNA SASAKI, MD and HIRONOBU MINAMI, MD, *National Cancer Center Hospital East, Tokyo, Japan*
TOMOHIDE TAMURA, MD and YASUHIRO SHIMADA, MD, *National Cancer Center Hospital, Tokyo, Japan*

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EXPRESSION OF c-*erb*B RECEPTORS, HEREGULIN AND OESTROGEN RECEPTOR IN HUMAN BREAST CELL LINES

Anna DEFAZIO*, Yeke-Eng CHEW, Rebecca L. SINI, Peter W. JAMES and Robert L. SUTHERLAND

Cancer Research Program, Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney, Australia

Members of the c-*erb*B family have been implicated in poor prognosis in breast cancer. Given the propensity for heterodimerisation within the *erb*B family, the pattern of co-expression of these receptors is likely to be as functionally important as aberrant expression of any given receptor alone. Therefore, the patterns of expression of the receptors, epidermal growth factor receptor (EGF-R), c-*erb*B-2, c-*erb*B-3, c-*erb*B-4, and one of the *erb*B ligands, heregulin (HRG), were examined in normal and malignant breast cell lines and compared with expression of oestrogen receptor (ER), a classical indicator of good prognosis. There was an inverse correlation between ER and EGF-R mRNA levels, as previously described, but no correlation between either of these receptors and c-*erb*B-2. c-*erb*B-3 expression was positively correlated with ER. In contrast, HRG expression was inversely related to ER. Expression of antisense-ER resulted in increased EGF-R mRNA, demonstrating a functional link between the expression of these 2 genes, however, there was no significant change in c-*erb*B-2 or c-*erb*B-3 mRNA, suggesting that ER is not directly involved in control of expression of these genes. A comparison of individual *erb*B receptors and HRG revealed that the majority of lines expressing increased levels of c-*erb*B-2 also expressed elevated levels of c-*erb*B-3 mRNA, and none of the cell lines that expressed both c-*erb*B-2 and either c-*erb*B-3 or c-*erb*B-4 expressed the ligand HRG. In summary, the levels of expression of c-*erb*B-1, -2, -3, and -4 varied in this series of breast cell lines, and the pattern of expression and the relationship of each growth factor receptor to the expression of ER was quite distinct. The lack of expression of HRG in cell lines that express receptors may be indicative of paracrine interactions between *erb*B ligands and their cognate receptors and may suggest that the ligand and receptors are expressed in different subtypes of breast epithelial cells from which the cell lines are derived. *Int. J. Cancer* 87:487–498, 2000.
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The epidermal growth factor receptor (EGF-R) or c-*erb*B family of receptor tyrosine kinases is frequently overexpressed in breast cancer and in various other epithelial carcinomas. To date, 4 members of this gene family have been described: EGF-R (c-*erb*B-1), c-*erb*B-2, c-*erb*B-3, and c-*erb*B-4 (Coussens *et al.*, 1985; Kraus *et al.*, 1989, Plowman *et al.*, 1990, 1993, Richter King *et al.*, 1985). Despite extensive structural homology, these receptors differ in their ligand specificities. Whereas EGF-R binds multiple distinct ligands that share an EGF-like motif, the heregulin (HRG) ligands of c-*erb*B-3 and c-*erb*B-4 arise from splice variants of a single gene. Collectively, this family of polypeptide growth factors has been termed neuregulins and include HRGs, neu differentiation factor, glial growth factors, and acetylcholine-receptor-inducing activity (Falls *et al.*, 1993; Holmes *et al.*, 1992; Peles *et al.*, 1992). Neuregulin-2 growth factors are encoded by a separate gene and are also ligands for c-*erb*B-3 and c-*erb*B-4 (Carraway *et al.*, 1997; Chang *et al.*, 1997). Epiregulin, an EGF-related ligand binds to EGF-R and c-*erb*B-4 (Komurasaki *et al.*, 1997). c-*erb*B-2 remains an orphan receptor with a ligand yet to be fully characterised.

A hallmark of this family of receptors is their propensity for heterodimerisation; ligand-induced *erb*B receptor heterodimerisation is hierarchical with *erb*B-2 being the preferred heterodimerizing partner of the other 3 *erb*B proteins (Graus-Porta *et al.*, 1997). Indeed, c-*erb*B-2 may act as an essential common subunit of *erb*B receptors rather than as a growth factor receptor in its own right (Karunagaran *et al.*, 1996).

While the expression level of each of the *erb*B receptors can influence cellular response to HRG, cells expressing the highest levels of *erb*B-2 have the greatest response to HRG (Aguilar *et al.*, 1999). c-*erb*B-3 constitutes a high-affinity HRG binding receptor only when co-expressed with c-*erb*B-2 (Sliwkowski *et al.*, 1994). A functional HRG receptor may therefore be viewed as a heterodimer of c-*erb*B-3 with c-*erb*B-2 where c-*erb*B-2 modulates affinity of c-*erb*B-3 for HRG binding and contributes an active tyrosine kinase domain. HRG also induces tyrosine phosphorylation of c-*erb*B-4 (Plowman *et al.*, 1993), however, a role of c-*erb*B-4 in HRG-mediated signal transduction in mammary epithelial cells has yet to be established (Chan *et al.*, 1995; Chen *et al.*, 1996).

There is extensive literature on the role of the *erb*B family in breast cancer. High levels of EGF-R expression are detected in breast tumours that have higher proliferation rates, higher metastatic potential, and in patients who have early recurrence of the disease (Sainsbury *et al.*, 1987; Toi *et al.*, 1990). Amplification and overexpression of c-*erb*B-2 have also been reported in a number of tumour types including breast, where most, but not all, studies have shown a direct correlation with poor clinical prognosis (Gullick, 1990; Quénel *et al.*, 1995; Seshadri *et al.*, 1993; Slamon *et al.*, 1987, 1989; Torregrosa *et al.*, 1997). c-*erb*B-2 has also been used as a target for therapeutic agents such as the humanised monoclonal antibody (MAb), Herceptin. Preliminary reports of Herceptin used alone or in combination with cisplatin have documented its efficacy against some breast tumours overexpressing c-*erb*B-2 (Pegram *et al.*, 1998).

Although c-*erb*B-3 is expressed in a significant proportion of breast cancers and the level of expression is variable, no relationship has been found between c-*erb*B-3 expression and patient survival (Lemoine *et al.*, 1992; Quinn *et al.*, 1994; Travis *et al.*, 1996). c-*erb*B-4 transcripts have been detected in breast tumours, however, there is little information on the relationship between expression of c-*erb*B-4 and clinical parameters in breast cancer (Sawyer *et al.*, 1998).

Given the propensity for heterodimerisation within the *erb*B family, the extent of co-expression of the receptors is likely to be a biologically important parameter. The expression of EGF-R and c-*erb*B-2 in breast tumour biopsies was significantly correlated in some clinical studies (Lundy *et al.*, 1991; Umekita *et al.*, 1992) but not others (Osaki *et al.*, 1992; Tauchi *et al.*, 1989; Toi *et al.*, 1994; Tsutsumi *et al.*, 1990). However, most studies have found that co-expression of c-*erb*B-2 and EGF-R had an additive adverse effect on prognosis in breast cancer (Harris *et al.*, 1989; Osaki *et al.*, 1992), and patients with tumours expressing both EGF-R and c-*erb*B-2 form a subgroup at particularly high risk for relapse (Toi *et al.*, 1994). Thus it may be that, in determining prognosis and

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*Correspondence to: Anna deFazio, Department of Gynaecological Oncology, Westmead Hospital, Westmead NSW 2145, Australia. Fax: 612-9845-7793. E-mail: annadf@westgate.wh.usyd.edu.au

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likelihood of response to therapy, the pattern of co-expression of these receptors is the important factor, rather than the aberrant expression of any given receptor alone.

The tissue distribution of c-*erb*B-3 is distinct from that of EGF-R and c-*erb*B-2 in normal adult and foetal tissue (Kraus *et al.*, 1989; Prigent *et al.*, 1992), and no relationship has been found between expression of c-*erb*B-3 and c-*erb*B-2 in breast cancers (Lemoine *et al.*, 1992; Travis *et al.*, 1996).

In breast tumours, oestrogen receptor (ER) content is a classical prognostic indicator, identifying a less-aggressive disease with a better clinical outcome and greater likelihood of response to endocrine therapy (McGuire, 1978). A marked inverse relationship between expression of ER and EGF-R has been described in breast cancer cell lines and breast tumour biopsies (Davidson *et al.*, 1987; Hall *et al.*, 1990; Koenders *et al.*, 1991; Lee *et al.*, 1990; Perez *et al.*, 1984; Sainsbury *et al.*, 1985), and we have previously shown that expression of ER and EGF-R is directly linked (deFazio *et al.*, 1997). However, while the inverse relationship between ER and EGF-R expression in breast cancer is quite clear, the relationship between ER and other members of the c-*erb*B family remains uncertain. Although a number of clinical studies found a correlation between hormone receptor status and c-*erb*B-2 amplification or overexpression, an equivalent number did not (Gullick, 1990; Lupu and Lippman, 1993; Nicholson *et al.*, 1993; Quénel *et al.*, 1995; Tauchi *et al.*, 1989). Some studies have found c-*erb*B-2 expression identifies certain subsets of patients, such as patients with ER-negative tumours with increased risk of death (Berns *et al.*, 1992); patients with ER-positive tumours likely to be resistant to chemotherapy (Allred *et al.*, 1992); or patients failing to respond to endocrine therapy on relapse (Wright *et al.*, 1992). Some *in vitro* studies have reported oestrogen treatment decreases expression of c-*erb*B-2 (Antoniotti *et al.*, 1992; Read *et al.*, 1990; Russell and Hung, 1992; Taverna *et al.*, 1994; Warri *et al.*, 1991), and increased expression of c-*erb*B-2 can abrogate the effects of anti-oestrogens (Benz *et al.*, 1992; Liu *et al.*, 1995). So while there appears to be some interaction between steroid hormone receptors and c-*erb*B-2, the nature of this interaction is not clear. No association has been found between c-*erb*B-3 and ER in breast cancer biopsies (Travis *et al.*, 1996), and the relationship between ER and c-*erb*B-4 has not been defined.

The relationship between ER and HRG expression is similarly unclear. One *in vitro* study showed that transfection of MCF-7 cells with heregulin-β2 resulted in disruption of ER function and development of oestrogen independence (Tang *et al.*, 1996), and a clinical study reported that a higher percentage of ER-negative tumours expressed HRG compared with ER-positive tumours (Normanno *et al.*, 1995), however, the findings were not statistically significant.

This study aimed to clarify the intrinsic relationship between expression of 4 members of the EGF-R/c-*erb*B family, EGF-R (c-*erb*B-1), c-*erb*B-2, -3, and -4, and HRG and to determine the relationship between these genes and ER in human breast cell lines.

MATERIAL AND METHODS

Cell culture

The sources of the breast cancer cell lines were as follows: BT-20, BT-474, BT-483, BT-459, Du-4475, Hs-578T, MDA-MB-134, -175, -361, -436, -453, -468, SK-BR-3, and ZR-75-1. Were obtained from the American Type Culture Collection (Rockville, MD). MCF-7, MDA-MB-157, MDA-MB-231, MDA-MB-330, and T-47D were supplied by EG & G Mason Research Institute (Worcester, MA) for the National Cancer Institute Breast Cancer Program Cell Culture Bank, as was the HBL-100 cell line. This cell line was derived from normal tissue but contains tandemly integrated SV-40 sequences (Caron de Fromentel *et al.*, 1985). Cultures were maintained in exponential growth in RPMI 1640 medium, supplemented with 6 mM glutamine, 10 μg/ml human insulin and 10% FCS (CSL, Parkville, Australia). Normal breast epithelial cell strains HMEC-1001-7 and HMEC-219-4 were obtained from Clonetics Corp. (San Diego, CA), and grown in the medium supplied by the manufacturer. Cells were free from *Mycoplasma* contamination (Gen-Probe T.C. Rapid Detection System, BioMedia, Doncaster, Australia).

Isolation and fractionation of RNA and DNA

Total RNA and genomic DNA was isolated from the same cell pellet by lysis in guanidinium isothiocyanate solution followed by CsCl centrifugation. Total RNA was isolated using standard methods (Sambrook *et al.*, 1989) and DNA was collected from the CsCl gradient, diluted in deionised water to a total of 10 ml, and extracted twice with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1). RNA and DNA were isolated from each cell line on a minimum of 2 independent occasions.

RNA (20 μg/sample) was separated by formaldehyde/agarose gel electrophoresis and transferred to Zeta-Probe nylon membranes (Bio-Rad, Richmond, CA) as previously described (Hall *et al.*, 1990). DNA was digested with *Eco*R I and fractionated on a 1% agarose gel in 1× TAE (40 mM Tris-acetate, 1 mM EDTA) and transferred to Zeta-Probe membranes (Bio-Rad) by capillary transfer in 0.4 M NaOH.

Probes

The 2.1-kb human ER cDNA was an *Eco*R I fragment of λOR8 encoding the open reading frame and a portion of the 3′ untranslated region (Walter *et al.*, 1985). The human EGF-R cDNA probe, encoding the extracellular ligand binding domain and the transmembrane region of EGF-R, was a 1.8-kb *Eco*R I fragment of the λ31 clone (Libermann *et al.*, 1985).

A 40-base single-stranded oligonucleotide to the translated exon 4 region of the c-*erb*B-2 gene used for Northern blot analysis was from Oncogene Science (Manhasset, NY). The c-*erb*B-2 cDNA probe used for Southern blot analysis was a 0.8-kb *Acc* I exon 4 fragment from the pMAC117 plasmid (Richter King *et al.*, 1988), a gift from Pam Russell, Oncology Research Centre, Prince of Wales Hospital (Randwick, NSW) used with permission from M. Kraus, National Cancer Institute (Bethesda, MD). The 0.65-kb probe to human c-*erb*B-3 carboxy terminal coding and 3′ untranslated sequence was also a kind gift from M. Kraus (Kraus *et al.*, 1989). The c-*erb*B-4 probe was an approx. 0.5-kb reverse transcriptase-PCR (RT-PCR) product from a cytoplasmic portion (amino acid residues 1,064–1,225) (Plowman *et al.*, 1993) of the receptor that displays low homology with other known members of the *erb*B family. The PCR primers were as follows: 5′-TTGTATACCGAGATGGAGGT-3′; 5′-ATGCCAGAGAAGGCCAAGA-3′. The template was total RNA from BT-474 cells. The HRG probe was a 0.2-kb fragment corresponding to amino acid residues 177–243 of the β1 isoform of HRG (encoding an EGF-like domain in the extracellular domain adjacent to the transmembrane region) isolated by RT-PCR using total RNA from MDA-MB-231 cells.

Equivalent loading of RNA samples for Northern blot analysis was confirmed by hybridization with an end-labelled oligonucleotide probe complementary to a highly conserved region of the 18S ribosomal RNA (nucleotide sequence 5′-CGGCATGTATTAGCTCTAGAATTACCACAG-3′ as previously described (Hall *et al.*, 1990)). A 2-kb *Eco*R I cDNA fragment of human fibroblast β-actin (Gunning *et al.*, 1983) was used as a control probe for Southern analysis.

Hybridization

The cDNA probes were labelled by nick-translation or random priming with (α-^{32}P)dCTP (3,000 Ci/mmol; Amersham, North Ryde, Australia) and oligonucleotides were end-labelled with (γ-^{32}P)ATP (5,000 Ci/mmol) to specific activities of 10^8–10^9 dpm/μg. Northern blot hybridization was performed as previously described (Hall *et al.*, 1990). Hybridization for Southern analysis was carried out at 65°C overnight at a probe concentration of 0.5–1 ng/ml in 0.5 M sodium phosphate, pH 6.9, 7% (w/v) SDS, 0.5% (v/v) Blotto (stock solution: 10% [w/v] instant skim milk powder + 0.2% sodium azide) and 1 mM EDTA. Membranes were

washed to a final stringency of 0.05× SSC (20× SSC: 3M sodium chloride, 0.3 M sodium citrate, pH 7.0) + 0.1% SDS for 30 min. Autoradiography was at −70°C when using intensifying screens, and allele sizes were determined by comparison with lambda *Hin*d III and pBR322 *Bst*I markers.

Quantitation

Autoradiographs resulting in a maximum peak height equal to or less than 2.0 absorbance units, in the absence of intensifying screens (unless specified), were quantitated using a Bio-Rad 620 video densitometer and 1D Analyst/Macintosh Data Analysis software.

Western blot analysis

Cell lysates from cells serum starved overnight in RPMI 1640 + 0.5% FCS were prepared as described (Margolis *et al.*, 1989) except that p-nitrophenyl phosphate was omitted from the lysis buffer. Equal amounts of total protein were separated by 6% SDS-PAGE. Proteins were visualized using the ECL detection system (Amersham), after incubation (2–4 hr, room temperature) with the primary antibody, c-*erb*B-4 rabbit polyclonal (G18) from Santa Cruz Biotechnology (Santa Cruz, CA).

Statistical analysis

Comparisons of receptor expression between cell lines were made using Kendall's rank correlation test (Statview 512+ software).

Antisense-ER transfection

An antisense-ER expression vector, pLENASER, was constructed by inserting the entire 1.8-kb ORF of the human ER cDNA in the reverse orientation into an expression vector under the control of the zinc-inducible human metallothionine II_A promoter (deFazio *et al.*, 1997). Exponentially growing MCF-7 cells were cotransfected with either 50 μg pLENASER or the parent vector, pLEN, and 5 μg pSV_2neo/150 cm^2 flask, using calcium phosphate precipitation as previously described (deFazio *et al.*, 1997). Transfected cells were selected by growth in G418, and antisense-ER expressing lines were cloned by limiting dilution and characterised (deFazio *et al.*, 1997). Zinc induction: cells (2 × 150 cm^2 flasks/treatment) were grown in phenol red-free RPMI 1640 medium, supplemented as described for stock maintenance, until cultures were 50–70% confluent, then changed to serum-free RPMI 1640 supplemented with glutamine, insulin, and 10 μg/ml transferrin 24 hr prior to addition of 50 μM $ZnSO_4$.

RESULTS

Relative expression of ER and EGF-R

Northern blot analysis showed hybridisation of the human ER cDNA to an RNA species of the expected molecular size of 6.4 kb in 10 of 19 breast cancer cell lines: MDA-MB-134 > MDA-MB-361 > MCF-7 > T-47D > BT-483 > BT-474 > ZR-75-1 > MDA-MB-175 > BT-20 > MDA-MB-468 as previously described (deFazio *et al.*, 1997) (Fig. 1a). Southern blot analysis was used to determine whether high mRNA expression was due to gene amplification. Cell line DNA digested with *Eco*R I and probed with ER cDNA showed no evidence of gene amplification or deletion in any of the cell lines examined in agreement with previous data on a subset of these cell lines (Watts *et al.*, 1992) (data not shown).

Two major transcripts of 10.5 and 5.8 kb were observed for EGF-R, the higher molecular weight mRNA species being the most abundant (Fig. 1a). Following prolonged exposure of autoradiographs, all cell lines were shown to express EGF-R mRNA except MDA-MB-134, MDA-MB-453, and Du-4475. MDA-MB-468 and BT-20 expressed the highest levels of EGF-R mRNA. In rank order, they were followed by the 2 normal cells lines, HMEC 1001-7 and HMEC 219-4, then SK-BR-3 ≅ MDA-MB-231 ≅ MDA-MB-330 ≅ MDA-MB-436 ≅ BT-549 > HBL-100 ≅ Hs-578T ≅ MDA-MB-157. The ER-positive cell lines uniformly expressed the lowest levels of EGF-R mRNA, confirming the previously described relationship between the level of expression of these 2 genes in breast cancer (Lee *et al.*, 1990).

The EGF-R gene was amplified in the only 2 breast cancer cell lines expressing higher levels of EGF-R mRNA than normal HMEC cell lines. EGF-R gene amplification was 6-fold in BT-20 and 8-fold in MDA-MB-468 compared with normal peripheral blood lymphocytes (PBLs) (Fig. 2), consistent with previous reports (Filmus *et al.*, 1985; Kraus *et al.*, 1987; Lebeau and Goubin, 1987). In addition to gene amplification, an *Eco*R I polymorphism was also detected. In the majority of cell lines, 3 bands of 7.7, 6.6, and 5.5 kb were seen, however, in MDA-MB-468 a polymorphic 6.3-kb band was found with concommitant loss of the 7.7-kb fragment. Analysis of DNA extracted from PBLs showed the same polymorphism to be present in 3 of 20 normal individuals (data not shown), indicating that this is likely to represent a normal EGF-R variant.

Expression and amplification of c-erbB-2

A single c-*erb*B-2 mRNA species of 4.6 kb was detected in all cell lines with prolonged exposure. Overexpression (that above normal breast cell lines HMEC 1001-7 and HMEC 219-4) was observed in 40% of the cell lines: BT-474 > SK-BR-3 > MDA-MB-361 > MDA-MB-453 > BT-483 ≅ ZR-75-1 ≅ MDA-MB-175 ≅ BT-20 (Fig. 1*a*). These findings are in general agreement with Kraus *et al.* (1987) with the exception of the BT-20 where this study revealed a small increase in expression above normal. The 4 cell lines with highest mRNA expression displayed c-*erb*B-2 gene amplification (Fig. 2): MDA-MB-361 (6-fold), SK-BR-3 (6-fold), BT-474 (4-fold), MDA-MB-453 (2-fold). Fold amplification compared with PBLs (shown in brackets) was determined by densitometry, and the results were in general agreement with Kraus *et al.* (1987).

Expression of c-erbB-3 mRNA

The previously reported mRNA transcript of 6.2 kb (Kraus *et al.*, 1989) was seen in most of the breast cell lines examined, but the relative expression of c-*erb*B-3 between cell lines varied more than 50-fold. The majority of breast cancer cell lines expressed higher levels of c-*erb*B-3 mRNA compared with the normal breast cell lines HMEC 1001-7 and HMEC 219-4. Highest expression was found in MDA-MB-361 ≅ MDA-MB-134 ≅ BT-483 > BT-474 ≅ MDA-MB-175 > MDA-MB-453 > ZR-75-1 > T-47D ≅ SK-BR-3 > MCF-7 ≅ BT-20 ≅ MDA-MB-468 > Du-4475 > MDA-MB-157 > Hs-578T ≅ HBL-100, BT-549, MDA-MB-436, MDA-MB-330, MDA-MB-231, HMEC 1001-7, HMEC 219-4. No evidence of c-*erb*B-3 gene amplification or rearrangement was observed (not shown), which is consistent with published data in breast cancer cell lines and primary cancer biopsies (Lemoine *et al.*, 1992). All ER-positive cell lines expressed moderate to high levels of c-*erb*B-3 mRNA and all of the high c-*erb*B-2 expressing cell lines also had increased expression of c-*erb*B-3.

Expression of c-erbB-4

c-*erb*B-4 mRNA was detected in only 3 of the 22 cell lines tested. c-*erb*B-4 mRNA was most abundant in ZR-75-1 with 5-fold less expression observed in the MDA-MB-361 cell line. c-*erb*B-4 mRNA was barely detectable in BT-474 but was clearly evident following prolonged autoradiographic exposure. Similar results were obtained by Western blot analysis, where highest expression of c-*erb*B-4 protein was seen in ZR-75-1, followed by MDA-MB-361, but c-*erb*B-4 was below the level of detection in 9 other cell lines tested (T-47D, MCF-7, BT-474, MDA-MB-453, MDA-MB-157, MDA-MB-330, MDA-MB-231, SK-BR-3, MDA-MB-468).

Expression of heregulins

Three HRG mRNA transcripts were detected, 6.6-kb and 2.5-kb species, representing the α, β1, and β2 splice variants, and 1.8 kb representing β3 (Holmes *et al.*, 1992). The majority of cell lines that expressed detectable levels of HRG expressed all 3 transcripts (Fig. 1*a*). One exception was MDA-MB-175 cells that expressed a single mRNA species of approximately 3.4 kb. HRG was most

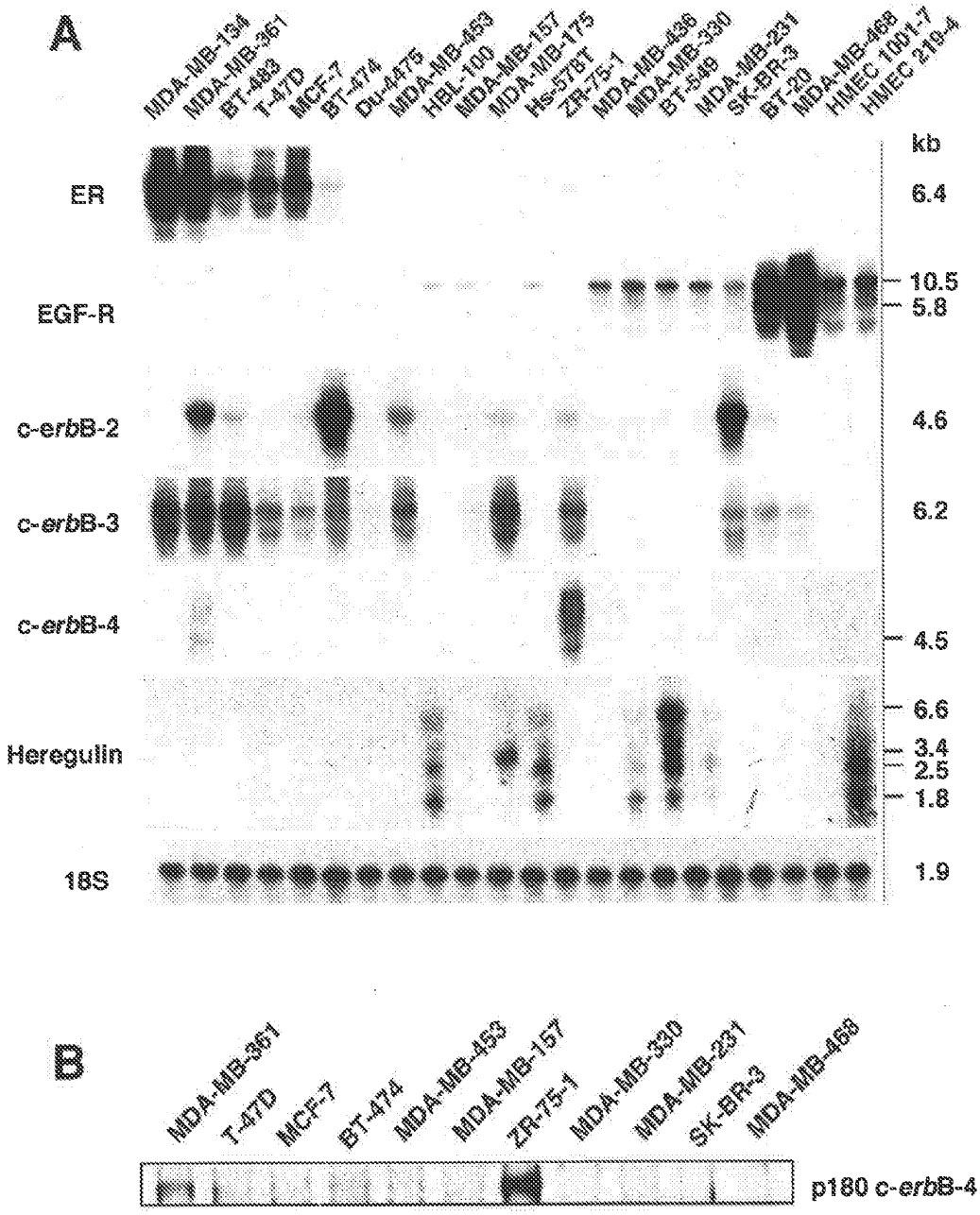


FIGURE 1 – mRNA expression of ER, EGF-R, *c-erbB-2*, *c-erbB-3*, *c-erbB-4*, and heregulin in 22 breast cell lines. (*a*) Total RNA (20 μg/lane) was electrophoresed and blots hybridised with cDNA probes to ER, EGF-R, *c-erbB-2*, *-3*, *-4*, and HRG. Blots were rehybridised with a control 18S rRNA oligonucleotide probe, and loading was shown to be equivalent in all lanes (representative blot shown). Autoradiograms were exposed for 48 hr for ER and EGF-R, 24 hr for *c-erbB-2* and *c-erbB-3*, and 15 min for 18S rRNA in the absence of intensifying screens. Heregulin was detected following exposure for 7 days with 2 intensifying screens (HMEC 1001-7 was not included in the heregulin blot). (*b*) Total protein prepared from cell lysates was separated by 5% SDS-PAGE. *c-erbB-4* was visualized using chemiluminescent immunodetection. (*c*) Autoradiograms from Northern blots were quantitated by densitometry, sorted in order of decreasing ER and increasing EGF-R levels, and the relative content of receptor mRNA in each cell line is presented as the area hybridizing with each probe.

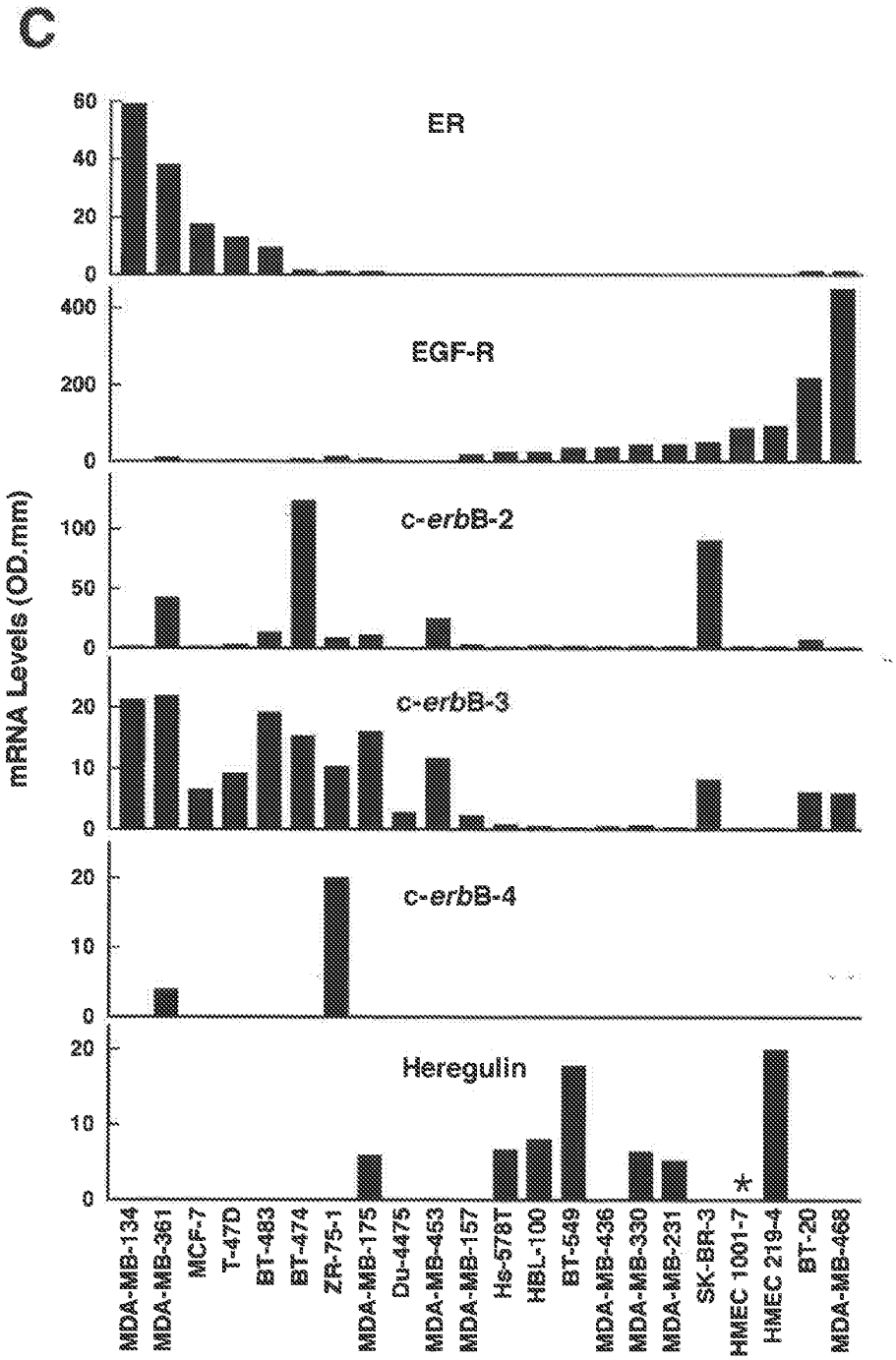


FIGURE 1 (CONTINUED)

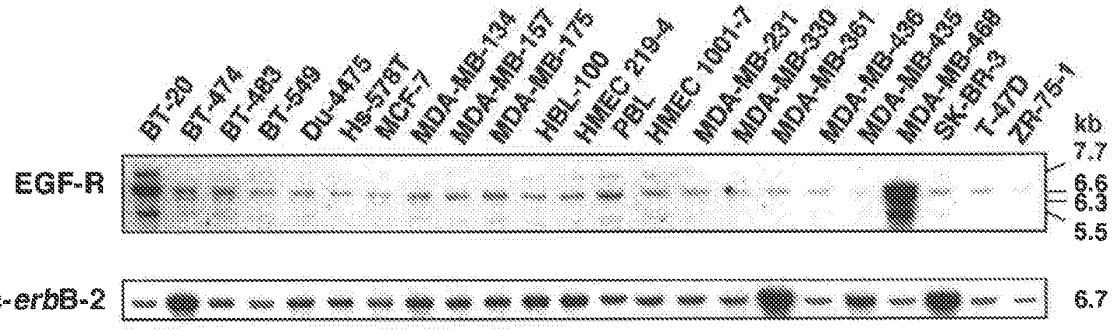


FIGURE 2 – Analysis of receptor gene amplification in breast cell lines. Total genomic DNA (10 μg per sample) was digested with *Eco*R I, size fractionated by electrophoresis and immobilised on a nylon membrane. Filters were hybridised with EGF-R and c-*erb*B-2 cDNA to determine fold amplification above PBL and with β-actin cDNA to control for gel loading and transfer, which were shown to be equivalent in all lanes (not shown). Autoradiographic exposure times in the presence of 2 intensifying screens were EGF-R, 5 days; c-*erb*B-2, 48 hr.

abundant in the normal breast cell line HMEC 219-4, followed closely by BT-549, and was expressed to a lesser extent in HBL-100, Hs-578T, MDA-MB-330, MDA-MB-231, and MDA-MB-175. HRG mRNA was not analysed in HMEC 1001-7, and levels were below the limits of detection in the remaining 14 cell lines.

Relationship between the expression of ER, erbB receptor genes and heregulin

The data obtained from densitometric analysis of the Northern blots described above are shown in Fig. 1c with the cell lines sorted according to ER and EGF-R expression levels. These data were analysed for potential correlations between the level of expression of *erb*B receptor and ER mRNA. The association between any 2 receptors was evaluated using Kendall's rank correlation test, which depends only on the rank and not the numerical value of the parameter and does not assume a linear relationship between the 2 parameters. These correlations are summarized in Table I, where the Kendall correlation coefficient, T, a measure of the extent of the relationship, and the p values are recorded. Analysis was also performed omitting cell lines shown to be amplified at the genomic level.

As previously shown, ER mRNA was significantly negatively correlated with expression of EGF-R mRNA ($p = 0.041$). Omission of BT-20 and MDA-MB-468, cell lines with EGF-R amplification, did not alter the overall result but did increase the level of statistical significance ($p = 0.002$). No relationship was found between c-*erb*B-2 mRNA levels and either ER or EGF-R.

c-*erb*B-3 mRNA expression was both positively correlated with expression of ER ($p = 0.0001$) and significantly negatively correlated with expression of EGF-R mRNA ($p = 0.002$). Omission of cell lines with EGF-R amplification (BT-20, MDA-MB-468) from the analysis again did not alter the overall result.

HRG expression was significantly inversely expressed with ER ($p = 0.004$) and was positively associated with EGF-R ($p = 0.048$). The only ER-positive cell line that expressed HRG was MDA-MB-175. Expression of HRG in this line may be abberant since the mRNA species detected was not of the expected size and conditioned medium from MDA-MB-175 cells failed to phosphorylate c-*erb*B-3 protein (data not shown).

c-*erb*B-3 also significantly correlated with expression of c-*erb*B-2 mRNA ($p = 0.003$), i.e., all cell lines that overexpressed c-*erb*B-2 also expressed elevated levels of c-*erb*B-3. c-*erb*B-4 correlations were not analysed, as it was only detected in 3 cell lines. It was noted, however, that all cell lines that expressed c-*erb*B-4 were ER-positive and did not express HRG.

HRG was significantly negatively correlated with c-*erb*B-3 expression ($p = 0.001$). There was no statistically significant correlation between the levels of HRG and c-*erb*B-2, however, none of the cell lines that had elevated expression of both c-*erb*B-2 and either

TABLE I – RELATIONSHIPS BETWEEN EXPRESSION OF ER, EGF-R, c-*erbB*-2, -3, AND HEREGULIN[1]

	ER	EGF-R	EGF-R[4]	c-*erbB*-2	c-*erbB*-2[5]	c-*erbB*-3	Heregulin
ER	1						
EGF-R	−0.32 $p = 0.041$[3]	1					
EGF-R[4]	−0.51 $p = 0.002$[3]	1	1				
c-*erb*B-2	0.21 $p = 0.171$	−0.23 $p = 0.14$	−0.22 $p = 0.18$	1			
c-*erb*B-2[5]	0.23 $p = 0.176$	−0.22 $p = 0.196$	−0.26 $p = 0.161$	1	1		
c-*erb*B-3	0.59 $p = 0.0001$[2]	−0.48 $p = 0.002$[3]	−0.57 $p = 0.004$[3]	0.46 $p = 0.003$[2]	0.37 $p = 0.033$[2]	1	
Heregulin	−0.46 $p = 0.004$[3]	0.31 $p = 0.048$[2]	0.47 $p = 0.005$[2]	−0.25 $p = 0.118$	−0.10 $p = 0.580$	−0.51 $p = 0.001$[3]	1

[1]Levels of mRNA determined by densitometric analysis of Northern blot autoradiograms were evaluated using Kendall's rank correlation test. Data are presented as the Kendall correlation coefficient, T (0 to ± 1), with the corresponding p values.–[2]Significantly, positively correlated $p < 0.05$.–[3]Significantly, inversely correlated $p < 0.05$.–[4]Analysis excluding cell lines with EGF-R gene amplification (BT-20, MDA-MB-468).–[5]Analysis excluding cell lines with c-*erb*B-2 gene amplification (MDA-MB-361, SK-BR-3, BT-474, MDA-MB-453).

c-*erb*B-3, or c-*erb*B-4 were found to express detectable levels of HRG.

Expression of antisense-ER did not significantly alter expression of c-erbB-2 or c-erbB-3

We have previously shown that the expression of ER and EGF-R is functionally linked in that a decrease in ER levels by ER-antisense RNA results in an increase in EGF-R mRNA expression (deFazio *et al.*, 1997). We therefore determined whether there was a similar relationship with other members of the *erb*B family. Although we had not observed a relationship between c-*erb*B-2 and either ER or EGF-R in our panel of cell lines, there are a number of reports in the literature that describe co-expression of c-*erb*B-2 and EGF-R and inverse expression of ER and c-*erb*B-2. To determine the relationship between ER and c-*erb*B-2 expression, c-*erb*B-2 mRNA levels were determined in MCF-7 cells transfected with an expression vector pLENASER, where antisense-ER was under the control of the zinc-inducible, human metallothionein II_A promoter (deFazio *et al.*, 1997). Time-dependent changes in gene expression following addition of zinc are shown in Fig. 3a. Induction of 1.8-kb antisense-ER RNA was observed within 0.5 hr, reaching a peak at 3–6 hr, and resulted in a 40% reduction in oestradiol (E_2) binding at 6 hr (deFazio *et al.*, 1997). Induction of antisense-ER was accompanied by an increase in EGF-R mRNA, apparent by 1 hr and reaching a maximum of 3-fold above control at 6 hr as previously described (deFazio *et al.*, 1997). There was no significant change in c-*erb*B-2 expression with induction of antisense-ER.

Induction of antisense-ER resulted in a small decrease in expression of c-*erb*B-3 mRNA at 3–12 hr. This result, coupled with co-expression of c-*erb*B-3 and ER in the panel of cell lines, was suggestive of regulation of c-*erb*B-3 by oestrogen. However, treatment of MCF-7 cells with oestrogen ± antioestrogens did not show oestrogen-specific regulation of c-*erb*B-3 mRNA (data not shown). The small decrease in c-*erb*B-3 mRNA following induction of antisense-ER is therefore probably not significant.

The lack of significant effect of zinc-induced antisense-ER RNA on c-*erb*B-2 and c-*erb*B-3 expression, in contrast with the effect on EGF-R, was corroborated by transfection of constitutively active antisense-ER under the control of the CMV promoter. MCF-7 cells were transfected with pCMVASER, and neomycin-resistant clones were isolated and characterised as previously described (deFazio *et al.*, 1997). The level of expression of antisense-ER RNA relative to endogenous ER mRNA, measured simultaneously by the double-stranded ER cDNA probe, was approximately equal (representative clone shown in Fig. 3b), and the number of E_2 binding sites was reduced by approximately 25% (deFazio *et al.*, 1997). Despite this modest reduction in ER, the expression of antisense-ER resulted in a 3-fold increase in the level of EGF-R mRNA, however, there was no effect on the expression of c-*erb*B-2 or c-*erb*B-3 mRNA levels.

DISCUSSION

This is one of the first comparative studies to evaluate expression of all 4 members of the *erb*B family of growth factor receptors, HRG, and ER in a large series of breast cell lines. The main findings of this study were: 1. each member of the *erb*B family of tyrosine kinase growth factor receptors has a unique pattern of expression; 2. c-*erb*B-3 was more highly expressed in ER-positive cell lines; 3. there was a negative correlation between expression of HRGs and expression of their receptors; c-*erb*B-3/c-*erb*B-2 and c-*erb*B-4/c-*erb*B-2; and 4. although ER is mechanistically involved in control of EGF-R expression, no data were found to support the direct involvement of ER in the basal expression of other members of the *erb*B family.

Epidermal growth factor receptor (c-erbB-1) is down-regulated in breast cancer cell lines

The 2 breast cancer lines with highest expression of EGF-R mRNA, BT-20 and MDA-MB-468, were shown to have EGF-R gene amplification as previously shown (Filmus *et al.*, 1985; Lebeau and Goubin, 1987). These results are consistent with clinical findings that EGF-R is amplified in some breast cancers, but it is not common. The cell lines with the highest EGF-R expression, in the absence of amplification, were the normal cell lines HMEC 1001-7 and HMEC 219-4. The corollary of this is that EGF-R is down-regulated in the majority of breast cancer cell lines. This finding may be counter-intuitive given the association between EGF-R expression and poor prognosis, however, it is in keeping with studies of primary breast cancer using sensitive radioimmunohistochemical techniques that showed that in the majority of tumours, EGF-R expression was lower than in normal cells (Dittadi *et al.*, 1993; Robertson *et al.*, 1996). The emergence of such data highlights the need to determine whether EGF-R expression per se has a role in breast tumour progression or whether relatively higher levels of EGF-R are only a reflection of ER negativity. These data also highlight the problems of ligand-binding analysis of EGF-R in tumour biopsies that may contain normal as well as malignant breast cells, and this potential complication may have contributed to inconsistent reports in the literature related to the role of EGF-R in breast cancer prognosis (Robertson *et al.*, 1996).

c-erbB-2 expression is independent of ER and EGF-R expression

There is increasing evidence that there is a link between c-*erb*B-2 and ovarian hormone receptor pathways, but the precise nature of this interaction is not clear. Ligand-induced activation of c-*erb*B-2 can lead to phosphorylation of ER protein and down-regulation of ER and ER transcripts (Pietras *et al.*, 1995; Sacedà *et al.*, 1996). Conversely, oestrogen treatment decreased expression of c-*erb*B-2 in breast cancer cell lines (Antoniotti *et al.*, 1992; Read *et al.*, 1990; Russell and Hung, 1992; Taverna *et al.*, 1994; Warri *et al.*, 1991), and under some circumstances the antioestrogen, tamoxifen, can induce expression of c-*erb*B-2 (Antoniotti *et al.*, 1992). Therefore, we asked whether the baseline expression of ER and c-*erb*B-2 is inversely correlated in breast cancer cells, as was found with ER and EGF-R. While a number of clinical studies found such a correlation between hormone receptor status and c-*erb*B-2 amplification or overexpression, an equivalent number failed to find a correlation with either ER or ER and PR (Gullick, 1990; Lupu and Lippman, 1993; Nicholson *et al.*, 1993; Quénel *et al.*, 1995; Tauchi *et al.*, 1989). In breast cell lines in culture, we found no significant correlation between ER and c-*erb*B-2 mRNA levels, and neither induced nor constitutive antisense-ER expression significantly altered c-*erb*B-2 mRNA levels in MCF-7 cells. It is therefore likely that the intrinsic level of ER expression is not a major component in the control of c-*erb*B-2 expression in breast cancer.

Although EGF-R and c-*erb*B-2 are both associated with poor prognosis in breast cancer, most studies have reported lack of statistically significant correlation between c-*erb*B-2 and EGF-R, in agreement with our findings in cell lines (Nicholson *et al.*, 1993; Nicholson *et al.*, 1994; Osaki *et al.*, 1992; Tauchi *et al.*, 1989; Toi *et al.*, 1994). In some studies, tumours have been substratified depending on the level of c-*erb*B-2 expression (Cuny *et al.*, 1994; Robertson *et al.*, 1996). In these studies, as in our cell line cohort, the highest level of c-*erb*B-2 expression was associated with gene amplification, however, significant overexpression was also found in the absence of gene amplification. Robertson *et al.* (1996) compared the expression of c-*erb*B-2 in the absence of gene amplification with EGF-R levels and found that expression of the 2 receptors was significantly inversely correlated. These data are in marked contrast with previous studies that had not differentiated tumours on the basis of gene amplification but are supported by the demonstration that EGF-R expression is decreased in breast cell lines engineered to overexpress c-*erb*B-2 (Aguilar *et al.*, 1999). We did not find a significant relationship between c-*erb*B-2 and EGF-R, either including or excluding cell lines with amplification of either c-*erb*B-2 or EGF-R. This lack of correlation may be a reflection of relatively small sample size or alternatively may support the view that expression of EGF-R and c-*erb*B-2 is con-

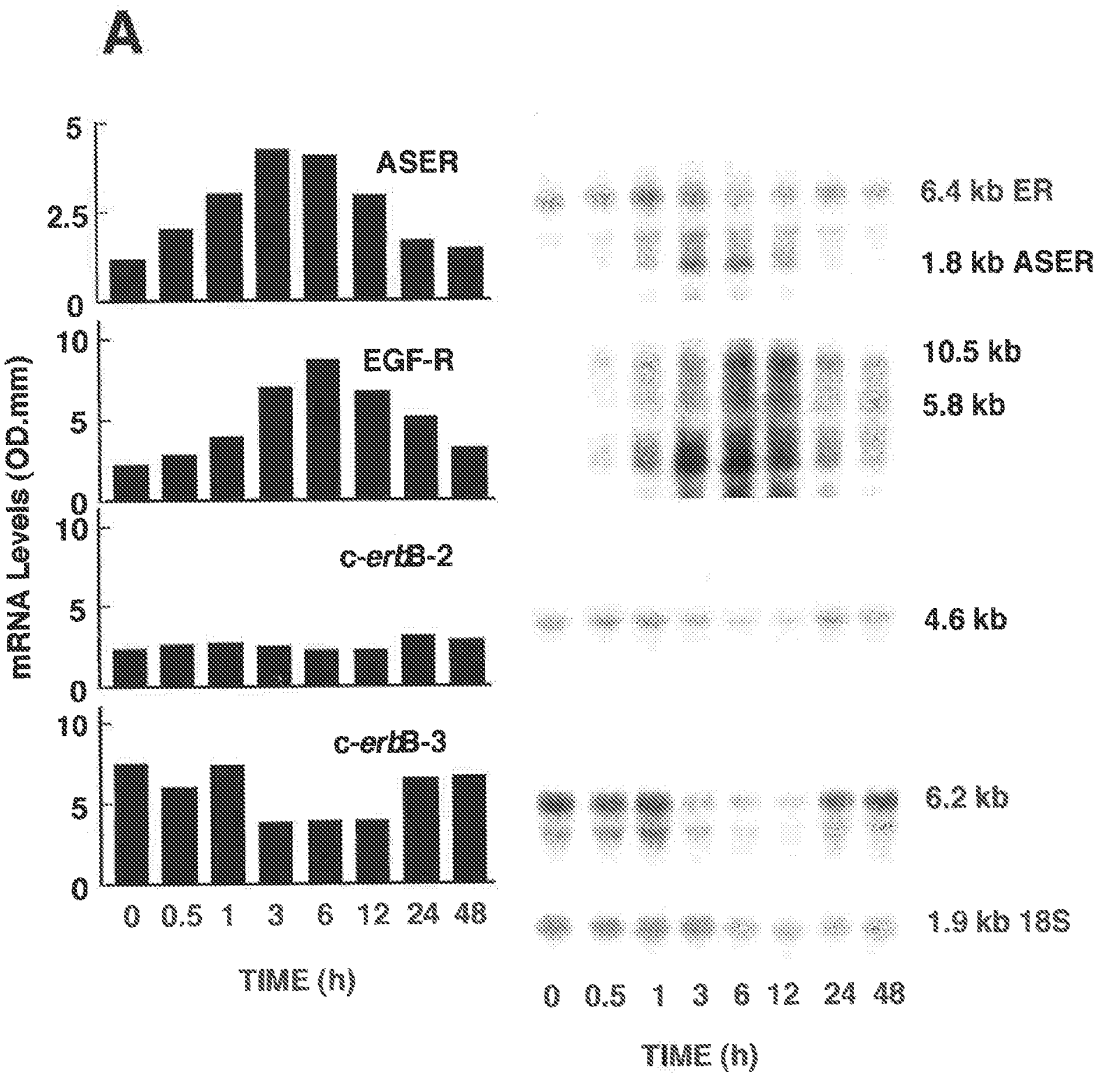


FIGURE 3 – Modulation of *erb*B gene expression following zinc induction of antisense-ER RNA. (*a*) RNA was isolated from MCF-7 cells transfected with antisense-ER (ASER) under the control of the zinc-inducible, human metallothionein II_A promoter at various times following addition of 50 μM $ZnSO_4$. Total RNA (20 μg/lane) was electrophoresed and blots hybridised with cDNA probes to ER, EGF-R, c-*erb*B-2, and -3. Blots were rehybridised with an 18S rRNA oligonucleotide probe to verify RNA gel loading and transfer to nylon membranes. Autoradiograms were exposed for 72 hr for ER, 48 hr for EGF-R, 6 days for c-*erb*B-2, 5 days for c-*erb*B-3, and 60 min for 18S rRNA. Autoradiograms were scanned densitometrically and the area of the resulting peaks were integrated. (*b*) Cells were transfected with pCMVASER and a constitutive antisense-ER (ASER) expression vector or a control vector (pCMV) were selected by growth in G418 and cloned by limiting dilution. Total RNA (20 μg/lane) was electrophoresed and blots hybridised with cDNA probes to ER, EGF-R, c-*erb*B-2, and c-*erb*B-3. Blots were rehybridised with a control 18S rRNA oligonucleotide probe to verify RNA gel loading and transfer to nylon membranes. Autoradiograms were exposed for 5 days for ER, 7 days for EGF-R, 4 days for c-*erb*B-2, 1 day for c-*erb*B-3, and 30 min for 18S rRNA.

trolled independently and that they are likely to have different physiological roles (Gompel *et al.*, 1996).

c-erbB-3 and ER are co-ordinantly expressed

c-*erb*B-3 was elevated in the majority of cancer cell lines tested, and mRNA levels were significantly correlated with ER. However, the relationship was not exclusive, that is, although all ER-positive cell lines expressed moderate to high levels of c-*erb*B-3, there were some ER-negative lines that also expressed elevated levels, suggesting that ER is not central to the control of basal c-*erb*B-3 expression. This conclusion was supported by lack of effect of constitutively expressed antisense-ER on c-*erb*B-3 expression and by failure to observe oestrogen-specific regulation of c-*erb*B-3 mRNA in MCF-7 cells in culture. Although one study found that *erb*B-3 expression was weakly associated with positive ER status and improved survival times over 10 years (Quinn *et al.*, 1994), the majority of studies failed to find a significant association between ER positivity and *erb*B-3 expression in breast tumours (Gasparini *et al.*, 1994; Lemoine *et al.*, 1992; Travis *et al.*, 1996).

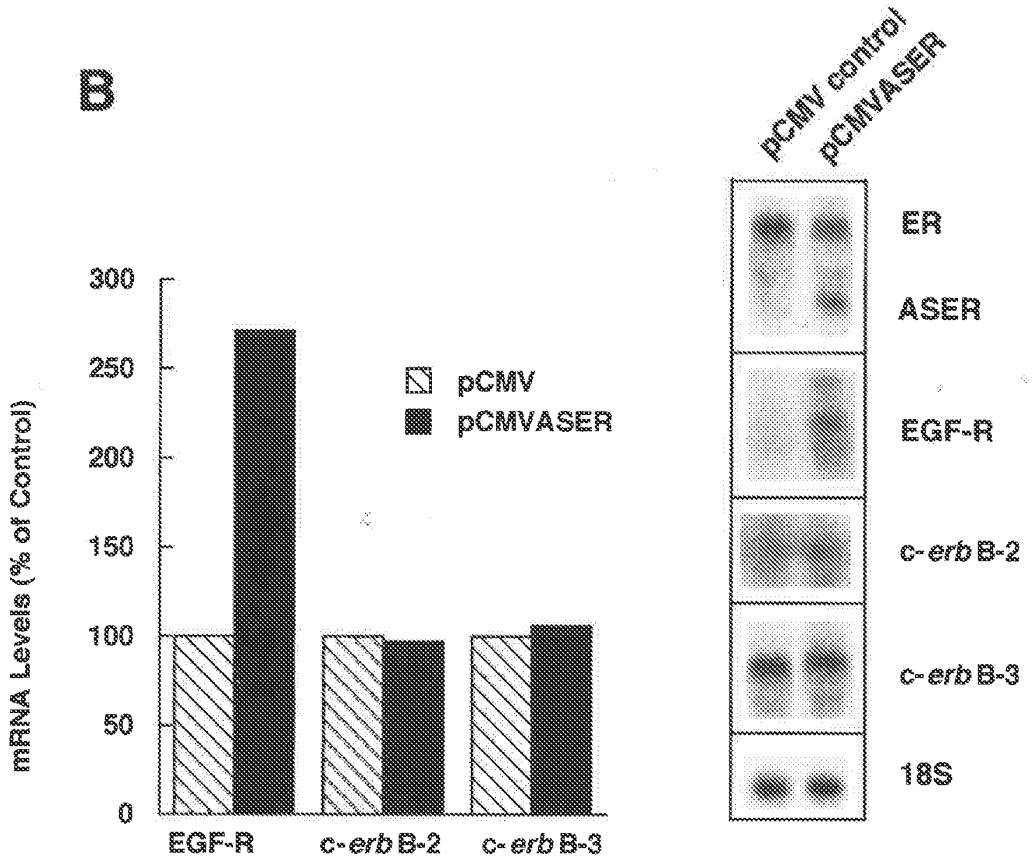


FIGURE 3 (CONTINUED)

A significant inverse correlation was found between c-*erb*B-3 and EGF-R in normal and malignant breast cell lines in agreement with the distinctly different pattern of distribution of the 2 receptors in normal tissues. c-*erb*B-3 is expressed at high level at sites where EGF-R is not detected, such as spinal ganglion cells, and conversely c-*erb*B-3 is absent from many tissues where EGF-R is prominent (Prigent *et al.*, 1992).

All cell lines with elevated levels of c-erbB-2 mRNA also expressed moderate to high levels of c-erbB-3

Heterodimerisation of c-*erb*B-3 and c-*erb*B-2 is required for a high-affinity HRG binding and subsequent signal transduction, and these 2 members of the *erb*B family cooperate in neoplastic transformation (Alimandi *et al.*, 1995; Lewis *et al.*, 1996; Sliwkowski *et al.*, 1994). It is therefore likely to be advantageous for tumour cell growth to co-express elevated levels of both receptors. We found that all cell lines with elevated levels of c-*erb*B-2 mRNA also expressed moderate to high levels of c-*erb*B-3, and a similar significant correlation between c-*erb*B-2 and c-*erb*B-3 has been found in both breast and ovarian tumours (Gasparini *et al.*, 1998; Scorcia *et al.*, 1998). One potential mechanism of co-expression of c-*erb*B-3 and c-*erb*B-2 is involvement of c-*erb*B-2 in control of expression of c-*erb*B-3 mRNA. This hypothesis is supported by the data of Aguilar *et al.* (1999), who have demonstrated an increase in c-*erb*B-3 expression in a proportion of breast cell lines engineered to overexpress c-*erb*B-2. A second potential mechanism is that transcription factors that control expression of the 2 receptors may be shared. One such factor is OB2-1, a transacting factor that has been reported to be involved in the transcription of both c-*erb*B-2 and -3 (Skinner and Hurst, 1993).

c-erbB-4 is not frequently expressed in breast cell lines

c-*erb*B-4 mRNA was only detected in 3 breast cancer cell lines, ZR-75-1, MDA-MB-361, and BT-474. To our knowledge, c-*erb*B-4 has not previously been examined in ZR-75-1 cells, and MDA-MB-361 have previously been reported to express intermediate levels of c-*erb*B-4 mRNA, measured by RT-PCR (Plowman *et al.*, 1993). Others have reported difficulty in detecting any c-*erb*B-4 in breast cancer cell lines by Northern blot analysis (Chan *et al.*, 1995) and the levels measured using RT-PCR are inconsistent, e.g., Plowman *et al.* (1993) reported MDA-MB-453 cells to be among those with the highest c-*erb*B-4 expression, whereas Chan *et al.* (1995) found none in this cell line. We therefore examined c-*erb*B-4 at the protein level in a panel of breast cell lines in addition to measuring mRNA levels by Northern blot analysis and found the results to be highly consistent. Therefore, while c-*erb*B-4 may be detected in additional cell lines using RT-PCR or other more sensitive methods, we did not find this receptor to be widely expressed at significant levels in malignant and normal breast cell lines and agree with Chan *et al.* (1995) that c-*erb*B-4 is likely to only play a minor role in HRG-mediated signal transduction in mammary cells. This data is also consistent with that of Aguilar *et al.* (1999), who reported that the expression of c-*erb*B-4 in breast cells, measured using a sensitive ELISA assay, is considerably lower than that of the other *erb*B receptors.

The cell lines that expressed c-*erb*B-4 were also ER positive, and although it is not possible to draw any conclusions based on such a small number of cell lines, ER and c-*erb*B-4 were also significantly positively correlated in mammary carcinoma specimens, suggesting that expression of c-*erb*B-4 may be associated with a more favourable outcome (Bacus *et al.*, 1996).

Heregulin is expressed in ER-negative breast cancer cell lines and is not expressed in cell lines with increased expression of c-erbB-2 or -3

HRG was found to be more frequently expressed in cell lines that were ER negative and expressed moderate levels of EGF-R receptor. In breast tumours, expression of ER is associated with a good prognosis and EGF-R is associated with a poorer prognosis, as discussed above. Therefore, the pattern of HRG expression observed in cell lines would suggest that relatively high levels of HRG may be associated with breast tumours with a more aggressive phenotype. A similar conclusion was drawn in other studies showing that overexpression of HRG by gene transfection resulted in oestrogen independence and increased metastatic ability (Lupu *et al.*, 1996; Tang *et al.*, 1996). In human breast carcinomas, a higher percentage of ER-negative tumours expressed HRG compared with ER-positive tumours (Normanno *et al.*, 1995), and in agreement with our data, HRG was expressed preferentially in ER-negative cells in a smaller panel of breast cancer cell lines (Normanno *et al.*, 1993). However, the precise role of HRG in breast cancer is far from clear. In a small patient cohort, HRG expression in peritumour stromal cells correlated with disease recurrence rather than staining in the tumour cells themselves (Visscher *et al.*, 1997). Such data suggest a paracrine role for HRG in breast cancer.

Highest expression of HRG was found in the HMEC 219-4 cell line, derived from normal mammary epithelium. HRGα is expressed in the normal mouse mammary gland; expression is increased during pregnancy and exogenously added HRGα increased ductal growth, side branching, and secretion, suggesting that HRGα is involved in differentiation of the mammary epithelium into milk-secreting lobuloalveoli (Jones *et al.*, 1996; Yang *et al.*, 1995).

None of the cell lines expressing HRG mRNA expressed appreciable levels of receptor mRNA, either c-*erb*B-3 or c-*erb*B-4, except for the cell line MDA-MB-175, which was found to express aberrant HRG. HRGs have been reported to promote growth, induce differentiation and inhibit proliferation depending on the concentration and cell lines examined (Lupu *et al.*, 1996). One reason for the inverse relationship between ligand and receptors may be that co-expression of high levels of both may not be conducive to cell growth, i.e., while HRGs are known to stimulate growth in the short term, constant stimulation of the pathway may lead to differentiation. This contention is not supported by the extensive study of Aguilar *et al.* (1999), who found growth stimulation in breast cell lines in both short- and long-term *in vitro* proliferation assays, in addition to stimulation of proliferation by HRG in tumours grown in nude mice, with no evidence of growth inhibition by HRGs. Therefore, another explanation for the pattern of exclusive expression of either ligand or receptors may be that HRGs function in a paracrine manner in the normal mammary gland and that epithelial cells within the mammary gland are phenotypically divergent, expressing either the ligand, HRG, or receptors, *erb*B-3/*erb*B-2, *erb*B-4/*erb*B-2. These different cell types may then give rise to tumours that retain some of these divergent features. This proposal is consistent with recent data showing that in the normal human breast the majority of proliferating cells are ER negative (Clarke *et al.*, 1997). Therefore, ER-negative (HRG-positive, EGF-R-positive) tumours may arise directly from ER-negative cells. Such a suggestion is contrary to the long-held belief that common epithelial breast tumours invariably arise from a small population of ER-positive cells and that loss of ER expression occurs during tumour progression.

All the breast cancer cell lines examined in this study expressed at least one member of the *erb*B family of receptors, supporting a central role for type I tyrosine kinase receptors in breast cancer biology and reflecting the interest in the *erb*B family and associated signal transduction pathways as attractive targets for cancer therapy. The pattern of expression of each receptor was found to be unique, and the HRG ligands were not co-expressed with their receptors, suggesting that they may act in a paracrine rather than autocrine manner. There is, however, a growing list of ligands that bind to the various members of the *erb*B family and their expression and function in breast cancer are yet to be fully investigated.

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appeared normal since none of them demonstrated any morphologic abnormality or rearranged bands (Fig. 2B, lanes 8, 9, 10, and 12). However, hybrid DNA sequences were markedly amplified and thus clearly detected by the PCR technique in samples obtained from patient 1 (Fig. 3C, lanes 4 through 6 and Fig. 4, lanes 5 and 6), indicating the presence of residual neoplastic cells carrying the t(14;18). Even though the hybrid 18q21–J_H DNA sequences were amplifiable in the pretreatment tumor sample obtained from patient 2 (Fig. 3C, lane 7), no hybrid DNA sequences were detected in the remission marrow sample by the PCR technique (Fig. 3C, lane 8). These findings indicate that the concentration of neoplastic cells carrying the t(14;18) was too low to be detected by our current techniques or the patient was completely free of tumor and there were no t(14;18) target DNA sequences present for amplification.

We have shown the feasibility of using the PCR technique to detect minimal numbers of neoplastic cells carrying a chromosomal translocation. Detection of small numbers of circulating monoclonal B cells by flow cytometry or clonal immunoglobulin gene rearrangement in patients with follicular lymphoma in remission has been reported (*1*, *16*). The sensitivity of our approach far exceeds the sensitivity limit achieved by conventional Southern blot analysis or the flow cytometric method. Detection of minimal neoplastic cells by means of chromosomal translocation and PCR will make it possible to address the following important biological and clinical questions that could not be answered before. Do patients in long-term remission have quiescent tumor cells with proliferative potential? Can detection of minimal residual tumor cells predict early relapse? Do patients with persistent minimal residual disease after prolonged treatment require non–cross-resistant therapy to prevent relapse? The answers will help in understanding tumor biology and designing strategies for cancer treatment.

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*erb*B-2 Is a Potent Oncogene When Overexpressed in NIH/3T3 Cells

PIER PAOLO DI FIORE, JACALYN H. PIERCE, MATTHIAS H. KRAUS, ORESTE SEGATTO, C. RICHTER KING, STUART A. AARONSON

A wide variety of human tumors contain an amplified or overexpressed *erb*B-2 gene, which encodes a growth factor receptor–like protein. When *erb*B-2 complementary DNA was expressed in NIH/3T3 cells under the control of the SV40 promoter, the gene lacked transforming activity despite expression of detectable levels of the *erb*B-2 protein. A further five- to tenfold increase in its expression under influence of the long terminal repeat of Moloney murine leukemia virus was associated with activation of *erb*B-2 as a potent oncogene. The high levels of the *erb*B-2 product associated with malignant transformation of NIH/3T3 cells were observed in human mammary tumor cells that overexpressed this gene. These findings demonstrate a new mechanism for acquisition of oncogenic properties by genes encoding growth factor receptor–like proteins and provide a functional basis for the role of their overexpression in the development of human malignancies.

EVIDENCE THAT RETROVIRAL ONCOgenes can encode proteins that are homologous to either growth factors or growth factor receptors has shed new light on the mechanisms by which cells become malignant. For example, v-*sis* is derived from the gene encoding one chain of the platelet-derived growth factor (*1*). In addition, v-*erb*B and v-*fms* are derived from the genes encoding the epidermal growth factor (EGF) (*2*) and colony-stimulating factor–1 (CSF-1) (*3*) receptors, respectively. A further linkage between growth control and oncogenes is the common tyrosine kinase activity of many growth factor receptors (*4*) and several viral oncogene products (*5*). These connections have motivated a search in nonvirally induced neoplasia for abnormalities of genes involved in the pathways by which growth factors stimulate normal cell growth.

Shih *et al.* identified a transforming gene in ethylnitrosourea (ENU)-induced rat neuroblastomas by means of the NIH/3T3 transfection assay (*6*). This oncogene, termed *neu*, was found to encode a product immunologically related to the *erb*B/EGF receptor (*7*) and to exhibit a cell surface location. Independently, we cloned and partially sequenced an *erb*B-related gene that was detected because of its amplification in a human mammary carcinoma (*8*). This same human sequence, designated *erb*B-2 (*9*, *10*) or HER-2 (*11*), was also cloned from normal genomic and complementary DNA (cDNA) libraries, respectively. Sequence analysis has shown that the structure of the predicted *erb*B-2/HER-2 product has significant homology to the EGF receptor (*9*, *11*). Several lines of evidence, including sequence analysis and chromosomal mapping, indicate that *neu* is an activated rat homolog of human *erb*B-2/HER-2 (*9*, *11*–*14*).

Thus far, the established mechanisms that activate genes encoding growth factor receptors to become oncogenes appear to involve structural alterations of their coding sequences. These include truncation or substitutions (*15*) as well as mutational alterations (*14*). While growth factor receptor–like genes with altered coding sequences have been readily detected as transforming genes by DNA transfection analysis, human tumors containing such amplified or overexpressed genes have not yielded transforming genes detectable by this approach (*8*, *10*). These findings have suggested that overexpression alone may convert the gene for a normal growth factor receptor into an oncogene. In the present studies, we have investigated the effects of overexpression of the normal coding sequence for the growth

Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, MD 20892.

Table 1. Transfection analysis of *erb*B-2 expression vectors.

DNA clone	Transformed foci/plate with DNA added (μg)*				Specific transforming activity† (FFU/pmol)
	1	0.1	0.01	0.001	
LTR-1/*erb*B-2	TMC	TMC	>100	7,3	4.1×10^6
LTR-2/*erb*B-2	TMC	TMC	30,21	4,1	2.0×10^6
SV40/*erb*B-2	0,0	0,0	0,0	0,0	$<10^5$
LTR/*ras*	TMC	TMC	45,50	6,3	3.6×10^6
pSV2/gpt	0,0	0,0	NT	NT	$<10^5$

*Transfection was performed with 40 μg of calf thymus DNA as carrier by means of the calcium phosphate precipitation technique (*27*). Focus formation on NIH/3T3 cells was scored at 14 to 21 days on duplicate plates. DNA added is shown as micrograms per plate. †Focus-forming units were adjusted to focus-forming units per picomole of cloned DNA added, based on the relative molecular weights of the respective plasmids. NT, not tested; TMC, too many to count.

factor receptor–like human *erb*B-2 gene on growth properties of NIH/3T3 cells. We demonstrate that levels of this gene product comparable to those observed in some human cancers are sufficient to induce neoplastic transformation.

To directly assess the effects of overexpression of the *erb*B-2 gene on cell growth properties, we assembled a full-length normal human *erb*B-2 clone from a series of overlapping clones previously described (*10*). Two nucleotide sequences for *erb*B-2 cDNA have been published (*9, 11*). Our sequence agrees with that of Coussens *et al.* (*11*) with the exception of a neutral change of isoleucine for valine at position 654 and six contiguous amino acid substitutions caused by a difference in reading frame between positions 518 and 523. In this latter region, our nucleotide sequence is identical to that published by Yamamoto *et al.* (*9*). An amino acid substitution of glutamic acid for valine in the putative transmembrane region of the normal rat homolog of *erb*B-2 has been shown to activate the transforming capacity of the *neu* oncogene in ENU-induced rat neuroblastomas (*14*). Our human *erb*B-2 cDNA contained the normal valine at this position.

Expression vectors based on the transcriptional initiation sequences of either the Moloney murine leukemia virus long terminal repeat (Moloney-MuLV LTR) or the SV40 early promoter were constructed in an attempt to express the *erb*B-2 cDNA at different levels in NIH/3T3 cells (Fig. 1) (*16*). Previous studies have indicated different strengths of LTR and the SV40 promoters in these cells (*17*). Upon transfection into NIH/3T3 cells, both LTR-1/*erb*B-2 and LTR-2/*erb*B-2 DNAs induced transformed foci at high efficiencies of 4.1×10^6 and 2.0×10^6 focus-forming units per picomole of DNA (FFU/pmol), respectively (Table 1). In striking contrast, the SV40/*erb*B-2 construct failed to induce any detectable morphological alteration of NIH/3T3 cells transfected under identical assay conditions.

Antiserum prepared against a peptide (anti-peptide) derived from amino acid residues 866 to 880, within the tyrosine kinase domain of the predicted human *erb*B-2 protein (*8, 9, 11*), was utilized to analyze the expression of the human *erb*B-2 protein in the transfectants. As shown in Fig. 2A, LTR-1/*erb*B-2 and LTR-2/*erb*B-2 transfectants each contained a 185-kD protein that was readily recognized by the anti-peptide but not preimmune serum; this recognition was specifically competed out by preabsorption of the anti-peptide with excess peptide. Cells transfected with the SV40/*erb*B-2 vector also produced the 185-kD protein, but at lesser amounts (Fig. 2A). Analysis of several clonally derived cell lines containing either LTR or SV40/*erb*B-2 constructs consistently revealed five- to tenfold higher levels of the 185-kD protein in cells containing the LTR expression vector (Fig. 2B). Since the SV40/*erb*B-2 construct lacked transforming activity, these results demonstrated that the higher levels of *erb*B-2 expression under LTR influence correlated with its ability to exert transforming activity.

The normal ligand for the *erb*B-2 receptor–like protein has yet to be identified. If present in serum, this ligand might be responsible for stimulating the overexpressed *erb*B-2 product and triggering its transforming ability. Thus, we investigated whether *erb*B-2–transformed cells maintained their altered phenotype when cultured in medium lacking serum (*18*). In this medium, LTR-1/*erb*B-2–transfected cells continued to exhibit a stable, transformed phenotype by growing as foci of densely packed cells.

To directly establish that a ligand-receptor interaction was not required for transformation by the *erb*B-2 protein, we engineered both LTR- and SV40-driven constructs such that *erb*B-2 sequences encoding the NH_2-terminal 621 amino acids were deleted (*19*). When the biological activities of these constructs were analyzed, the LTR-driven truncated *erb*B-2 cDNA, designated LTR-1/Δ*N*-*erb*B-2, exhibited a transforming activity of 3.5×10^5 FFU/pmol, almost tenfold greater than that of LTR-1/*erb*B-2. The transforming efficiencies of the *erb*B-2 and truncated *erb*B-2 coding sequences under LTR influence were at least comparable to those of known oncogenes such as v-H-*ras* and avian v-*erb*B (*20*). Moreover, the truncated *erb*B-2 sequence under the control of an SV40 promoter demonstrated a readily detectable transforming ability of around

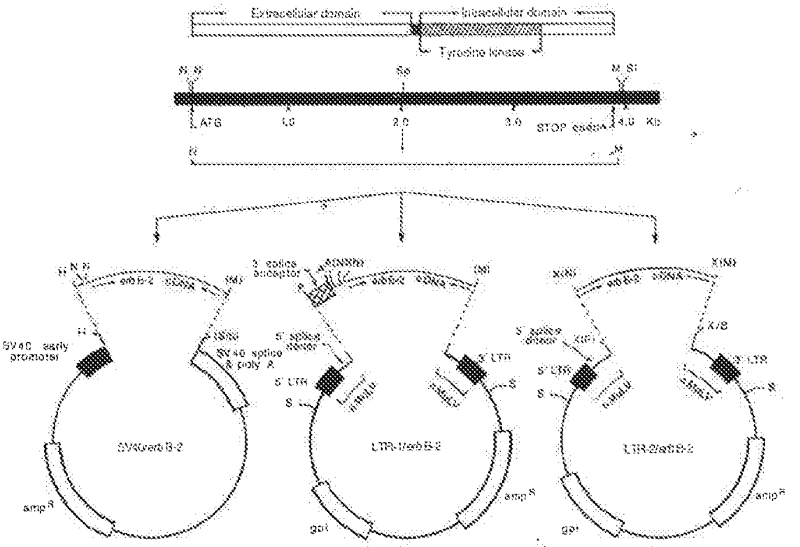


Fig. 1. Construction of expression vectors for the human *erb*B-2 cDNA. A Nco I–Mst II fragment encompassing the entire *erb*B-2 open reading frame was cloned under the transcriptional control of either the SV40 early promoter or Moloney-MuLV LTR. Crosshatched box (LTR-1/*erb*B-2), *erb*A–*erb*B intergenic region of pAFH containing the 3' splice acceptor site. Abbreviations: N, Nco I; Sp, Sph I; M, Mst II; St, Stu I; H, Hind III; Sm, Sma I; P, Pst I; B, Bam HI; and X, Xho I. Sites indicated in parenthesis were not reconstituted after the cloning procedures.

Table 2. Anchorage-independent growth and tumorigenicity of *erb*B-2-transformed cells.

DNA transfectant*	Colony-forming efficiency in agar (%)†	Cell number for 50% tumor incidence‡
LTR-1/*erb*B-2	45	10^3
LTR-1/ΔN*erb*B-2	70	$<10^3$
SV40/*erb*B-2	<0.01	$>10^6$
LTR/*erb*B	20	5×10^4
LTR/*ras*	35	10^3
pSV2/gpt	<0.01	$>10^6$

*All transfectants were isolated from plates that received 1 μg of cloned DNA and were selected by their ability to grow in the presence of killer HAT medium (*28*). †Cells were plated at tenfold serial dilutions in 0.33% soft agar medium containing 10% calf serum. Visible colonies comprising >100 cells were scored at 14 days. ‡NFR nude mice were inoculated subcutaneously with each cell line. Ten mice were tested at cell concentrations ranging from 10^6 to 10^2 cells per mouse. Tumor formation was monitored at least twice weekly for up to 30 days.

3.9×10^2 FFU/pmol. The largest and most actively growing foci were observed with the truncated *erb*B-2 coding sequence under LTR influence (Fig. 3). All of these findings suggested that NH_2-terminal truncation, if anything, increased the transforming activity of the *erb*B-2 protein.

To investigate expression of the truncated *erb*B-2 protein under SV40 and LTR influence, mass cell populations transformed by either construct were subjected to immunoprecipitation analysis with the anti-*erb*B-2 peptide serum. This antiserum specifically detected protein(s) migrating in the range of 80 to 90 kD (Fig. 2C). Both full-length and truncated *erb*B-2 proteins were larger than their predicted amino acid lengths by about 45 and 15 to 20 kD, respectively. This is likely due to post-translational modification by glycosylation, which has been reported for the rat homolog of *erb*B-2 (*21*). Cells transformed by the SV40/Δ*Nerb*B-2 construct expressed five- to tenfold lower levels of the truncated protein than did LTR/Δ*Nerb*B-2 transformants (Fig. 2C). These results further implied that NH_2-terminal truncation constitutively activated transforming activity of the *erb*B-2 gene at a lower level of expression. Both full-length and truncated *erb*B-2 proteins segregated with the subcellular membrane fraction, suggesting that their sites of action were localized to the cell membrane (*22*).

To compare the growth properties of NIH/3T3 cells transfected by these genes, we analyzed the transfectants for anchorage-independent growth in culture. The colony-forming efficiencies of both LTR-1/*erb*B-2 and LTR-1/Δ*Nerb*B-2 transfectants were very high and comparable to those of LTR-driven v-H-*ras* and v-*erb*B transfectants (Table 2). Moreover, the LTR-1/*erb*B-2 transfectants were as malignant in vivo as cells transformed by the highly potent v-H-*ras* oncogene and 50-fold more tumorigenic than cells transfected with v-*erb*B. The LTR-1/Δ*Nerb*B-2–transfected cells were the most potent of all, inducing tumors within 3 weeks in all mice inoculated with 10^2 cells. In contrast, the SV40/*erb*B-2 transfectant failed to display anchorage-independent growth in vitro and did not grow as tumors in nude mice even when 10^6 cells were injected (Table 2).

The *erb*B-2 gene has been reported to be amplified or overexpressed (or both) in a wide variety of human malignancies (*8, 10, 13, 23*). We sought to compare the level of overexpression of the *erb*B-2–encoded 185-kD protein in human mammary tumor cell lines possessing amplified *erb*B-2 genes with that of NIH/3T3 cells transformed by the *erb*B-2 coding sequence. Human mammary tumor cell lines, SK-BR-3 and MBA-MB361, each exhibit *erb*B-2 gene amplification and overexpress a single normal-sized *erb*B-2 transcript (*10*). The antiserum against the *erb*B-2 product detected several discrete protein species ranging in size from 150 to 185 kD in extracts of MDA-MB361 and SK-BR-3 mammary tumor cell lines, as well as in LTR/*erb*B-2 NIH/3T3 transformants (Fig. 4). The relative levels of the 185-kD *erb*B-2 product were similar in each cell line and markedly elevated over that expressed by MCF-7 cells, a mammary adenocarcinoma cell line displaying no *erb*B-2 gene amplification or overexpression (Fig. 4). Thus, human mammary tumor cells that overexpressed the *erb*B-2 gene had levels of the *erb*B-2 gene product capable of inducing malignant transformation in a model system.

Our present studies demonstrate that the human *erb*B-2 gene can be activated as an oncogene by its overexpression in NIH/3T3 cells. The level of the *erb*B-2 product was shown to be critical in determining its trans-

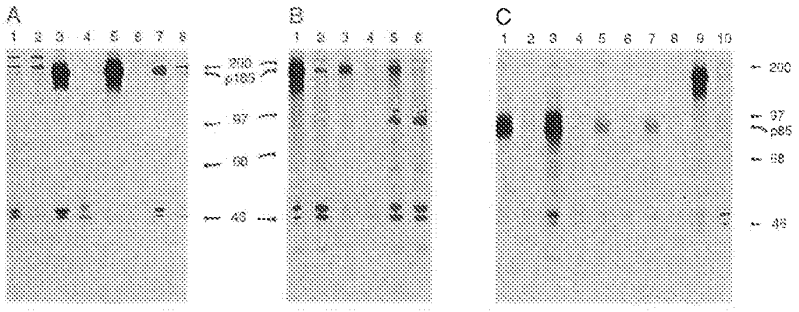


Fig. 2. Immunoprecipitation and electrophoretic analysis of *erb*B-2–specific products in *erb*B-2–transfected NIH/3T3 cells under LTR or SV40 promoter control. LTR-1/*erb*B-2, LTR-2/*erb*B-2, and LTR/Δ*Nerb*B-2 transfectants were isolated from plates that received 1 μg of cloned DNA and were selected by their ability to grow in the presence of killer HAT medium (*28*). SV40/*erb*B-2 and SV40/Δ*Nerb*B-2 transfectants were obtained by cotransfecting the cloned DNA (1 μg) with pSV2/gpt at a molar ratio of 20:1 (SV40/*erb*B-2:pSV2/gpt) and selecting for growth in killer HAT medium (*28*). (**A**) Cell extracts from [^{35}S]methionine-labeled mass populations of LTR- or SV40-driven *erb*B-2 transfectants were treated with rabbit anti-*erb*B-2 peptide serum directed against amino acid residues 866 to 880 (lanes 1, 3, 5, and 7) or preimmune serum (lanes 2, 4, 6, and 8). Lanes 1 and 2, NIH/3T3; lanes 3 and 4, LTR-1/*erb*B-2; lanes 5 and 6, LTR-2/*erb*B-2; lanes 7 and 8, SV40/*erb*B-2. (**B**) [^{35}S]methionine-labeled extracts from clonally derived LTR- or SV40-driven *erb*B-2 cultures were immunoprecipitated with the above-mentioned antibody alone (lanes 1, 3, and 5) or with antibody that had been preadsorbed for 30 minutes with the specific competing peptide (lanes 2, 4, and 6). Lanes 1 and 2, LTR-1/*erb*B-2 cl1; lanes 3 and 4, SV40/*erb*B-2 cl1; lanes 5 and 6, SV40/*erb*B-2 cl2. (**C**) [^{35}S]methionine extracts of mass populations of cells transfected with the NH_2-truncated *erb*B-2 under either LTR or SV40 influence and marker-selected, as indicated above, were immunoprecipitated with antibody alone (lanes 1, 3, 5, 7, and 9) or antibody-preadsorbed with the specific competing peptide (lanes 2, 4, 6, 8, and 10). Lanes 1 and 2, LTR-1/Δ*Nerb*B-2 mass population #1; lanes 3 and 4, LTR-1/Δ*Nerb*B-2 mass population #2; lanes 5 and 6, SV40/Δ*Nerb*B-2 mass population #1; lanes 7 and 8, SV40/Δ*Nerb*B-2 mass population #2; lanes 9 and 10, LTR-1/*erb*B-2. Sizes are shown in kilodaltons.

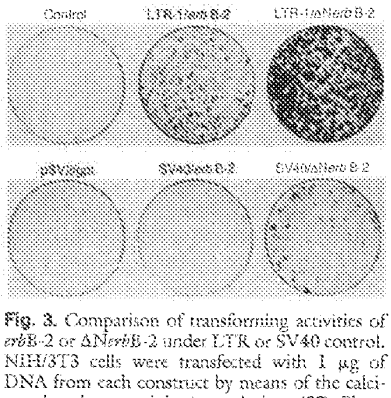


Fig. 3. Comparison of transforming activities of *erb*B-2 or Δ*Nerb*B-2 under LTR or SV40 control. NIH/3T3 cells were transfected with 1 μg of DNA from each construct by means of the calcium phosphate precipitation technique (*27*). Plates were stained with hematoxylin at 14 days after transfection. Control, calf thymus DNA. pSV2/gpt was also used as a negative control.

forming ability. An SV40-driven *erb*B-2 cDNA construct lacked detectable focus-forming ability despite the fact that NIH/3T3 cells containing this construct exhibited readily detectable levels of the *erb*B-2 protein. When the same *erb*B-2 cDNA was placed under LTR control, a further five- to tenfold increase in expression of the *erb*B-2 product was associated with the acquisition of transforming properties by the gene. Previous studies have indicated that a 50-fold amplification of the normal rat homolog of *erb*B-2 under SV40 promoter influence was insufficient to induce the malignant phenotype (*24*). Whether its gene product was expressed at as high a level as that of our transforming LTR-*erb*B-2 construct or whether normal human *erb*B-2 is inherently more active as a transforming gene than its rat homolog when overexpressed remains to be elucidated.

An increased number of growth factor receptors may cause transformation by raising the level of constitutive tyrosine kinase activity (*4*) to a threshold required for growth stimulation or by facilitating receptor-receptor interactions that may be a prerequisite for their activation. As the ligand for the *erb*B-2 protein has yet to be identified, it is possible that *erb*B-2 transformed cells themselves might produce this ligand; thus, the receptor might be chronically stimulated. Since NH_2-terminal truncation of the *erb*B-2 coding sequence removed its extracellular ligand-binding domain, the need for a ligand for the truncated protein can be excluded. This protein was shown to transform when expressed at significantly lower level than that required for activity of the full-length *erb*B-2 product. Investigation of the tyrosine kinase activity and processing of the truncated as compared to the normal *erb*B-2 receptor protein should provide insight into the basis for its enhanced transforming activity.

The v-*erb*B oncogene, which represents a truncated version of the avian EGF receptor, was found to be considerably less effective than either full-length or truncated *erb*B-2 coding sequences in inducing the neoplastic phenotype. Preliminary evidence indicates that the human EGF receptor coding sequence is also much less active as a transforming gene when overexpressed in NIH/3T3 cells (*22*). Thus, the growth signals generated by these two related receptors appear to differ in some way. Investigation of recombinants between the EGF receptor and *erb*B-2 coding sequences should make it possible to identify those regions that confer more potent transforming activity to the *erb*B-2 protein.

There is considerable evidence that genes encoding growth factor receptors can be activated as transforming genes by structural alterations in their coding sequences (*3, 13, 15*). In contrast, abnormalities involving growth factor receptors in human malignancies appear most commonly to involve their overexpression in the absence of structural alterations (*8, 10, 13, 23, 25, 26*). In addition, amplification of *erb*B-2 has been reported to be an important prognostic indicator of a more aggressive phenotype in mammary adenocarcinomas (*26*). Our results demonstrate that human tumors with *erb*B-2 gene amplification can express the *erb*B-2 gene product at very high levels comparable to LTR/*erb*B-2–transformed NIH/3T3 cells. Thus, our studies establish a mechanistic basis for amplification of the growth factor receptor gene as representing a causal driving force in the clonal evolution of a tumor cell rather than being an incidental consequence of tumorigenesis.

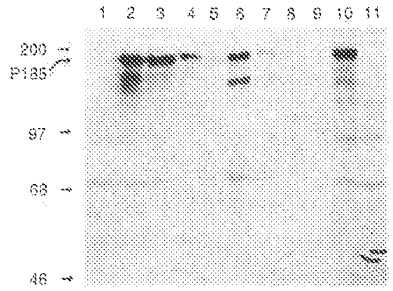


Fig. 4. Comparison of the levels of *erb*B-2 proteins in LTR-1/*erb*B-2–transformed NIH/3T3 cells and human mammary tumor lines by immunoblot analysis. Varying amounts of total cellular protein were separated by electrophoresis and transferred to nitrocellulose filters. The *erb*B-2 protein was detected with rabbit anti–*erb*B-2 peptide serum coupled to ^{125}I-labeled protein A as described (*10*). Lane 1, NIH/3T3 (40 μg); lane 2, LTR-1/*erb*B-2 (40 μg); lane 3, LTR-2/*erb*B-2 (40 μg); lane 4, LTR-2/*erb*B-2 (13 μg); lane 5, LTR-2/*erb*B-2 (4.5 μg), lane 6, SK-BR-3 (40 μg); lane 7, SK-ER-3 (13 μg); lane 8, SK-BR-3 (4.5 μg); lane 9, SK-BR-3 (1.5 μg); lane 10, MDA-MB361 (40 μg); and lane 11, MCF-7 (40 μg). Sizes are shown in kilodaltons.

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19. To construct expression vectors for 5′-truncated *erb*B-2 cDNA, the Sph I–Sma I fragment of the *erb*B-2 cDNA was cloned into the LTR-1 or SV40-based expression vectors previously described (Fig. 1) (*16*). The translation of the truncated *erb*B-2 protein begins at a methionine 32 amino acids proximal to the transmembrane region. The Sph I–Sma I fragment was cloned into the Hind III–Sma I of pSV2/*neo* vector (described in the legend to Fig. 1) to obtain SV40/Δ*Nerb*B-2. Joining between the Hind III and Sph I sites at the 5′ end of the coding sequence was obtained with an oligonucleotide of the following structure: 5′-AGCTTCAGGCCCCAGACCACTGCATGGCATG-3′. This oligonucleotide contained an in-frame ATG to initiate the translation of the truncated *erb*B-2 protein. LTR-1/Δ*Nerb*B-2 was engineered essentially following the same strategy outlined in Fig. 1 for construction of LTR-1/*erb*B-2. The joining between the Apa I site and the 5′ Sph I site of the *erb*B-2 cDNA was obtained using the following adapter oligonucleotide: 5′-CAGACCACTGCATGGCATG-3′. This oligonucleotide contained an in-frame ATG to direct the synthesis of an *erb*B-2 protein. All of the above constructions were sequenced in the junction regions to verify that the predicted structure was achieved after the recombination procedures.
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29. C. Kinsley provided excellent technical assistance.

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Variable Occurrence of the *nrd*B Intron in the T-Even Phages Suggests Intron Mobility

JOAN PEDERSEN-LANE AND MARLENE BELFORT

The bacteriophage T4 *nrd*B gene, encoding nucleoside diphosphate reductase subunit B, contains a self-splicing group I intervening sequence. The *nrd*B intron was shown to be absent from the genomes of the closely related T-even phages T2 and T6. Evidence for variable intron distribution was provided by autocatalytic ^{32}P-guanosine 5′-triphosphate labeling of T-even RNAs, DNA and RNA hybridization analyses, and DNA sequencing studies. The results indicate the nonessential nature of the intron in *nrd*B expression and phage viability. Furthermore, they suggest that either precise intron loss from T2 and T6 or lateral intron acquisition by T4 occurred since the evolution of these phages from a common ancestor. Intron movement in the course of T-even phage divergence raises provocative questions about the origin of these self-splicing elements in prokaryotes.

WHILE SELF-SPLICING RNAs ARE remarkable for their autocatalytic properties (*1*), there is mounting evidence implicating these introns as mobile elements. There are several examples where the presence of eukaryotic group I introns is not uniform in equivalent genes of closely related species [for example, the large ribosomal RNA (rRNA) of *Tetrahymena* (*2*)] or even in different strains of the same species [for example, the large rRNA of *Saccharomyces cerevisiae* mitochondria (*3*)]. In the latter case site-specific transposition of the intron from intron$^+$ to intron$^-$ copies of the gene, with the aid of an intron-encoded endonuclease, has been demonstrated (*3*). Furthermore, there is evidence (based on conserved features of sequence and structure) for the transfer of group I introns between different fungal mitochondrial genomes (*4*) and between fungal mitochondrial genes and the *Tetrahymena* nucleus (*5*). Surprisingly, the protein potentially encoded by the intron open reading frame (ORF) of gene *td* in bacteriophage T4 (*6*) shares homology with intron ORFs of three group I mitochondrial intervening sequences of filamentous fungi (*7*). This observation led to speculations on a recent genetic exchange between these fungi and the genome of T4 (*7*).

In the face of these provocative observations that argue in favor of intron mobility, we have investigated the distribution of the group I *nrd*B intron of phage T4 (*8, 9*) in the T-even phage family. We provide evidence that the intron in the T4 *nrd*B gene, which encodes the B subunit of ribonucleoside diphosphate reductase, is absent from the genomes of T2 and T6. These results indicate the dispensability of the *nrd*B intron for T-even phage viability and imply movement of the T4 *nrd*B intron since the divergence of the closely related T-even phages from their common ancestor.

The existence of multiple T4 genes encoding group I self-splicing RNAs was inferred by labeling T4 RNA with ^{32}P-guanosine 5-triphosphate (GTP) under self-splicing conditions (*8*). In addition to a labeled band corresponding to the previously identified *td* intron (*6, 10*), several other labeled species were seen. The most intense of these other bands corresponds to the 0.6-kb *nrd*B intron (*8*). The profile of labeled bands was strikingly different in a similar autocatalytic ^{32}P-GTP labeling experiment with T2 and T6 RNAs (Fig. 1). While the *td* band appeared to be present in all three phages, the 0.6-kb *nrd*B species was apparently absent from T2 and T6 RNA. The *td* intron is indeed homologous throughout the T-even phages, as demonstrated by hybridization and dideoxy primer-extension analysis of pre–messenger RNA (mRNA) and splice products (*11*). In contrast, similar experiments argue in favor of the variable occurrence of the *nrd*B intron in the T-even phage family.

Dot-blot analysis revealed that several *nrd*B intron–specific probes that gave a strong hybridization signal with T4 DNA or RNA did not hybridize to T2 or T6 DNA or RNA (Fig. 2, probes 3 and 4). This was true for end-labeled oligonucleotide probes directed against the intron ORF (probe 4) as well as for probes that hybridized to noncoding sequences believed to form part of the active splicing conformation (probe 3). Similar results were obtained with a nick-translated intron probe (see legend to Fig. 2C). The *nrd*B intervening sequence thus appears to be absent from the genomes of T2 and T6. In contrast, the *td* intron-specific control probe hybridized throughout, except to a T4 phage construct that has a *td* deletion (Fig. 2A, probe *td*i). These data, suggesting the absence of the *nrd*B intron from T2 and T6, are in agreement with the results obtained with a synthetic

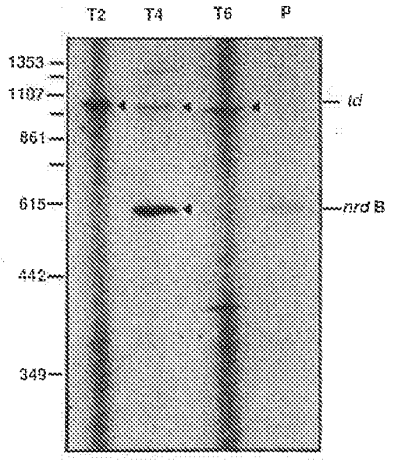


Fig. 1. Labeling of T2, T4, and T6 RNAs with ^{32}P-GTP. RNA was extracted from T2-, T4- or T6-infected *Escherichia coli* B 12 minutes after infection or from cells transformed with plasmid pJSK6 after transcriptional activation of the intron-containing *nrd*B gene (*8*). After incubating the deproteinized RNA with α-[^{32}P]GTP at 42°C for 1 hour under self-splicing conditions (*8, 18*), samples were separated on a 5% acrylamide gel alongside denatured DNA size standards (a 123-bp ladder) used to infer species lengths (left). The position of the *td* intron was indicated by a *td* control clone and that of the *nrd*B intron from the band in lane P, which contains labeled RNA from pJSK6. The absence of an *nrd*B band of equivalent intensity or mobility from the T2 and T6 lanes should be noted. Unmarked bands in the T4 and T6 lanes have not been identified and may well represent other group I introns.

Wadsworth Center for Laboratories and Research and School of Public Health Sciences of the State University of New York at Albany, New York State Department of Health, Albany, NY 12201.

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Antibody-targeted leucinostatin A

Franco Dosio[a], Maurizio Ricci[b], Paola Brusa[a], Carlo Rossi[b], Luigi Cattel[a,*]

[a]*Istituto di Chimica Farmaceutica Applicata, University of Turin, Turin, Italy*

[b]*Istituto di Chimica Farmaceutica e Tecnica Farmaceutica, University of Perugia, Perugia, Italy*

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Abstract

This paper describes chemical modification of the toxic agent Leucinostatin A and its conjugation with a monoclonal antibody. The molecule, isolated from a culture filtrate of *Paecilomyces marquandii* (Massee) Hughes, is a nonapeptide antibiotic with cytotoxic and phytotoxic properties. To evaluate its toxicity, and to improve its specificity as an antitumor agent, the molecule was conjugated to the monoclonal antibody AR-3, specific to human colorectal and ovarian carcinomas. The targeting ability of AR-3, bearing different amounts of toxic agent, was tested on related and unrelated cell lines. Stability of the ester linkage between Leucinostatin A and AR-3 was also studied.

In this study we show that coupling of Leucinostatin A with a tumor-directed monoclonal antibody is a practical way to increase both the cytotoxicity and selectivity of the chemotherapeutic agent.

Keywords: Leucinostatin A; Monoclonal antibody; Drug-targeting; Antitumoral agent

1. Introduction

Monoclonal antibodies (mAbs) have potential as targeting vehicles for the specific delivery of cytotoxic chemotherapeutic agents, toxic peptides, biological response modifiers and therapeutic radionuclidics [1].

So far, both high molecular weight toxins (MW 30–60 kDa) such as ribosome inactivating proteins [2–4] and also small molecules (MW about 1×10^3 daltons) such as the antitumor drugs doxorubicin and vinca alkaloids [5,6] and polypeptide-like compounds such as cyclosporin [7] have been linked to mAbs.

The nonapeptide antibiotic Leucinostatin A 1 (Fig. 1) is a cytotoxic and phytotoxic molecule (MW 1217) isolated from culture filtrates of *Paecilomyces marquandii* (Massee) Hughes [8,9]. It is remarkably active against gram-positive bacteria, yeast and fungi [9–11]; and shows antitumor activity in solid Ehrlich carcinoma and murine leukaemic cell lines [10]. Leucinostatin A is quite toxic in vivo, the LD_{50} ranging between 0.8 mg/kg (i.p.) and 5.4 mg/kg (oral) [11]. The cytotoxic effect has been ascribed to inhibition of mitochondrial ATP synthesis leading to uncoupling of oxidative phosphorylation [12]. More recently [13] it has been shown that Leucinostatin A has ionophoric properties facilitating transport of cations through the plasma membrane.

In the present work our aim was to enhance both the antitumor activity and the specificity of Leucinostatin A by conjugating it to the monoclonal antibody AR-3, able to recognize human ovarian or colorectal carcinoma cells [14]. This approach has the further

* Correspondence to: Prof. Luigi Cattel, Istituto di Chimica Farmaceutica Applicata, c.so Raffaello 31, 10125 Torino, tel (++39).11.6707697, fax (++39).11.6707695, email dosio@silver.ch.unito.it

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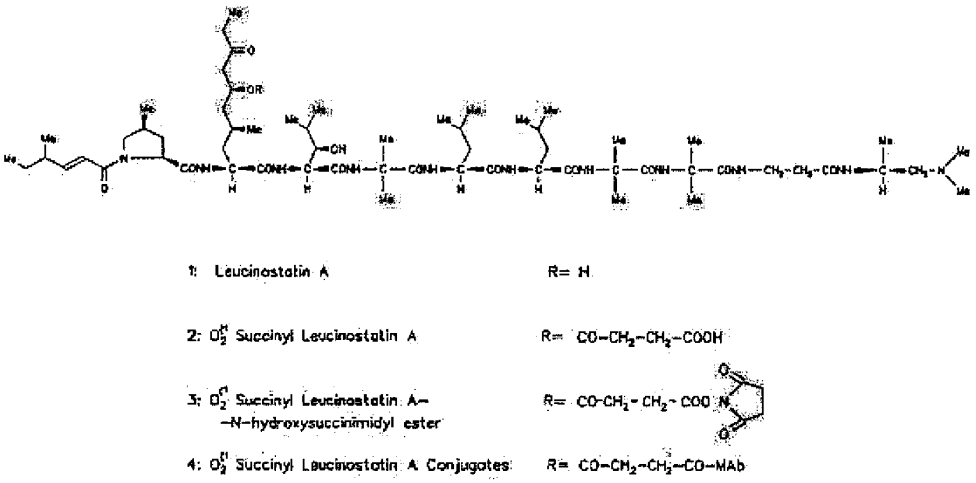


Fig. 1. Leucinostatin A and derivatives.

advantage of prolonging the biological half-life of the lipophilic Leucinostatin A, for which a rapid in vivo degradation can be expected [10]. Furthermore, conjugation with AR-3 antibody has been shown to lower the blood clearance of the ricin toxin [15].

We report here increased antitumour activity and increased selectivity of Leucinostatin A for the target human adenocarcinoma cell line HT-29 after conjugation with AR-3.

2. Material and methods

2.1. Leucinostatin A extraction and purification

Leucinostatin A (1) (molecular weight 1218.6) was obtained from benzene extracts of *Paecilomyces marquandii* (Massee) Hughes culture broth by extensive flash chromatography fractionation on silica gel columns in a nitrogen atmosphere. $CHCl_3$ was used as eluent, and to this CH_3OH and NH_3 were added in increasing proportions up to a final composition $CHCl_3$-CH_3OH-NH_3 90:9:1. After solvent evaporation the white residue was crystallized several times using dry acetone and ethyl acetate. Further details of the procedure are reported elsewhere [1].

2.2. Derivatization of Leucinostatin with succinic acid

Fifty mg of 1 (41 μmol) were dissolved in 400 μl of anhydrous N,N-dimethylformamide (DMF). To 48 mg of succinic acid (410 μmol) previously dried, dissolved in 300 μl of DMF and triethylamine, 116 μl (80 μmol) of the DMF Leucinostatin solution were added. The solution was stirred, chilled at −20°C, and 70 μl of isobutylchloroformate were added dropwise. The reaction mixture was maintained first at −20°C for 30 min and then at 4°C. The mixed anhydride was added to the 1 solution, the temperature was raised to 50°C and the reaction was stirred and maintained for 72 h at 50°C. Under these conditions no insoluble material was present. After TLC evaluation (Rf 1 0.67, Rf Succinyl-Leucinostatin A (2) 0.4 in $CHCl_3$-CH_3OH-NH_3 80:18.5:1.5), the reaction mixture was extracted with 2×5 ml of water and 2×5 ml of 5% $NaHCO_3$. Compound 2 was purified from unreacted 1 by preparative silica gel TLC (Merck, Milan, Italy), using $CHCl_3$-MeOH-NH_3 (80:18:2) as eluent to give 29 mg of 2 (yield 52%). To evaluate the NMR and mass spectra the compound was further purified by flash chromatography using $CHCl_3$-CH_3OH-NH_3 (93:6:0.9) as eluent under a stream of nitrogen. The appropriate fractions,

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previously monitored by NMR, were recombined to yield pure compound 2 (6 mg).

H-NMR ($CDCl_3$) δ: 0.85–1.53 (m, 54 H, two primary, 10 secondary and 6 tertiary C-CH_3 signals), 3.11 (s, 6H, N(CH_3)$_2$) 6.8 (1H, dd, J = 16 and 6 Hz), 6.33 (1H, d, J = 6 Hz, CH-CH=*CH*-CO-), 4.92 (dd, 1H, J = 11 and 2.5 Hz). FAB-MS: m/z 1318(M+), 1300 (M+-H_2O).

To obtain labelled 2 a similar preparation was done in the presence of 200 μl of ^{14}C-succinic acid (47.8 mCi/mmol) (Amersham Int., Buckinghamshire, UK). The ^{14}C-succinylated Leucinostatin A had a specific activity of 61 μCi/mmol, as measured in a scintillation LS-5000TD beta-counter (Beckman Instr., Fullerton, CA, USA). The purity of the compound was checked using a System 200 Imaging Radioscanner (Canberra-Packard Instr., Downers grove, IL, USA).

2.3. Conjugation of succinyl-Leucinostatin A with monoclonal antibody

Compound 2 was dissolved in 800 μl of anhydrous DMF and stored at −20°C. To an aliquot of 0.7 mg (0.52 μmol) of 2, 20 μl, with a radioactivity of 80000 cpm, was added 0.1 mg of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride in 10 μl, and 0.06 mg of N-hydroxysuccinimide in 10 μl. After 30 min at 20°C, 20 μl of the crude N-hydroxysuccinimidyl ester of succinyl-Leucinostatin A (3) were added to 2 mg of monoclonal antibody AR-3 (13.3 nmol) in 0.3 ml of 0.1 M phosphate buffer, pH 7.5, 0.1 M NaCl (20 molar excess of 3). The reaction was maintained at 20°C with stirring for 30 min and then at 4°C for 24 to 65 h. After centrifugation to remove a mixture of protein and unreacted 3, the supernatant was purified by gel centrifugation (4 ml P6DG BioRad gel (Bio-Rad Lab., Richmond, Ca, USA)) and the derivatized antibody was collected and stored at 4°C. By evaluation of the recovered protein, using UV absorption at 280 nm with $E^{0.1\%} = 1.4$ and radioactivity measurements, the degree of derivatization was determined.

2.4. Conjugation of succinyl-Leucinostatin A with bovine IgG

Using the above procedure, a solution of bovine IgG (10 mg/1.5 ml) was derivatized. In this case, to check the degree of derivatization as a function of time, several samples were withdrawn and purified by gel centrifugation (2 ml of gel for 0.1 ml of reaction mixture). Three different time/temperature protocols were used to vary the degree of derivatization. Different molar excess ratios of crude 3 were used (10, 20 and 40).

2.5. ELISAs

HT-29 (human colorectal adenocarcinoma) or MeWo (human melanoma) cell lines were seeded in 96 well microtiter plates (Greiner GmbH, Kremsmunster, Austria) and maintained in RPMI 1640 medium containing 10% foetal calf serum (FCS) and 0.1% antibiotics, at 37°C in a 5% CO_2 humidified atmosphere until a monolayer was formed. On fixed cells, serial dilutions of AR-3 and conjugates (100 μl) were added. After washing, the wells were treated with peroxidase-labelled goat anti-mouse IgG and the affinity capability of the conjugates (4) were correlated with the colour developed (405 nm with a Tiertek Multiscan MK II (Flow Lab., USA)) by incubating a solution of ABTS (2–2'-azinobis-(3-ethylbenz-thiazolinesulfonic acid) and H_2O_2 (KPL lab., USA) with cells.

2.6. Inhibition of cellular protein synthesis

The method of Cattel et al. [3] appropriately modified was followed. 3×10^4/well of HT-29 and MeWo cells, maintained as described above, were seeded in microtiter plates and incubated for 3 h to allow cells adhesion. Various concentrations of antibody (in 100 μl), and AR-3 conjugates 4 were added in triplicate and incubated for 72 h. The supernatants were removed and after washing the cells were incubated for 16 h with 200 μl of medium containing 1 μCi of ^{3}H-Leucine (58 Ci/mmole) (Amersham). The cells were removed from the plates using Nonidet P-40 (Sigma, St. Louis, MI, USA) and the incorporated radioactivity measured in the beta-counter. The results were expressed as percentage ^{3}H-Leucine incorporation with respect to the control (i.e. absence of inhibitor, 150000 CPM for HT-29 and 60000 CPM for MeWo), background values being subtracted.

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2.7. Stability of the ester linkage between Leucinostatin A and succinic acid

Radiolabelled **2** (3 μl of DMF solution) was treated in a 96 well microtiter plate with 200 μl each of sterile FCS, RPMI 1640 with 10% of FCS, and mouse serum and incubated at 37°C in a humidified atmosphere. At different times the solutions were extracted with $CHCl_3$ (3×2 ml) with MeOH. Samples were taken from 40 min to 98 h. The extracts were evaporated and resuspended in 100 μl of MeOH; 10 μl were measured in the beta counter, while 90 μl were applied to TLC plate (0.25 mm, Merck) and eluted with $CHCl_3$-MeOH-NH_3 (90:18:2). The radioactive spots were detected with a System 200 Imaging Scanner (Rf 2 0.4, Rf succinic acid 0.01). The ratios of intensities of radioactive spots were measured by integration using NSCAN software (Packard). The experiments were done in triplicate.

2.8. Stability of Leucinostatin A-AR-3 conjugate in tissue culture medium

Fifty μl of a sterile solution of conjugate **4** (6,7 Leucinostatin A groups/mAb) were incubated in 500 μl of RPMI 1640 with 20% FCS in a 24 well microtiter plate at 37°C in a humidified atmosphere for 72 h. The solutions were treated with 20% trichloroacetic acid (TCA) and washed three times with 5% TCA. The protein pellet was washed with dry acetone and counted in the beta-counter.

3. Results and discussion

Leucinostatins are members of a large class of peptide antibiotics that incorporate a variety of modified aminoacids and blocking groups.

X-ray diffraction studies [16] and conformational behaviour in solution [17] have shown that Leucinostatin A folds in a regular right-handed -helix having a positive-charged ammonium ion at the C-terminal, a hydrophobic core and two arms made by a hydrophilic decanoyl residue and a hydrophobic N-terminal moiety respectively.

The overall shape of Leucinostatin A makes the molecule poorly soluble in water as well as chemically rather unreactive.

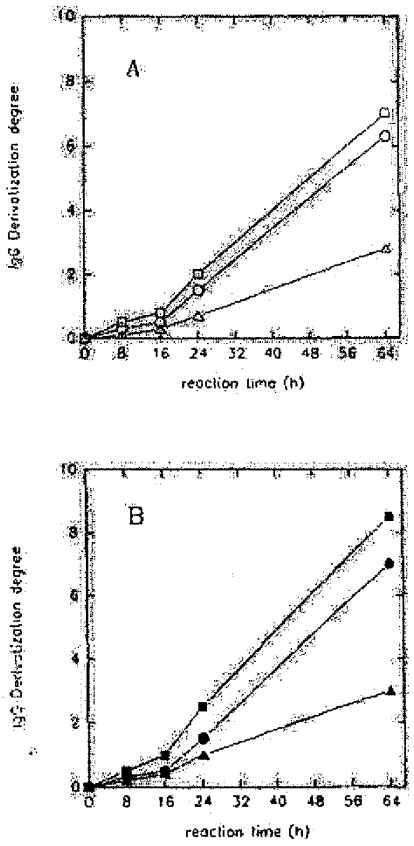


Fig. 2. Time-dependent derivatization of bovine IgG by reaction with different amounts of **3** (molar excess 10 fold, triangle; 20 fold, circle; 40 fold, square) using the carbodiimide method, as described in Material and Methods. Panel A reaction at 4°C; panel B at 20°C.

Generally, a drug to be conjugated with an antibody requires a suitable reactive group for covalent attachment to the linker. We chose the partially reactive hydroxyl group present on the 6-hydroxydecanoyl residue as starting-point for linkage with a succinyl spacer group.

The succinylation of Leucinostatin A was accomplished by preparing the reactive mixed succinic anhydride through reaction with isobutylchloroformate. The

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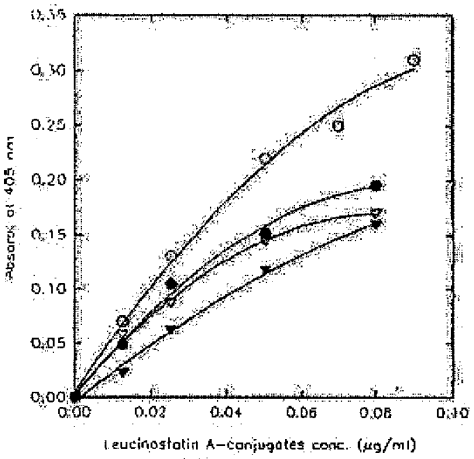


Fig. 3. Immunoenzymatic assay of the specific capacity of AR-3 and Leucinostatin A-conjugates on HT-29 target cells. Symbols: AR-3 (○) derivatized with 1.0 (●), 3.7 (△) and 6.7 (▲) groups of Leucinostatin A.

mixed anhydride intermediate reacted with Leucinostatin A only after several h at 50° giving the succinyl derivative **2** in good yield (52%).

The ^{1}H-NMR spectrum of **2** showed, beside a signal pattern similar to that found for Leucinostatin A, a signal at 4.92 ppm indicating the presence of an ester function.

The ^{13}C-NMR spectra disclosed 62 signals typical of pure Leucinostatin A together with four new resonance signals attesting the presence of a succinyl group. The structure of **2** was also confirmed by mass spectra.

To determine the degree of the antibody conjugation, labelled **2** was also prepared by using ^{14}C-labelled-succinic acid. The next step was covalent coupling of **2** to AR-3 while maintaining the solubility of the resulting immunoconjugate **4** as well as the activity of the mAb and the drug.

We previously tested the availability of the ε-amino group of lysine residues in AR-3 for covalent linkage with N-hydroxysuccinimide heterobifunctional reagents [3]. Thus, to conjugate the succinyl-derivative **2** to the mAb, we decided to convert the carboxylate group of the drug into the N-hydroxysuccinimide derivative **3**.

The reaction was performed in DMF, in which all reagents were completely soluble. The active intermediate was not isolated and the mixture was directly added to the protein solution. Initially we selected bovine IgG as a model to test the formation of a stabile amide bond between the ε-amino lysine groups and **3** (Fig. 2). Due to the low solubility of **3** in water, the solution appeared cloudy. To obtain a sufficient degree of derivatization (at least 1 residue of **3** per moles IgG) the reaction was continued for 24 h at room temperature.

A 1:1 molar ratio in the conjugate **4** was obtained even if the reaction proceeded at 0° in the presence of

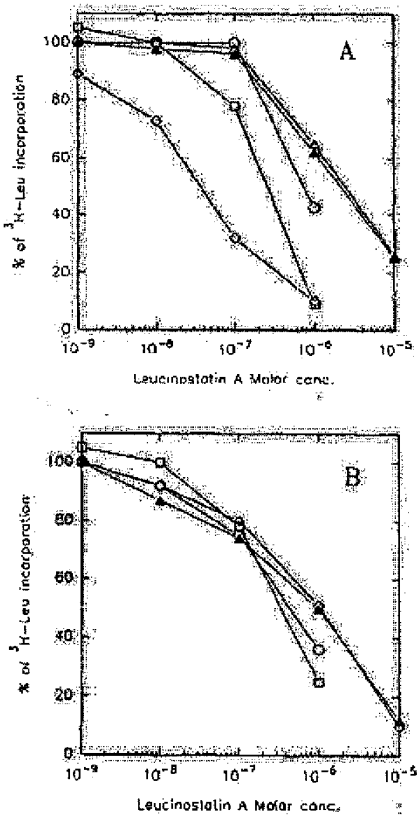


Fig. 4. Cytotoxic activity on target (panel A) and control (panel B) cell lines. The percentage of activity is expressed as ^{3}H-Leucine incorporation with respect to the internal control. Symbols: AR-3 derivatized with 1.0 (○), 3.7 (□) and 6.7 (◇) groups of Leucinostatin A. Derivative **2** is shown as △ and **1** as ▲.

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Table 1
Cytotoxic effects of test substances as inhibition of ^{3}H-leucine incorporation

Test substance	Toxicity to cells with the appropriate antigen				Toxicity to cells lacking the appropriate antigen				
	Test cells	(S) ID_{50} conjugate	(T) ID_{50} free drug	Toxicity factor	Test cells	(U) ID_{50} conjugate	(V) ID_{50} free drug	(N) Normalizing factor	Selectivity factor
Conjugate 4 (6.7 drug groups)	HT-29	0.05	3.6	72	MeWo	0.5	1.0	4.5	45
Conjugate 4 (3.7 drug groups)	HT-29	0.6	3.6	6	MeWo	0.6	0.8	4.5	4.5

ID_{50} = concentration (in μM) needed for 50% inhibition of radioactivity uptake.
"Toxicity factor" – compares the cytotoxicity of 4 with free 1 (T/S).
"Selectivity factor" – compares the cytotoxicity of 4 on antigen-bearing cells with that of antigen-lacking cells (NU/S).
"Normalizing factor" – expressed as T/V.

a much higher molar excess of the reactive molecule. Consequently, to prepare the AR-3 conjugate 4, a coupling procedure was designed which applied a 40 molar excess of 3 and maintained the temperature for 30 min at 20° and then 24 or 65 h at 4°.

Under these conditions the ability of the immunoconjugate to recognize the targeted membrane antigens was maintained as shown by the ELISA evaluation (Fig. 3). In fact, when one or 6.5 Leucinostatin A groups were introduced into the protein, the decrease in antibody specificity was only 5% and 12%, respectively.

All the derivatized antibodies where tested by electrophoresis in denaturing conditions to check the formation of high molecular weight polymers; this showed that the proportion of dimerized AR-3 was less than 7%. To test the cytotoxicity of conjugate 4, the protein synthesis inhibition assay [3] was used since Leucinostatin A has been shown to suppress protein synthesis in L 1210 murine leukaemic cells at a concentration of 10 μg/ml [10], probably as a consequence of strong disorganisation of cell membranes.

To evaluate the selectivity of conjugate 4 we compared protein synthesis inhibition both on targeted human colorectal carcinoma cells and on unrelated human melanoma MeWo cell line. Inhibition of ^{3}H-Leucine incorporation was measured and plotted as a function of conjugate concentration (Fig. 4). With antigen-positive HT-29 cells the toxicity of derivative 3 was increased 72 times when linked (6.7 Leucinostatin A groups/mAb) to AR-3. With antigen negative MeWo cells there was an increase in toxicity of 1.5 times, which we do not consider significant.

To determine the selectivity ratio between the cell lines, the cytotoxicity of 4 (6,7 Leucinostatin A groups/mAb) was compared on antigen-bearing and antigen-lacking cell lines. This was done by normalising the IC_{50} values of 3 on MeWo to the corresponding values on antigen-bearing HT-29 cells. A ratio of 45 was obtained (Table 1).

The toxicity of the conjugate was also found to be strictly related to the number of molecules of Leucinostatin A bound. Highest activity was shown by the conjugate having a mean of 6.7 groups of 2 linked to the antibody. Reducing the ratio to 3.7 lowered both the cytotoxicity and the selectivity. Thus 4 behaves like many anti-cancer drugs for which a critical intracellular concentration is needed to achieve a sufficient cytotoxicity [19].

The activity shown by the mAb-toxin conjugate [4] toward the HT-29 carcinoma cell line showed that the linkage between the mAb and the Leucinostatin A-succinyl moiety was cleaved once the immunoconjugate entered the target cell, possibly via mediated endocytosis, in a way that regenerated the free drug in active form. However, it is also possible that some conjugate internalization occurred by non-specific pinocytosis. Such non-specific cytotoxicity has been observed with a number of antibody-drug conjugates [20–22].

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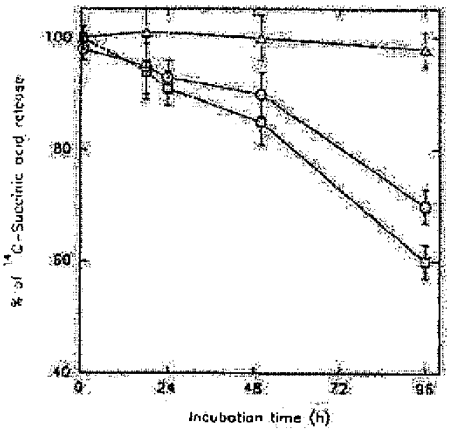


Fig. 5. Release of ^{14}C-succinic acid from succinylated Leucinostatin A (4). The percentage of ester cleavage was measured by radioactive count and TLC analysis of compound 2 after incubation in foetal calf serum (○), mouse serum (□) and RPMI with 10% foetal calf serum (△).

To determine whether this partial non-specificity was due to clearance of the conjugate during the cellular test time, the stability of the conjugate was checked by incubation of labelled 4 with complete culture medium for the same length of time as that of the protein inhibition assay. After 72 h at 37° in a controlled atmosphere at least 90% of the radioactivity was recovered associated with the protein fraction.

Succinylation had no effect on the cytotoxicity of Leucinostatin A. Thus, the succinyl derivative 3 could be considered a good pro-drug candidate since the succinyl derivatization of 1 enhanced the water solubility of the drug without affecting its biological activity. It was also sufficiently stable in mouse or foetal calf serum, where only 10–18% of the toxic moiety was released after 48 h (Fig. 5).

In conclusion we obtained a covalent conjugate between a monoclonal antibody and the cytotoxic polypeptide Leucinostatin A which retained both the targeting specificity of the antibody and the biological activity of the drug, furthermore the coupling procedure employed consisted of mild reactive steps.

Conjugate 4 induced a local rise of drug concentration resulting in a 72-fold increase in toxicity. Furthermore, the conjugate seemed to be preferentially target-specific as shown by a selectivity factor of 45.

These results show that coupling of Leucinostatin A with a tumor-directed mAb is a very practical method to increase both the cytotoxicity and the selectivity of this chemotherapeutic agent.

Acknowledgements

We thank Sorin Biomedica Saluggia (Dr. C. Bonino, Dr. V. Rossi) for the gift of AR-3 monoclonal antibody and for the protein radiolabelling. This work was supported by MURST 40%–60%, by Associazione Italiana per la Ricerca sul Cancro and CNR grants (Piano Finalizzato II, Chimica Fine e Secondaria).

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REVIEWS

Antibody—Drug Conjugates: Linking Cytotoxic Payloads to Monoclonal Antibodies

Laurent Ducry* and Bernhard Stump

Lonza Ltd, CH-3930 Visp, Switzerland. Received May 4, 2009; Revised Manuscript Received July 7, 2009

Antibody—drug conjugates (ADCs) combine the specificity of monoclonal antibodies (mAbs) with the potency of cytotoxic molecules, thereby taking advantage of the best characteristics of both components. Along with the development of the mAbs and cytotoxins, the design of chemical linkers to covalently bind these building blocks is making rapid progress but remains challenging. Recent advances have resulted in linkers having increased stability in the bloodstream while allowing efficient payload release within the tumor cell.

INTRODUCTION

After cardiovascular diseases, cancer represents the second most common cause of death in the Western world and is a major area of focus for the pharmaceutical industry. Surgery and radiotherapy are generally used when a tumor is localized to a certain tissue, but chemotherapy is needed when metastasis has occurred. Despite extensive research, most anticancer drugs have nonspecific toxicity. By targeting the cell cycle and thereby killing rapidly proliferating cells, they do not explicitly discriminate between healthy and tumor tissues and only gain a limited selectivity for malignant cells. Such cytotoxic drugs have a narrow therapeutic window, which limits their efficacy and results in severe side effects. Due to a lack of selectivity, drug concentrations that would eradicate the tumor can often not be used. In addition, tumors can develop resistance against anticancer drugs after prolonged treatment. Therefore, achieving improved tumor selectivity through targeting of cytotoxic drugs to the cancer cells is needed.

A promising approach to achieving a more selective treatment is targeted prodrug therapy (*1*). Antibody—drug conjugates (ADCs) are ideal candidates for such prodrugs. ADCs are monoclonal antibodies (mAbs) linked to cell-killing drugs. Thanks to their high binding specificity for tumor-specific antigens, mAbs can be used as vehicles to target cell-killing payloads to tumor cells. Unique or overexpressed, tumor-specific antigens can be found in a wide range of human tumor cells (*2*). Some mAbs have the ability to recognize and specifically bind to these tumor-associated antigens. They can be used as single agents for the treatment of cancer through binding to cancer-cell-specific antigens and induction of an immunological response against the target cancer cell (*3*). However, therapeutic efficacy is often limited by the extent to which the antibody leads to cell death. Monoclonal antibodies are extremely discriminating for their targets but sometimes therapeutically ineffective on their own. The insufficient efficiency of most naked mAbs in cancer therapy has been circumvented by arming the immunoglobulin with radioactive isotopes (*4*) or cytotoxic drugs (*5*—*7*) (Figure 1), yielding highly specific ADCs.

ADCs can be viewed as sophisticated delivery systems for antitumor cytotoxic drugs. The mAb guides the toxin precursor

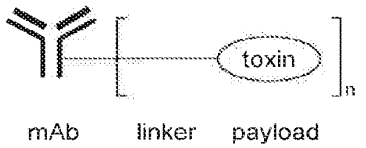


Figure 1. Schematic representation of an antibody—drug conjugate.

to the target cancer cell, where the prodrug can be converted chemically and/or enzymatically to the parent drug and unfold its cytotoxic activity. The ADC is exposed to different conditions on its journey from the blood vessels to the molecular target in the tumor tissue. The mode of action at cellular or molecular level is complex, with each step bringing its own challenges:

a. Circulation. The ADC must behave like a naked antibody when circulating in the plasma. In particular, the linker must be stable in the bloodstream to limit the damage to healthy tissue. Decomposition or decay would release the cytotoxin before being delivered to the target site.

b. Antigen Binding. It is necessary that the conjugated mAb retains high immunoaffinity. Attaching the cytotoxic compound to the mAb must thus not disturb its binding specificity.

c. Internalization. A sufficient intracellular concentration of the drug must be achieved. This is challenging because antigen targets on cell surfaces are often present in limited numbers and the internalization process for antigen—antibody complexes is frequently inefficient.

d. Drug Release. Once internalized, the ADC has to efficiently release the original cytotoxic drug in its active form inside the tumor cell.

e. Drug Action. The inherent potency of the released drug must be sufficient to kill the tumor cell, even at low concentration. To achieve significant cytotoxicity, use of very potent drugs with subnanomolar IC_{50} (as free drug) becomes necessary. It has been shown that compounds found to be too toxic when tested as a stand-alone chemotherapy agent are suitable candidates for ADC payloads. These toxins can be 100 to 1000 times more cytotoxic than traditional anticancer agents.

The molar loading of the drug on the mAb can be tuned to achieve the desired potency. Because of points (c) and (d), linkage of several drugs is generally necessary to achieve significant cytotoxicity. However, if too many cytotoxic molecules are attached

* laurent.ducry@lonza.com.

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Chart 1

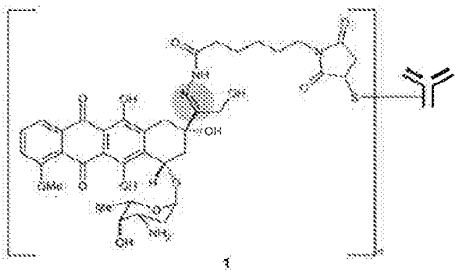
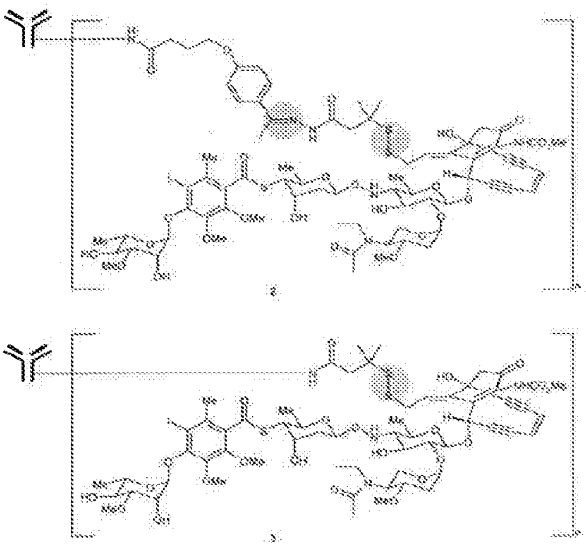


Chart 2



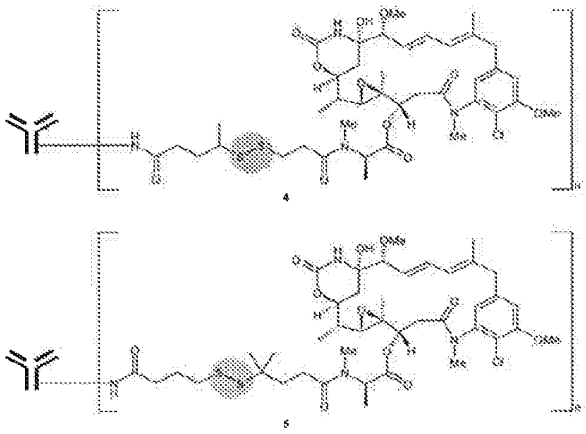
to the antibody, the body may recognize the conjugate as a damaged form of the protein and quickly clears it from the system, proving points (a) and/or (b) to be problematic. The extent of drug substitution may also affect pharmacokinetics, so that most of the time 2−4 drugs per antibody give the best therapeutic window (*8*, *9*). To avoid the interference with the antigen recognition, the drugs are ideally linked at the Fc or constant region of the mAb that does not participate in binding to the antigen (point (b)). To regain the full cytotoxic potential of the drugs, cleavable linkers releasing the drug molecule at the target site in unmodified form were developed to address point (d), although these also affect the stability of the construct. Therefore, the right balance between plasma stability and efficient drug release at the target cell has to be found.

The insights gained during ADC development within the past decade have resulted in a number of new strategies and drugs (*4*−*7*). This review focuses on linker technologies used to attach low molecular weight cytotoxic drugs to mAbs. The linker is a small but central part of ADCs and as such accounts for stability in circulation, favorable pharmacokinetic properties, and efficient release of toxic agent at the tumor site.

CHEMICALLY LABILE LINKERS

Selective linker cleavage and payload release is based upon the differential properties between the plasma and some cytoplasmic compartments. Linkers were chosen that were stable in the blood's neutral pH environment but were getting cleaved

Chart 3



once the ADC entered the lower pH environment found inside a cell. The focus in the early days of ADC development was on acid-cleavable hydrazone linkers that were relatively stable at neutral pH (bloodstream pH 7.3−7.5), but were undergoing hydrolysis once internalized into the mildly acidic endosomes (pH 5.0−6.5) and lysosomes (pH 4.5−5.0).

In the 1990s, Bristol-Myers Squibb scientists covalently linked doxorubicin, an intercalating agent that blocks DNA replication and is used as chemotherapeutic agent, to the humanized mAb BR96 (*10*). Doxorubicin was connected over a (6-maleimidocaproyl)hydrazone linker to cysteine residues of the Lewis-Y specific mAb. The preclinical activities of BR96-doxorubicin (*1*, Chart 1) were remarkable, but despite the use of eight drugs per mAb molecule, the amount of conjugate needed to achieve these effects *in vivo* were high (>100 mg/kg). This was presumably due to the relatively low potency of doxorubicin (IC_{50} of 0.1−0.2 μM for human carcinoma lines (*11*), whereas subnanomolar activities are now typically seen for ADC payloads). Moreover, the half-life of the drug in the blood was approximately 43 h, which is short compared to the half-lives of several days to weeks of the naked BR96 mAb in humans. The compound later failed to show sufficient clinical efficacy.

The first and so far sole mAb linked to a cytotoxic payload that has been given regulatory approval is Wyeth's gemtuzumab ozogamicin (**2**, Mylotarg; Chart 2) (*12*−*14*). The drug was approved by the US Food and Drug Administration (FDA) in 2000 for use in patients over 60 suffering from relapsed acute myelocytic leukemia (AML), the most common form of leukemia in adults. Gemtuzumab ozogamicin (**2**) consists of *N*-acetyl-γ-calicheamicin (an enediyne antibiotic that binds to the minor groove of DNA and causes double-strand breaks leading to apoptosis) covalently attached to humanized anti-CD33 IgG4 κ antibody (hP67.6) via a bifunctional linker. The 4-(4-acetylphenoxy)butanoic acid moiety allows for attachment to surface-exposed lysines of the antibody over an amide bond and forms an acyl hydrazone linkage with *N*-acetyl-γ-calicheamicin dimethyl hydrazide. Upon internalization of the ADC, the calicheamicin prodrug is released by hydrolysis of the hydrazone in lysosomes of the CD33-positive target cells. The hydrolyzability of an unconjugated intermediate at 37 °C over 24 h increased from 6% at pH 7.4 to 97% at pH 4.5 (*15*). The enediyne drug then gets activated by reductive cleavage of the disulfide bond. This disulfide linkage has been stabilized by two methyl groups to prevent premature release of calicheamicin by circulating reduced thiols, such as glutathione. In most preclinical models, the hydrazone linkage produced ADCs with higher potency than the corresponding amide-bearing conjugate **3**, providing evidence that with this mAb the disulfide alone is insufficient for

Scheme 1. Presumed Release Mechanism of MMAE (7) from ADC 6 Incorporating a Valine-Citrulline Peptidic Linker

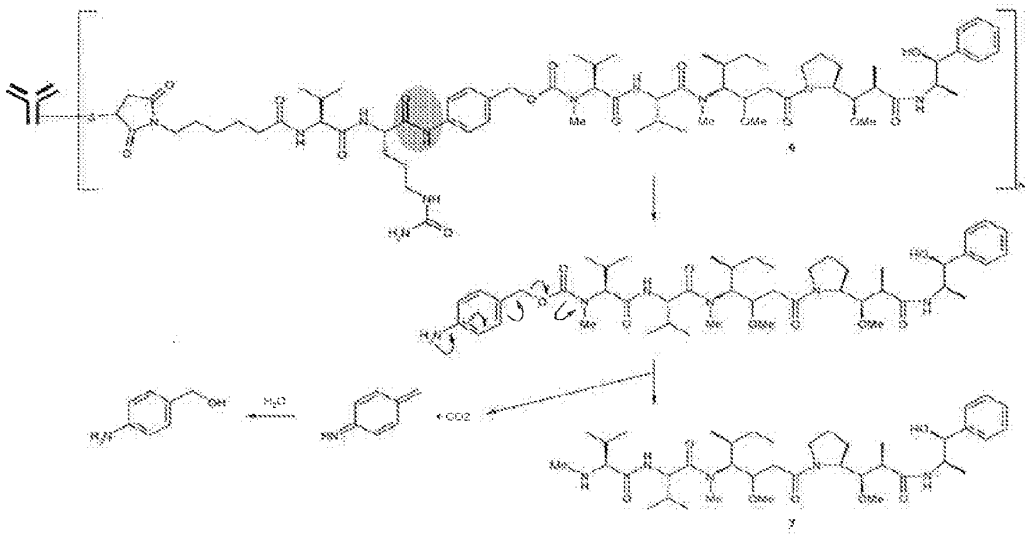
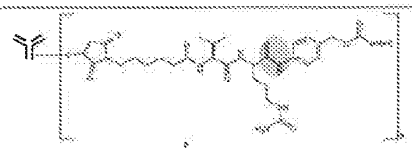
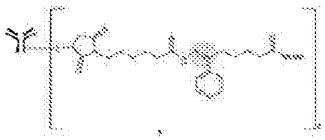


Table 1. Compared Stabilities of Enzyme-Labile and Chemically Labile Linkers in ADCs with MMAE Conjugated to cBR96 mAb

ADC	Linker	Half-live mouse plasma	Half-live human plasma	Maximum tolerated dose
	Valine-citrulline	30 days*	230 days*	30 mg/kg
	Phenylalanine-lysine	12.5 days*	80 days*	n/a
	Hydrazone	2.1 days	2.6 days	15 mg/kg

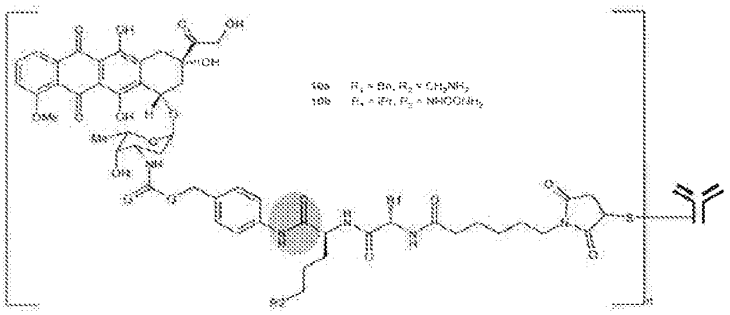
* projected half-life

efficient release of the drug in the target cell. Interestingly, with the murine mAb CTM01, ADC 3 showed activities equal to or even greater than that of the corresponding hydrazone conjugate 2 in several *in vitro* and *in vivo* tumor models, revealing that this conclusion is not general (*16*). In clinical trials, however, compound 3 had only limited evidence of activity.

Mylotarg has met limited successes due to a narrow therapeutic window. The linker technology based on pH-dependent release mechanism is possibly not sufficiently stable and too much of the drug is being released in the bloodstream, as pharmacokinetic data have shown that the mean half-life of Mylotarg is 72 h (*15*). Nonetheless, the development of CMC-544 (inotuzumab ozogamicin), which is a humanized anti-CD22 mAb identically attached to *N*-acetyl-γ-calicheamicin dimethyl hydrazide via the acid-labile 4-(4′-acetylphenoxy)butanoic acid linker is ongoing at Wyeth. Although this ADC is closely related to Mylotarg, good stability was shown in both human plasma and serum (rate of hydrolysis of 1.5−2%/day over 4 days) (*17, 18*).

Disulfide bonds can be an alternative to acid-labile hydrazone linkers. In this case, the release is attributed to the high intracellular concentration of glutathione. This thiol-containing tripeptide is present in micromolar concentrations in the blood, whereas its concentration in the cytoplasma is in the millimolar range (up to 1000-fold higher). This is especially true for tumor cells, where irregular blood flow leads to hypoxic state (decreased oxygen level), resulting in enhanced activity of reductive enzymes and therefore even higher glutathione concentrations. In the absence of sulfhydryl groups, disulfide

Chart 4



bonds are thermodynamically stable and provide reasonably good stability in the bloodstream.

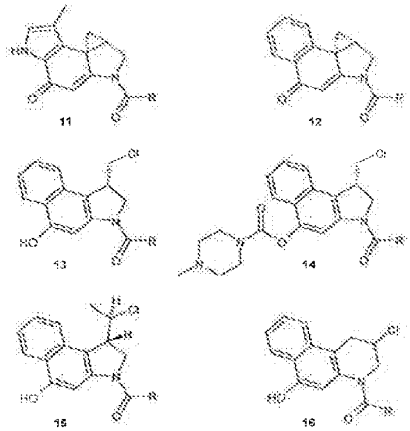
An example of an intracellularly cleavable disulfide-based linker was already discussed above (see "amide" conjugate 3). Other examples are taxoid ADC payloads, conjugated via a disulfide-bearing 4-mercaptopentanoate linker to mAbs directed against the epidermal growth factor receptor (*19*). However, the most significant examples are ADCs where the immunoglobulin is connected over the same type of linker to ImmunoGen's drugs DM1 or DM4 (*20*−*22*). These potent thiol-containing maytansine analogues are antimitotic agents that bind to tubulin and inhibit microtubule assembly. After internalization of the ADC via antigen-mediated endocytosis and delivery to lysosomes by vesicular trafficking, the mAb is believed to be degraded, affording lysine derivatives linked to the toxin (*22*). Further intracellular modifications like cleavage of the disulfide linker through a disulfide exchange, and finally thiol methylation presumably catalyzed by intracellular methyltransferases, generate potent derivatives of DM1 or DM4 (*22*). These methylated drugs are uncharged, enabling them to pass back out through cell membranes to broaden their attack; the drug not only kills from inside the cell, but also knocks out adjacent cells within the tumor that are not targeted by the antibody (*23*). The linker of compound 4 (Chart 3) nevertheless proved to be relatively unstable *in vivo* (half-life of 47 h in mice) (*24*). Replacement of DM1 by DM4 structurally leads to the introduction of two methyl substituents on the carbon atom geminal to the disulfide bond, thereby limiting the accessibility for reducing agents. Consistent with these considerations, ADC 5 exhibits an increased half-life of 102 h in mice.

ENZYME-LABILE LINKERS

Chemically labile linkers often suffer from limited plasma stability. An alternative approach to achieve better control of the drug release involves linking a drug to an antibody via a peptide linkage. The free drug can be specifically cleaved from the carrier by the action of lysosomal proteases (e.g., cathepsin or plasmin) present at elevated levels in certain tumor tissues (*25*). Peptidic bonds are expected to have good serum stability, as proteases are normally not active outside cells because of unfavorable pH conditions and inhibition by serum protease inhibitors.

Seattle Genetics is using a maleimido-containing dipeptide linker to conjugate cysteine residues of mAbs with monomethyl auristatin E (MMAE, 7) and F (MMAF), two auristatin derivatives that bind to tubulin and inhibit microtubule assembly. A valine-citrulline linker was designed to provide high plasma stability (half-lives in mice and monkey of 6.0 and 9.6 days, respectively) as well as a cleavage site for proteases (*26*). Efficient hydrolysis of the dipeptide linker by cathepsin B has been established *in vitro*. After enzymatic

Chart 5



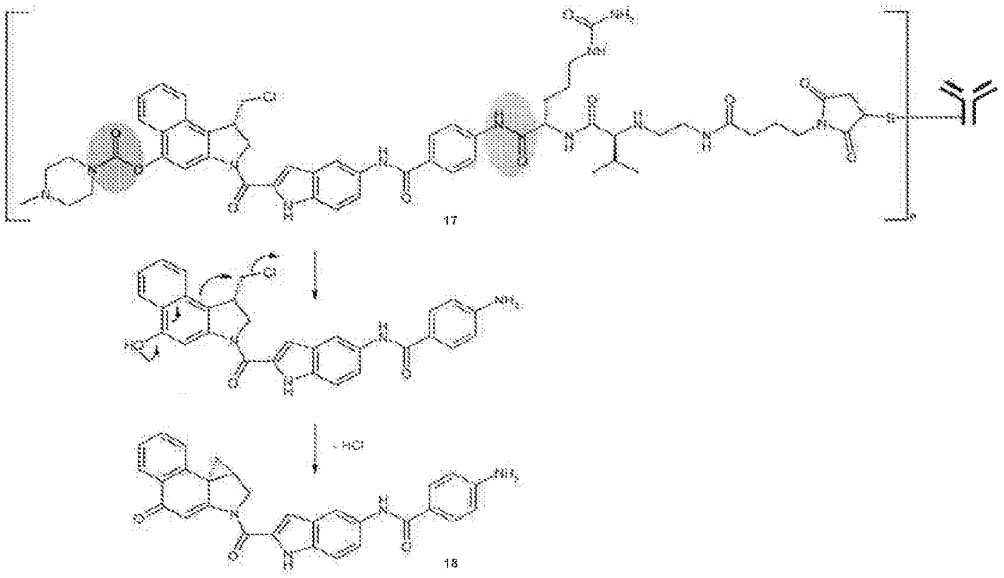
cleavage, 1,6-elimination of the strongly electron-donating 4-aminobenzyl group will occur, releasing MMAE in its active form (Scheme 1). This self-eliminating spacer is needed to spatially separate the drug from the site of enzymatic cleavage (*27*). Otherwise, the bulky payload has a negative influence on the kinetics of the peptide hydrolysis, as the access to the enzyme's active site can be affected.

Comparison of plasma stabilities of ADCs bearing enzyme-labile valine-citrulline or phenylalanine-lysine linkers (6 and 8, respectively) with ADC 9 equipped with a chemically labile hydrazone linker connecting MMAE with the cBR96 mAb showed impressively the advantage of the peptide linkage (Table 1): in human plasma, the ADC 6, featuring a peptidic valine-citrulline-derived linker, exhibits an almost 100-fold increased stability compared to its hydrazone relative 9 and the *in vitro* specificity was increased (*26*). The peptidic linker has a half-life of 6 days in mice compared to 2 days for the hydrazone linker (3-fold improvement), resulting in a lower *in vivo* toxicity (*28*). Several ADCs incorporating a peptidic linker are in clinical development, with the most advanced being cAC10-vcMMAE (SGN-35) for CD30-positive hematologic malignancies (*26*, *29*, *30*).

In addition to *N*-terminus linked MMAE and MMAF, attachment of dipeptide linkers at the C-terminus was studied as a way to vary the rate of drug release and thus ADC potency and tolerability (*26*, *31*). For MMAF, it could be shown that linking the mAb over a dipeptide linker to the C-terminus of the drug can lead to further improvement of the therapeutic window.

The superiority of enzyme-labile linkers was also shown for doxorubicin derivatives conjugated to mAbs (*11*, *32*). With this

Scheme 2. Presumed Release Mechanism of CBI-Containing Drug 18 from ADC 17



strategy, the immunological specificity of BR96 mAb−peptide−doxorubicin conjugates **10a** and **10b** (Chart 4) was significantly improved compared to that of the corresponding hydrazone-based conjugate **1** (*11*), demonstrating again the importance of the linker structure for the ADC properties.

Peptide-containing linkers have been successfully used with CC-1065-derived toxins. CC-1065, duocarmycins, and other cyclopropapyrroloind-4-one (CPI, **11**; Chart 5) derivatives are potent minor-groove binding DNA alkylating agents. According to Boger et al., cyclopropabenzindol-4-one analogues (CBI, **12**) are chemically more stable, biologically more potent, and synthetically more accessible than their parent compounds incorporating the natural CPI alkylation subunit (*33*). In analogy to a duocarmycin prodrug (*34*), the seco-CBI precursor **13** was developed. This compound, which was postulated to show improved pharmacokinetics, will provide the CBI analogue **12** via Winstein cyclization (*35*). A further improvement was achieved by protecting the phenolic hydroxyl group with an enzyme-labile carbamate, as in derivative **14**, decreasing the toxicity of the prodrug while increasing its water solubility by introduction of an additional basic nitrogen (*36*).

Using their UltiMAb technology, Medarex conjugated compound **14** to a mAb via a peptidic linker (*37*). Conceptually, this approach is a double prodrug strategy. It does not only improve the drug's therapeutic efficacy by conjugating it to a tumor-specific mAb, but additionally employs a prodrug of the cytotoxic agent that has reduced potency and thus minimizes side effects before being released intracellularly by carboxyesterases. The two activating steps needed to trigger the release of the active drug (Scheme 2) may lead to an enlarged therapeutic window.

Tietze et al. have reported that the CBI prodrugs **15** and **16** exhibit lower toxicities due to the reduced reactivity of the secondary chloride compared to the primary chloride present in **13** and **14** (*39*−*41*). Such derivatives with secondary chlorides (see, for example, ADC **19**; Chart 6) are used in Syntarga's SpaceLink conjugation technology (*42*). To our knowledge, no data are yet published on the linker stability.

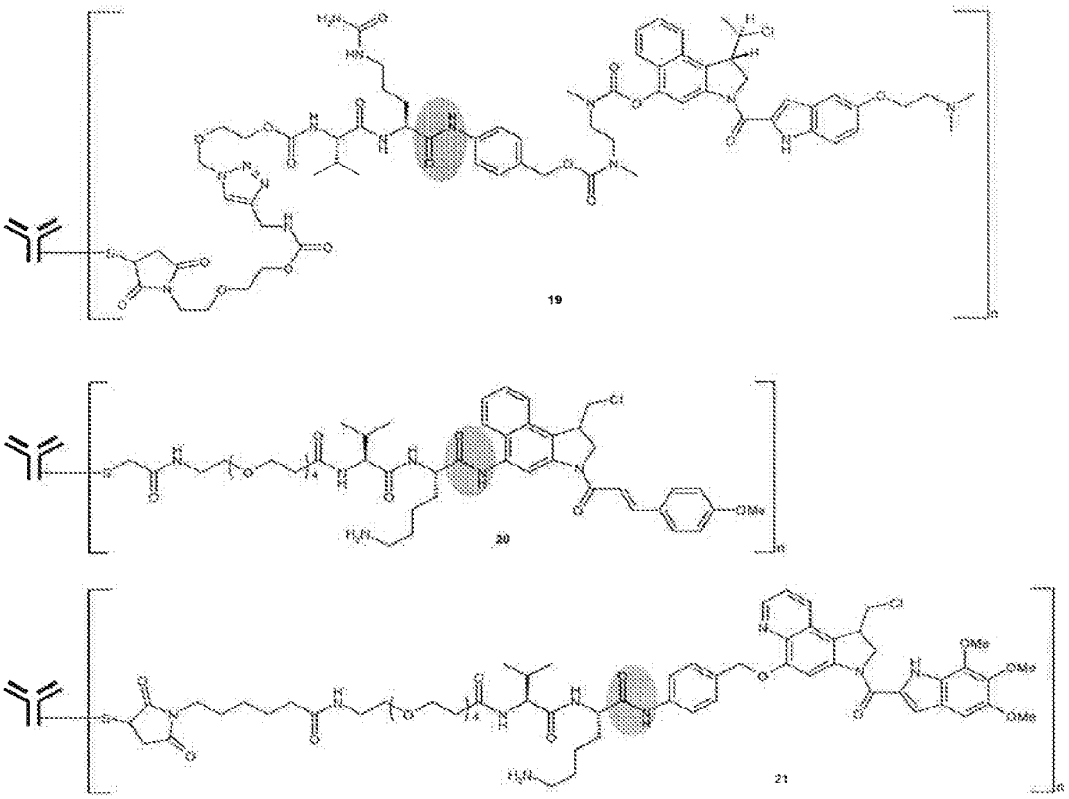
To extend its ADC technology to drugs with a complementary mode of action, Seattle Genetics has developed ADCs **20** and **21** (Chart 6) containing an amino-CBI and a hydroxy aza-CBI payload, respectively (*43*). Because of the hydrophobicity of this class of molecules, attention was focused on developing hydrophilic peptide-linker derivatives that prevented aggregation. This was achieved by use of a more hydrophilic valine-lysine sequence (compared to valine-citrulline as in ADC **6**) and incorporating a tetra(ethylene glycol) unit between the mAb and the peptide linker. The direct attachment of the linker to the amine of the CBI building block (as in **20**) or over a self-immolative *p*-aminobenzyl spacer to the corresponding hydroxyl group of the aza-CBI toxin (as in **21**) prohibits the spontaneous formation of the active toxin in the plasma. Only the enzymatic cleavage of the linker after internalization of the ADC into the cancer cell triggers the release of the prodrug that can now be transformed into the DNA-alkylating cyclopropyl derivative via a Winstein cyclization.

Another type of enzyme-labile linker was recently reported by Seattle Genetics (*44*). In this case, a carbohydrate moiety serves as the cleavage site. Its hydrophilic nature should limit aggregation. Interestingly, the β-glucoronide moiety in ADC **22** is not directly linking the ADC with the payload. Nevertheless, cleavage by β-glucuronidase triggers the 1,6-elimination of the spacer liberating the free drug **23** (Scheme 3). This carbohydrate motif was also used in ADCs bearing Camptothecin analogues (*45*).

NONCLEAVABLE LINKERS

ImmunoGen has reported the fortuitous discovery of a noncleavable thioether bond to link DM1 to mAbs (*22*, *46*). Derivative **24** was actually prepared as a control experiment, but surprisingly, this noncleavable thioether linked ADC also proved very potent. The release mechanism is believed to occur via internalization of the ADC followed by degradation of the mAb component in the lysosome, resulting in the release of the maytansinoid drug **25** still attached via the linker to a lysine residue. This chemical modification of the drug did not diminish its cytotoxic potential. This form of the drug is, however, charged and presumably not able to diffuse into neighboring cells. Hence, it cannot kill adjacent tumor cells

Chart 6

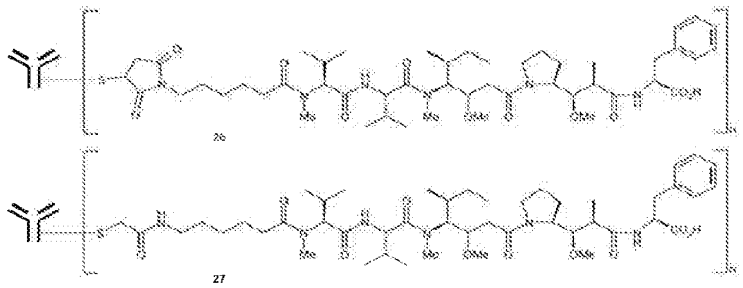


that do not express the target antigen (antigen-negative cells). Besides this by-stander effect (*23*), there is an additional reason why cleavable linkers may have a broader efficacy. Although internalization is often the common initial activation process for both cleavable and noncleavable linkers (*22*), ADCs with cleavable linkers may also be active even when they are poorly internalized (*47*). One possible explanation is that, when the linker is cleaved in extracellular space, the free drug subsequently permeates the cell to reach its target. This mechanism is not operating for ADCs incorporating noncleavable linkers, which must be internalized and degraded within the cell to become active. They are thus dependent on the biology of the target cell.

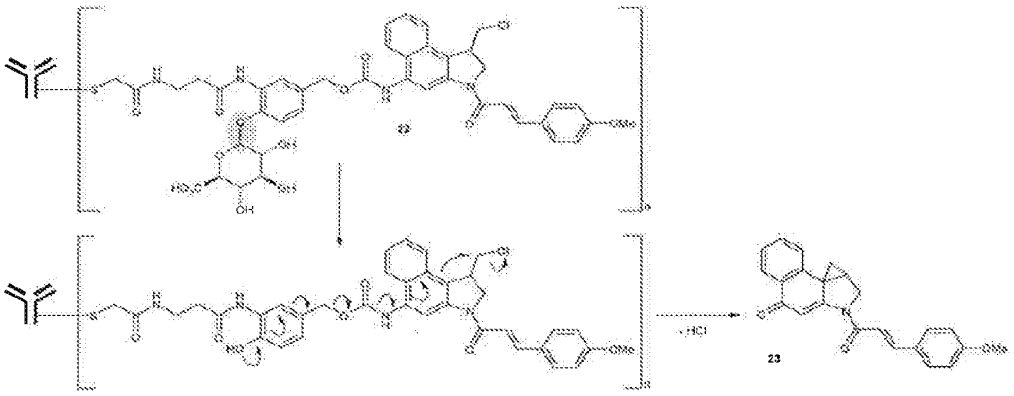
The *in vivo* stability of the thioether-linked ADC **24** proved, with a half-life of 134 h in mice, superior to that of compounds **4** and **5** (47 and 102 h, respectively). ADCs with noncleavable linkers were found to be better tolerated (*47*), presumably resulting in an improved therapeutic index. The use of noncleavable linkers has thus become an important feature of ImmunoGen TAP (tumor-activated prodrug) conjugation technology. This is best exemplified by the promising clinical results obtained by Genentech's trastuzumab-DM1 (T-DM1) for HER2-positive metastatic breast cancer (*48*).

Seattle Genetics made the same finding with MMAF, namely, that the cleavable dipeptide linker (as in **6**) could be replaced by a noncleavable thioether bond (as in ADC **26**; Chart 7) while retaining the *in vivo* activity (*49*). Mass spectrometry showed that the released drug was a cysteine-adduct of MMAF, presumably resulting from mAb degradation within lysosomes as proposed for maytansinoid conjugate **24**. MMAE, although closely related structurally, was not

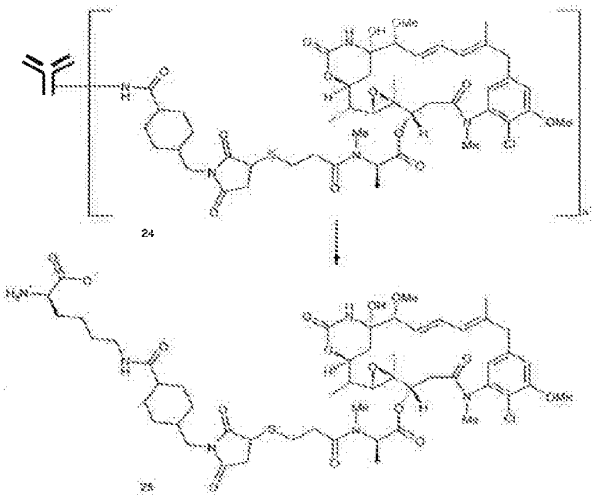
Chart 7



Scheme 3. Postulated Release Mechanism of CBI-Containing Drug 23 from ADC 22



Scheme 4. Release of Maytansine Drug-Derivative 25 through Lysosomal Degradation of ADC 24



active when attached in this manner. Only MMAF can sustain significant modification to the *N*-terminal position and still retain its potency. Recently, it was shown that a slow transfer of the drug from the ADC to albumin cysteines in the plasma occurs with alkyl-maleimide ADCs (*50*). To counteract this effect, the linker stability was enhanced by replacing the maleimide (as in ADC **26**) with an acetamide functionality (as in ADC **27**). This type of ADC led to no apparent degradation in the bloodstream for up to 14 days.

CONCLUSION

A promising approach to improve the potency of antibody-based cancer therapies is to conjugate the mAb with cytotoxic chemotherapeutic drugs. In the foreseeable future, ADCs will eventually fulfill the promise of specific delivery of cytotoxic drugs to tumor cells (targeted therapy), thus avoiding the dose-limiting toxicity of chemotherapy that occurs as a result of its effects on normal cells. The linker technology profoundly impacts ADC potency, specificity, and safety. Early ADC linkers were commonly derived from acid-labile hydrazones, which were designed to be cleaved inside target cancer cells, but inevitably underwent cleavage at nontarget sites. Newer linkers feature hindered disulfides and peptidic moieties that have achieved greater *in vivo* stability. To spare nontarget tissues from chemotherapeutic damage, noncleavable linkers were recently developed. They are thus far the most stable linkers used in ADCs. This recent development may shift some of the future challenge in ADC development from the linker technology toward finding cytotoxic drugs that retain their potency when bearing a covalently attached linker. The improved linker stability and reduced toxicity, however, come together with greater target dependence than in the case of cleavable linkers. ADCs with noncleavable linkers must be internalized and degraded within the cell, whereas compounds with cleavable linkers may be active against targets that are poorly internalized through extracellular drug release and drug entry into tumor cells. Similarly, killing of bystander antigen-negative cells through targeting of antigen-positive cells (collateral toxicity) is presumably only possible with cleavable linkers. The Lonza ADC-team thus believes that no general linker design exists for all ADCs or cancer types. Despite significant progress, each mAb must be examined separately in order to optimize a specific delivery system.

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CLONING OF THE MURINE *TROP2* GENE: CONSERVATION OF A PIP_2-BINDING SEQUENCE IN THE CYTOPLASMIC DOMAIN OF TROP-2

Tarek EL SEWEDY[1], Mara FORNARO[1,2] and Saverio ALBERTI[1]*

[1]*Unit of Experimental Oncology, Department of Cell Biology and Oncology, Istituto di Ricerche Farmacologiche Mario Negri—Consorzio Mario Negri Sud, Santa Maria Imbaro (Chieti), Italy*

Trop-2 is a novel calcium signal transducer expressed at high levels by most human carcinomas. To develop an animal model to study the function of this molecule *in vivo*, we have cloned the murine *Trop2* gene. Using human *TROP2* primers, we amplified by PCR a segment of murine *Trop2*. This was used as a probe to clone a full-length gene by hybridization of a genomic library. The cloned murine *Trop2* gene is functional, as indicated by sequencing and by expression after transfection. The murine Trop2 is 87.4% similar to its human homologue, with the highest conservation in the extracellular region between residues 86 and 157. Essentially all cysteines are conserved between the human and the murine genes, suggesting conservation of the Trop2 disulfide bridges and of its overall structure. Intriguingly, the cytoplasmic tail of Trop2 shows a highly conserved phosphatidylinositol 4,5-bisphosphate (PIP_2)-binding sequence, which overlaps with a protein kinase C phosphorylation site. Thus, we speculate that PIP_2 might regulate the phosphorylation state of Trop2 and play a role in its signal transduction. Murine Trop2 mRNA is detected in normal kidney, lung, ovary and testis, similarly to the human gene. Interestingly, the highest levels of expression are found in immortalized keratinocytes. Since Trop2 is undetectable in undifferentiated spindle cell carcinomas, this suggests a preferential expression at early stages of tumor progression. *Int. J. Cancer* 75:324–330, 1998.
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The human Trop2/GA733-1/EGP-1 is a cell-surface glycoprotein expressed at high levels by the majority of human carcinomas but scarce in or absent from most normal tissues. The most relevant exceptions are multi-stratified epithelia and the placental trophoblast (Fornaro *et al.*, 1995; Lipinski *et al.*, 1981; Pradet *et al.*, 1984; Miotti *et al.*, 1987; Alberti *et al.*, 1992; Stein *et al.*, 1993). Following standard nomenclature rules, the human gene is called *TROP2* (Fornaro *et al.*, 1995), whereas the murine gene is indicated as *Trop2* (Morse, 1992). Trop2 is used for both the human and the murine proteins. Trop2 is phosphorylated by protein kinase C on serine 303 (Basu *et al.*, 1995) and thus was proposed to have a role as a signal transducer. Cross-linking Trop2 with antibodies causes a transient increase of intracellular calcium levels (data not shown), indicating that Trop2 is a cell-surface receptor that might recognize specific ligand(s) and transduce a calcium signal upon binding. The homologue Trop1/KSA/GA733-2 (Miotti *et al.*, 1987; Strnad *et al.*, 1989; Linnenbach *et al.*, 1993) has been suggested to be an adhesion molecule (Litvinov *et al.*, 1994), and a similar role might be played by Trop2. Different adhesion molecules, *e.g.*, integrins, can transduce calcium signals (Schwartz *et al.*, 1993) and regulate several responses linked to adhesion to a substrate, including cell growth (Assoian, 1997). A similar potential role of Trop2 would be consistent with the high expression of this molecule in tumor cells and with the finding that human cancer cells express an oncogenic chimeric mRNA between cyclin D1, a master regulator of the G_1-S transition in the cell cycle (Baldin *et al.*, 1993), and *TROP2* (data not shown).

Although the above findings suggest a potential role of Trop2 in tumor growth, *in vivo* evidence is missing. Thus, to develop an animal model to study the function of this molecule *in vivo*, we undertook cloning of the murine *Trop2* gene. A murine gene was considered necessary for these studies since the homologous human and murine Trop1 proteins (Miotti *et al.*, 1987; Strnad *et al.*, 1989; Linnenbach *et al.*, 1993; Bergsagel *et al.*, 1992) show 18% of non-conserved residues (Bergsagel *et al.*, 1992) and a similar divergence has to be expected for Trop2. Thus, the use of a murine gene would have avoided possible species-restricted Trop2/ligand(s) interactions. Determination of the murine *Trop2* sequence also was expected to define the most evolutionarily conserved regions of Trop2. These are likely to correspond to the most functionally relevant regions of this molecule and would be potential targets for functional and mutagenetic studies. Finally, the use of a murine *Trop2* probe would allow definition of its expression pattern in rodents and comparison with the human gene, as a further test of functional conservation.

We describe the cloning of the murine *Trop2* gene. We show that the structure and pattern of expression of Trop2 are conserved across species and identify conserved regions that are expected to play a crucial role in the function of this molecule. In particular, we show that the cytoplasmic tail of Trop2 has a highly conserved phosphatidylinositol 4,5-bis phosphate (PIP_2)-binding sequence (Yu *et al.*, 1992; Pitcher *et al.*, 1996), suggesting a PIP_2-regulated signaling of Trop2.

MATERIALS AND METHODS

Cells

The murine C5N immortalized keratinocytes and the A5 and Carc-B spindle cell carcinomas (Diaz-Guerra *et al.*, 1992; Burns *et al.*, 1991) were kind gifts of Drs. G. Portella (Naples, Italy) and A. Balmain (Glasgow, UK), respectively. C5N, A5, Carc-B, L, F9, F9-C1 (Strickland and Mahdavi, 1978), 3LL lung carcinoma cells and the mouse fibroblast L cells were maintained in DMEM (GIBCO, Grand Island, NY) supplemented with glutamine, antibiotics and 10% FCS (GIBCO).

PCR

Oligonucleotide primers were synthesized by Duotech (Milano, Italy). Twenty-four different combinations of 20-bp-long human *TROP2* non-degenerate primers were tested for amplification of murine *Trop2* by PCR. Thirty cycles of denaturation at 94°C for 1 min, annealing at temperatures between 45° and 55°C for 1 min and 1 min synthesis at 72°C were performed using a thermocycler (Perkin-Elmer Cetus, Branchburg, NJ). Murine genomic DNA was used as template since *Trop2* was expected to be without introns, like its human counterpart (Fornaro *et al.*, 1995). One pair of these primers (F3, R3) (Table I) amplified a 166 bp band of murine *Trop2*. Four degenerate oligonucleotide primers, F1, F2, R1 and R2 (Table I), were subsequently designed within 2 regions highly conserved between Trop1 and Trop2 (Fornaro *et al.*, 1995). These

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[2]Present address: Department of Pathology, School of Medicine, Yale University, New Haven, CT, USA.

*Correspondence to: Consorzio Mario Negri Sud, 66030 Santa Maria Imbaro (Chieti), Italy. Fax: (39) 872 578.240. E-mail: alberti@cmns.mnegri.it

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were used in a nested PCR amplification. PCR products were analyzed by Southern blotting, using as probes the corresponding regions of the human *TROP2* gene. Hybridizing bands were purified, subcloned into pBluescript SK$^+$ (Stratagene, La Jolla, CA) and sequenced.

Genomic cloning

Several phage, cosmid or YAC mouse genomic libraries were screened by PCR or by hybridization, using as probes the human *TROP2* or the murine *Trop2* fragments amplified by PCR. A successful screening was performed on a murine genomic 129SV lambda FixII phage library (kindly donated by Dr. M. Davis, Stanford, CA), using as a probe the F2-R2 murine *Trop2* PCR fragment (Table I, Fig. 1). Filters were hybridized at high stringency (43°C, 5× SSPE and 50% formamide) and washed at 55°C in 0.1× SSPE (Sambrook *et al.*, 1989). One positive clone was isolated and purified by 2 successive rounds of screening. The *Trop2* phage DNA was analyzed by Southern blot using various probes scattered along the human *TROP2* open reading frame (ORF). A 2.1 kb Xba I segment that contained the entire murine *Trop2* ORF was isolated and sequenced.

DNA sequencing

DNA sequencing was performed following the method of Sanger (Sambrook *et al.*, 1989) using T7 Sequenase kits from USB (Cleveland, OH).

Southern and Northern blot analyses

Total RNA was extracted from murine cell lines. Poly-A$^+$ mRNA was extracted from fresh C57BL/6 mouse organs snap-frozen in liquid nitrogen (Sambrook *et al.*, 1989). Genomic DNA from the different sources was also extracted (Sambrook *et al.*, 1989; Alberti and Fornaro, 1990). Restriction enzymes were purchased from New England Biolabs (Beverly, MA) or Boehringer-Mannheim (Mannheim, Germany). Southern and Northern hybridizations were performed at high stringency (Sambrook *et al.*, 1989). Ten micrograms of DNA, 20 μg of total RNA or 1.5 μg of poly-A$^+$ RNA were loaded per lane. Reproducible DNA loading, transfer and hybridization were verified by the comparable signal intensity among different cellular sources of DNA (see below).

Production of recombinant murine Trop2 in Escherichia coli

A recombinant murine Trop2 was synthesized. Briefly, the F2-R2 *Trop2* segment was subcloned in the galactose-inducible pQE-10 vector (Qiagen, Chatsworth, CA). The correctness of the resulting ORF was confirmed by DNA sequencing. Synthesis of the recombinant murine Trop2 was induced by 0.2 mM IPTG for 5 hr. The 6 histidine-tagged recombinant murine Trop2 was purified by affinity chromatography on a chelated Ni^{2+} column (Qiagen) following the manufacturer's instructions. The purity of the recombinant Trop2 protein was verified by PAGE.

Anti-murine Trop2 anti-sera

Rabbit anti-mouse Trop2 anti-sera were produced as described (Ausubel *et al.*, 1959). Briefly, the recombinant murine Trop2 protein was cross-linked to keyhole limpet hemocyanin (KLH) (Sigma, St. Louis, MO) with glutaraldehyde following standard procedures (Ausubel *et al.*, 1959). The murine Trop2/KLH was emulsified in complete Freund's adjuvant and injected s.c. in rabbits (500 μg per animal). Subsequent immunizations in incomplete Freund's adjuvant were performed at 4-week intervals. Procedures involving animals and their care were conducted in conformity with the institutional guidelines and with national and international laws and guidelines.

Western blot analysis

Western blots were performed as described (Ausubel *et al.*, 1959). Briefly, cell membrane preparations from 10^7 murine *Trop2* transfected or control cells were analyzed by SDS-PAGE and transferred to nitrocellulose filters. Filters were pre-hybridized in 5% milk for 18 hr and hybridized in the same solution with anti-murine Trop2 anti-serum for 1 hr at room temperature. Filters were washed for 20 min and further incubated with goat anti-rabbit peroxidase (Calbiochem, La Jolla, CA). After washing, antibody binding was revealed by enhanced chemiluminescence (ECL; Amersham, Aylesbury, UK).

DNA transfection

Murine *Trop2* was subcloned in the mammalian expression plasmid pBJ1-neo (Lin *et al.*, 1990; kindly supplied by Dr. M. Davis). DNA transfection was performed by calcium phosphate co-precipitation (Alberti and Herzenberg, 1988). Transfected cells were selected in medium containing 500 μg/ml geneticine.

Computer sequence analysis

DNA sequences were analyzed using Genetics Computer Group programs (Devereux *et al.*, 1984). Peptide diagonal analysis was performed with the COMPARE and DOTPLOT programs. Multi-sequence conservation analyses between members of the Trop family were performed with the LINEUP, PILEUP and BOX-SHADE programs.

TABLE I – PRIMERS USED IN THE PCR AMPLIFICATION OF THE MOUSE *TROP2*

Primer	Sequence
F1	AGCTCTAGATCSWGRACAAYGATGGNCT[1]
F2	CGATCTAGABTAYGAYCCYGACTGCGA
F3	GTCTGAGTGGTTGAAGGCGC
R1	CCAAAGCTTCCACCACRATGACRGCGA
R2	GACAAGCTTGABVCCGGCBGTGAGGC
R3	GCGCCAGTGCAACCAGACGT

[1]Degenerate bases are indicated following the IUB-GCG code (Devereux *et al.*, 1984): S, C or G; W, or T; R, A or G; Y, C or T; B, C or G or T; V, A or C or G; Xba I or Hind III restriction sites are underlined.

RESULTS

PCR amplification of murine Trop2

Low-stringency hybridization of murine genomic libraries with human *TROP2* led to the isolation of several spuriously hybridizing clones (data not shown). Thus, we searched for a murine *Trop2* probe to be used in high-stringency library screenings. We amplified by PCR segments of murine *Trop2* and verified their identity by sequencing. A pair of human primers (F3, R3; Table I) amplified a band of 166 bp corresponding to the mouse coding region between residues 110 and 165 (Fig. 1). A 553 bp murine *Trop2* fragment was amplified using 4 degenerate oligonucleotides, F1, F2, R1, R2 (Table I, Fig. 1), in a nested PCR. After sequencing, the 553 bp band was used as a probe in library screening.

Cloning of genomic murine Trop2

After an initial failure to isolate by hybridization murine *Trop2* from several genomic libraries (Super-CosI cosmids [Stratagene], Charon-4 and RIII phages, and YACs [Genome Systems, St. Louis, MO]), we screened by PCR a 129SV genomic library in lambda FIX II and showed that it contained a murine *Trop2*. Five hundred thousand plaques from this library were plated, transferred to nitrocellulose filters and hybridized with the murine F2-R2 segment at high stringency. After 3 rounds of screening, we isolated one murine *Trop2* clone. High-stringency Southern blots identified a single 2.1 band that contained the entire coding region, as confirmed by sequencing (Fig. 1).

Sequence analysis

The murine *Trop2* gene is intronless, like its human counterpart (Fornaro *et al.*, 1995), supporting the hypothesis that *TROP2* derives from a retro-position of *TROP1* in the genome (Linnenbach *et al.*, 1993). The murine *Trop2* ORF encodes a protein of 317 amino acids that is 79.2% identical and 87.4% similar to human Trop2. The hydrophobic plot of murine Trop2 corresponds to that

```
   1  CGTGAGAGTCGGGAGCTGGGCCTCTGGACAAACTCCTGAGGTGCACTAGGAAAGGTGCACGCCCTGGTTC   70
  71  TGAGCTATGAGGAGGGGTGCGGCCAGGTAAGTACTGGTTTCCTCTATGGGAATTTTGGTCAGCAGATGGC  140
 141  AGGAGCAATCTCCCCTGCCTGATTCTATCTCAGCAGGCTCAGGGCTGCCGGACTTCTTCCGCGGCACCTG  210
 211  GGAATAGGGAGCGCCACCCAAATTCCATTGAGTGAGTCACTAGCTAACCAGTCCTACAGGTACAGGAGCT  280
 281  TATAAGAGCTGGAGGGAAAGCCCGGCAGGAGTCGCCTGCAGTGTCCTACCCAGCCTGAATCCTTACGAAT  350
 351  CCCTGCCTCTGGTCTGTAGTTGAGGTCCATTCTACTCCACCCCACCATGGCGAGGGGCTTGGATCTAGCA  420
                                                  M  A  R  G  L  D  L  A    8

 421  CCGCTGCTACTGCTACTGCTGGCGATGGCGACCCGCTTTTGCACGGCTCAGAGCAACTGTACATGCCCCA  490
   9  P  L  L  L  L  L  L  A  M  A  T  R  F  C  T  A  Q  S  N  C  T  C  P  T   32

 491  CCAACAAGATGACGGTCTGCGACACAAATGGCCCAGGCGGGGTCTGCCAATGTCGGGCAATGGGCTCACA  560
  33    N  K  M  T  V  C  D  T  N  G  P  G  G  V  C  Q  C  R  A  M  G  S  Q   55

 561  GGTATTGGTCGACTGCTCCACGCTAACTTCCAAGTGCCTGCTGCTCAAGGCGCGCATGAGCGCCCGGAAG  630
  55  V  L  V  D  C  S  T  L  T  S  K  C  L  L  L  K  A  R  M  S  A  R  K   78
                                 ┌>P1                 ┌>P2
 631  AGCGGCCGCAGCCTGGTGATGCCGAGCGAGCACGCGATACTGGACAACGATGGCCTCTACGACCCGGAGT  700
  79  S  G  R  S  L  V  M  P  S  E  H  A  I  L  D  N  D  G  L  Y  D  P  E  C  102
                              ┌>P3
 701  GTGACGACAAGGGCCGCTTCAAGGCGCGCCAGTGCAACCAGACCTCGGTGTGCTGGTGCGTAAACTCGGT  770
 102    D  D  K  G  R  F  K  A  R  Q  C  N  Q  T  S  V  C  W  C  V  N  S  V  125

 771  GGGCGTGCGCCGCACGGACAAGGGAGACCAAAGCCTGCGCTGCGACGAAGTGGTGCGAACCCACCACATC  840
 125  G  V  R  R  T  D  K  G  D  Q  S  L  R  C  D  E  V  V  R  T  H  H  I   148
                                                 R3<┐
 841  CTCATTGAGTTGCGCCACCGCCCGACCGACCGAGCCTTCAACCACTCTGACCTAGACTCCGAGCTGCGGC  910
 149  L  I  E  L  R  H  R  P  T  D  R  A  F  N  H  S  D  L  D  S  E  L  R  R  172

 911  GGCTCTTCCAAGAACGCTACAAGCTGCACCCCAGCTTCCTATCCGTGCTACACTATGAGGAGCCCACCAT  980
 172    L  F  Q  E  R  Y  K  L  H  P  S  F  L  S  V  V  H  Y  E  E  P  T  I  195

 981  TCAGATAGAGCTTCGGCAGAACGTGTCGCAGAAGGGCTTGAGAGACGTGGACATCGCTGATGCCGCCTAC 1050
 195  Q  I  E  L  R  Q  N  V  S  Q  K  G  L  R  D  V  D  I  A  D  A  A  Y   218

1051  TACTTCGAAAGGGACATTAAAGGCGAGTCACTGTTCATGGGCCGCCGCGGCCTGGACGTCGAGGTGCGTG 1120
 219  Y  F  E  R  D  I  K  G  E  S  L  F  M  G  R  R  G  L  D  V  Q  V  R  G  241

1121  GGGAACCCCTGCATGTGGAGCGGACCGTCATCTACTACCTGGACGAGAAGCCCCCCCAGTTCTCCATGAA 1190
 242    E  P  L  H  V  E  R  T  V  I  Y  Y  L  D  E  K  P  P  Q  F  S  M  K  265
                                 R2<┐                R1<┐
1191  GCGCCTCACCGCCGCGCTCATTGCCGTCATCGCTGTCGTCTCGGTAGCGGTAGTGGCTGGTGTGGTGGTC 1260
 265  R  L  T  A  G  V  I  A  V  I  A  V  V  S  V  A  V  V  A  G  V  V  V...  288

1261  TTGGTGCTCACCAAACGGAGGAAGTCGGGCAAATACAAAAAGGTGGAGCTTAAGGAGCTGGGGGAGATGA 1330
 289  L...V...V  T  K  R  R  K  S  G  K  Y  K  K  V  E  L  K  E  L  G  E  M  R  312

1331  GAAGCGAACCTAGCTTGTAGGTTTCCTGTAGGATGCCCCGACTTCCTCGGCACCTCAGACCAGATGGGTT 1400
 313    S  E  P  S  L                                                        317

1401  GGCCTGTTAATTCTCAGTCAGGAGGTAATTTTCCTCCCAGCTTTGACCTCTTCCTTCTCTCTCCACAAAC 1470
1471  AAGTTCTCTAGAGCG  1485
```

FIGURE 1 -- Nucleotide and amino acid sequence of the cloned murine *Trop2* gene (accession number Y08830). Consensus TATA box and SP1-binding sites are doubly underlined. Potential glycosylation sites are underlined. The position of the primers used for PCR and the direction of amplification are indicated by arrows. The transmembrane region is indicated by a dotted underline. Residue numbers are shown on the side.

of human Trop2 and predicts a type I transmembrane protein with a hydrophobic leader peptide, devoid of the poly-proline stretch present in the human molecule (Fig. 2), and one transmembrane domain (Malthiery and Lissitzky, 1987). Comparison of the Trop1 and Trop2 polypeptide sequences identifies 3 regions that are conserved in all Trop molecules and that appear to characterize structurally the Trop family (Fig. 3*a*). Two of these regions are extracellular, while the third includes the transmembrane and cytoplasmic regions. Like all other Trop molecules (Linnenbach *et al.*, 1993; Bergsagel *et al.*, 1992), the murine Trop-2 cysteine-rich domain contains an EGF and a thyroglobulin repeat (Malthiery and Lissitzky, 1987) and 12 of its 13 extracellular cysteines are conserved with the human Trop2, indicating a similar folding and 3-dimensional structure. Remarkably, the only additional cysteine is in the leader sequence, which is expected to be cleaved from the mature protein. The highest conservation between human and

murine Trop2 is in the extracellular segment between residues 86 and 157 (Figs. 2, 3*b*).

The region of highest conservation in the cytoplasmic domain encompasses a sequence that is highly homologous to PIP_2-binding sites of gelsolin (Yu *et al.*, 1992) and of the G protein-coupled receptor kinase 4 (GRK4) (Pitcher *et al.*, 1996) (Fig. 4). Intriguingly, this PIP_2-binding site is not present in Trop1 molecules. A serine phosphorylation site (Basu *et al.*, 1995) and a potential tyrosine phosphorylation site (Cooper *et al.*, 1984) also are conserved in the human and murine Trop2 cytoplasmic regions (Figs. 1, 2).

Tissue and cell line expression of murine Trop2

We measured the mRNA levels of murine *Trop2* in several fresh tissues and cell lines. The murine *Trop2* 1.8 kb mRNA transcript was detected in kidney, lung, ovary and testis (Fig. 5*a*). Interestingly, quite high levels of expression of murine *Trop2* were detected in a murine immortalized keratinocyte C5N cell line,

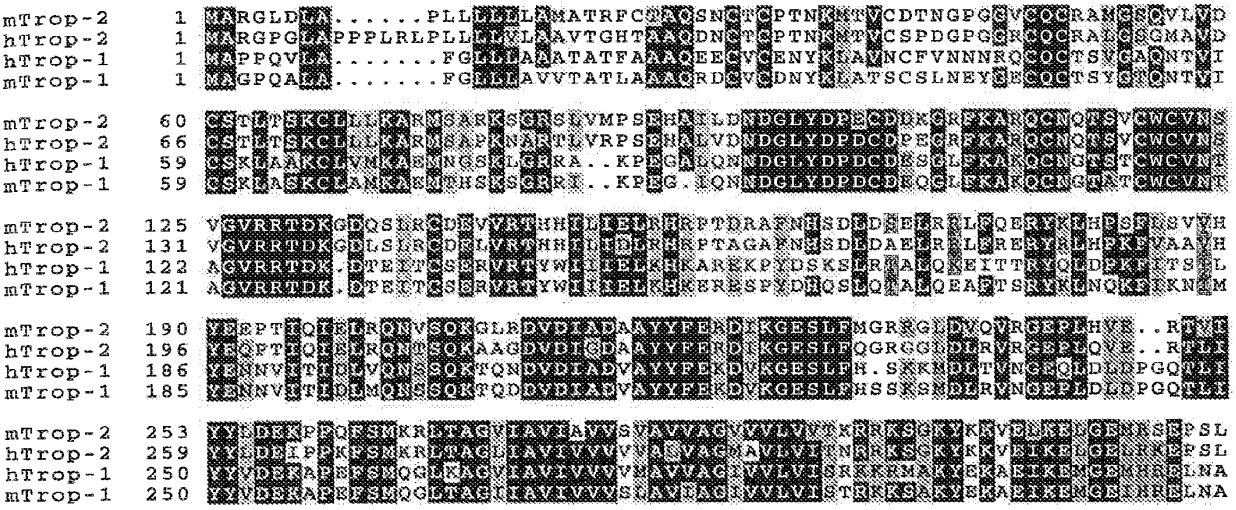


FIGURE 2 -- Multiple peptide sequence alignment of the different members of the Trop family. Murine (m) Trop2, human (h) Trop-2 (X77753), hTrop1 (M32306), mTrop1 (M76124). Identical or similar amino acids shared by 3 or more sequences are shaded in black or gray, respectively. Residue numbers are shown on the side.

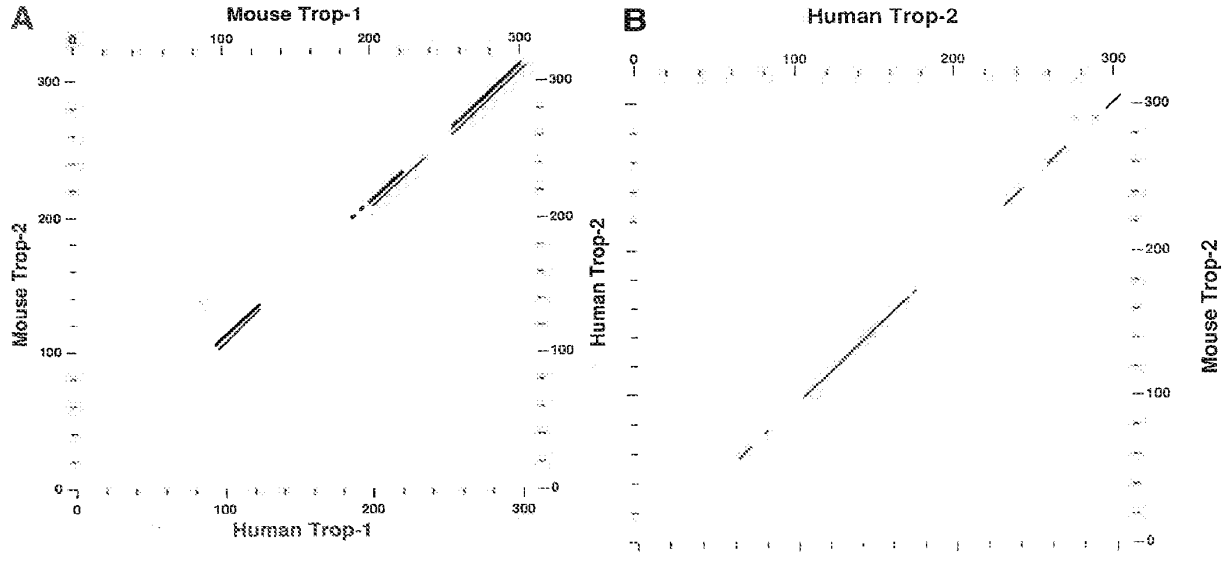


FIGURE 3 -- Conserved regions in the Trop protein family. *(a)* Compound dot-plot comparison of Trop2 *vs.* Trop1 in humans or mice. Thick lines represent regions of homology between human molecules, while thin lines indicate regions of homology between murine molecules. This analysis was performed using a window of 30 residues and a stringency of 27. *(b)* Dot-plot comparison between murine and human Trop2 peptide sequences. The highest conservation is observed in one extracellular domain. The comparison was done using a window of 30 residues and a stringency of 40 (Devereux *et al.*, 1984).

hTrop-2	302	K	S	G	.	K	Y	K	K	308
mTrop-2	296	K	S	G	.	K	Y	K	K	302
GRK4	22	K	S	G	.	R	S	K	K	29
Gelsolin	162	K	S	G	L	K	Y	K	K	169

FIGURE 4 – The PIP_2-binding motif in Trop2, GRK4, gamma splice variant (U33055) and gelsolin (P06396). The dot indicates a 1 amino acid gap.

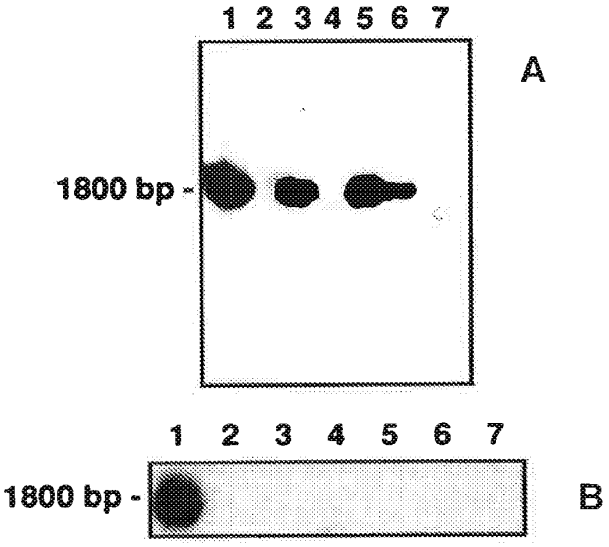


FIGURE 5 – Expression of the murine *Trop2* gene. *(a)* Northern blot of fresh murine tissues. Lane 1, kidney; lane 2, heart; lane 3, lung; lane 4, spleen; lane 5, ovary; lane 6, testis; lane 7, liver. *(b)* Northern blot of murine cell lines. Lane 1, C5N; lane 2, A5; lane 3, Case-B; lane 4, F9; lane 5, F9C1; lane 6, L cells; lane 7, 3LL. Filters were hybridized with an F2-R2 murine *Trop2* probe.

whereas all spindle cell carcinomas tested lacked detectable *Trop2* expression (Fig. 5*b*).

Expressibility of cloned murine Trop2

Stable transfectants for murine *Trop2* were produced in murine L cells, and expression was confirmed by Northern blot (data not shown). Western blot analysis of plasma membrane preparations from these transfectants specifically identifies a 45 kDa protein (Fig. 6), proving that our murine *Trop2* clone is functional and can be expressed efficiently after transfection. The molecular weight of the transfected mouse Trop2 is about 10 kDa larger than predicted from the amino acid sequence. This probably is caused by glycosylation of one or more of the 4 potential glycosylation sites (Devereux *et al.*, 1984) (Fig. 1), as previously demonstrated for the human molecule (Alberti *et al.*, 1992).

Murine Trop2 *is a single-copy gene*

Southern blot analysis of murine genomic DNA digested with XbaI and hybridized at high stringency with the coding region of the murine *Trop2* reveals a single 2.1 kb band (Fig. 7), as predicted by the restriction map of the *Trop2* clone. Cutting genomic DNA with other restriction enzymes consistently produces only one or a few murine *Trop2* bands, which show an overall intensity comparable to other single-copy genes hybridized to probes of similar

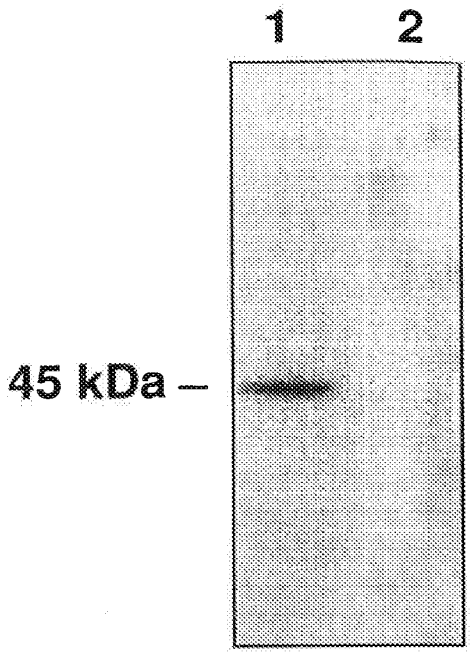


FIGURE 6 – Expressibility of the murine *Trop2* gene. Western blot analysis of murine *Trop2* transfectants. Lane 1, murine *Trop2*-transfected L cells; lane 2, L cells transfected with the selectable marker alone.

length (data not shown). The comparable signal intensity among different cellular sources of DNA excludes also the occurrence of gene amplification (Alberti *et al.*, 1994). Taken together, these findings indicate that *Trop2* is a single-copy gene in mice.

DISCUSSION

Human Trop2/GA733-1/EGP-1 is a cell-surface glycoprotein (Alberti *et al.*, 1992) expressed at high levels by the majority of human carcinomas (Lipinski *et al.*, 1981; Miotti *et al.*, 1987; Stein *et al.*, 1993). Phosphorylation of its cytoplasmic tail by protein kinase C (Basu *et al.*, 1995) and the intracellular calcium signaling caused by cross-linking with monoclonal antibodies (data not shown) indicate that Trop2 is a novel cell-surface signal transducer. These findings together with the high expression of this molecule in tumor cells suggest a potential role of Trop2 in tumor development.

To study the function of Trop2 *in vivo* in an animal model, we have cloned the murine *Trop2* gene. Two sets of primers corresponding to highly conserved regions in Trop molecules were used in a nested PCR and amplified a 553 bp murine *Trop2* segment. This was used to screen several phage and cosmid mouse genomic libraries by hybridization, leading to isolation of a full-length genomic *Trop2* from a 129SV murine library. *Trop2* is a single-copy gene in mice, as was previously shown in humans (Fornaro *et al.*, 1995). The cloned murine *Trop2* is a functional gene, as shown by sequencing and by expression of the murine Trop2 protein in transfected L cells. This genomic *Trop2* clone is syngenic with the murine ES embryonal carcinoma cells used in gene-targeting experiments to obtain knock-out mice (Deng and Capecchi, 1992); thus, it is suitable for *Trop2* gene-ablation experiments.

Murine *Trop2* encodes a type I transmembrane protein of 317 amino acids. The position and number of the extracellular cysteines of murine and human Trop2 are highly conserved, suggesting

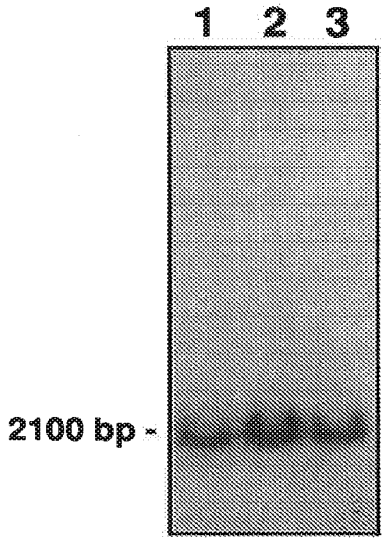


FIGURE 7 – Murine *Trop2* is a single-copy gene. Southern blot analysis of murine genomic DNA isolated from: lane 1, mouse tail; lane 2, F9 teratocarcinoma cell line; lane 3, L fibrosarcoma cell line. DNA was digested with Xba I.

conservation of disulfide bonds, of overall folding and of 3-dimensional structure. Sequence homology between murine and human Trop2 is particularly high in one extracellular domain, suggesting a critical function of this region of the molecule, *e.g.*, in the binding to Trop2 ligand(s). Intriguingly, the cytoplasmic tail of human and murine Trop2 contains a PIP_2-binding consensus sequence that is almost identical to a PIP_2-binding site found in gelsolin (Yu *et al.*, 1992) or GRK4 (Pitcher *et al.*, 1996) (Fig. 4). Gelsolin is an actin filament-severing and -capping protein (Yu *et al.*, 1992), and GRKs are cytoplasmic kinases that phosphorylate G protein-coupled receptors (Pitcher *et al.*, 1996). PIP_2 can regulate the binding of gelsolin and GRK4 to other cytoplasmic molecules (Yu *et al.*, 1992; Lambrechts *et al.*, 1997) or to the plasma membrane (Pitcher *et al.*, 1996). Thus, it might play a similar role for Trop2. Quite interestingly, the PIP_2-binding sequence of Trop2 encompasses a serine that can be phosphorylated by protein kinase C (Basu *et al.*, 1995). Thus, PIP_2 might regulate protein kinase C-mediated phosphorylation of Trop2 (Basu *et al.*, 1995). Consistently, a Trop2-like PIP_2-binding site in neurogranin can be phosphorylated by protein kinase C and the binding of PIP_2 increases the efficiency of phosphorylation (Lu and Chen, 1997).

We have shown that murine Trop-2 is transcribed in kidney, lung, ovary and testis, quite similarly to its human counterpart (Fornaro *et al.*, 1995; Lipinski *et al.*, 1981; Fradet *et al.*, 1984; Miotti *et al.*, 1987; Alberti *et al.*, 1992; Stein *et al.*, 1993). High levels of expression have been found in immortalized keratinocytes, whereas spindle cell carcinomas, *i.e.*, highly malignant cells at an advanced stage of tumor progression (Diaz-Guerra *et al.*, 1992; Burns *et al.*, 1991), do not express Trop2. These preliminary findings suggest that Trop2 is expressed preferentially at early stages of cell transformation. We are now testing this possibility by quantifying the levels of murine Trop2 in a larger set of murine cell lines at different stages of tumor progression and by comparing the growth and invasion properties of tumor cells that normally do not express Trop2 when they are transfected with the murine gene.

ACKNOWLEDGEMENTS

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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
PD20-9009WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/JP2015/002020	10 April 2015 (10-04-2015)	10 April 2014 (10-04-2014)
Applicant		
DAIICHI SANKYO COMPANY, LIMITED		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language,** the international search was carried out on the basis of:
      [X] the international application in the language in which it was filed
      [ ] a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
   b. [ ] This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).
   c. [X] With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. [ ] **Certain claims were found unsearchable** (See Box No. II)

3. [ ] **Unity of invention is lacking** (see Box No III)

4. With regard to the **title,**
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   [X] the text is approved as submitted by the applicant
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2015/002020

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

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2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/JP2015/002020

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/48
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2012/019024 A2 (IMMUNOGEN INC [US]; SETIADY JULIANTO [US]; SKALETSKAYA ANNA [US]; RUI) 9 February 2012 (2012-02-09) cited in the application Claims 1, 40	1-38
A	WO 2012/064733 A2 (MEDIMMUNE LLC [US]; DIMASI NAZZARENO [US]) 18 May 2012 (2012-05-18) Figure 11; page 120, example 8	1-38
X,P	WO 2014/061277 A1 (DAIICHI SANKYO CO LTD [JP]) 24 April 2014 (2014-04-24) claims 1, 50-54	1-38

☐ Further documents are listed in the continuation of Box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
9 July 2015	20/07/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bettio, Andrea

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/JP2015/002020

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2012019024 A2	09-02-2012	NONE	
WO 2012064733 A2	18-05-2012	EP 2638066 A2	18-09-2013
		JP 2014505463 A	06-03-2014
		US 2013330350 A1	12-12-2013
		WO 2012064733 A2	18-05-2012
WO 2014061277 A1	24-04-2014	TW 201420118 A	01-06-2014
		WO 2014061277 A1	24-04-2014

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A Phase II Study of Intravenous Exatecan Mesylate (DX-8951f) Administered Daily for 5 Days Every 3 Weeks to Patients with Metastatic Breast Carcinoma

Francisco J. Esteva, M.D., Ph.D.[1,2]
Edgardo Rivera, M.D.[1]
Massimo Cristofanilli, M.D.[1]
Vicente Valero, M.D.[1]
Melanie Royce, M.D.[1]
Anil Duggal, M.D.[3]
Philippe Colucci, Ph.D.[4]
Robert DeJager, M.D.[3]
Gabriel N. Hortobagyi, M.D.[1]

[1]Department of Breast Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas.

[2]Department of Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas.

[3]Daiichi Pharmaceutical Corporation, Montvale, New Jersey.

[4]MDS Pharma Services, Montreal, Quebec, Canada.

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Address for reprints: Francisco J. Esteva, M.D., Ph.D., The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Box 424, Houston, TX 77030; Fax: (713) 745-5768; E-mail: festeva@mdanderson.org

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BACKGROUND. The objective of the current study was to determine the antitumor activity, safety, and pharmacokinetic (PK) profile of exatecan mesylate in patients with anthracycline-resistant and taxane-resistant, metastatic breast carcinoma.

METHODS. All patients had clinical evidence of metastatic breast carcinoma; disease resistance or progression after chemotherapy that included anthracyclines and taxanes; no prior chemotherapy with camptothecin derivatives; and bidimensionally measurable disease. The starting dose of exatecan mesylate was either 0.5 mg/m^2 per day or 0.3 mg/m^2 per day, depending on prior chemotherapy exposure. PK blood samples were collected from each patient during the first course of therapy.

RESULTS. Thirty-nine patients received a total of 172 courses of therapy (median, 4 courses; range, 1–16 courses). Three patients (7.7%) had a partial response, and 20 patients (51.3%) had either a minor response or stable disease. Approximately 20% of patients had stable disease for 6 months or longer. The median time to disease progression was 3 months, and the median survival was 14 months. The most frequent severe adverse event was neutropenia. The most frequent severe (Grade 3–4) nonhematologic toxicities were fatigue, nausea, headache, myalgia, constipation, emesis, and paresthesias in 28%, 10%, 10%, 8%, 8%, 5%, and 5% of patients, respectively. Exatecan mesylate displayed linear PK characteristics at the doses administered. The average plasma clearance, total volume of distribution, and terminal elimination half-life were approximately 1.4 L per hour per m^2, 12 L/m^2, and 8 hours, respectively.

CONCLUSIONS. Exatecan mesylate had moderate activity in patients with anthracycline-refractory and taxane-refractory, metastatic breast carcinoma. The toxicity profile of exatecan mesylate was acceptable, and it appeared to have linear PK characteristics on the basis of multiple dose administration. *Cancer* 2003;98:900–7. © *2003 American Cancer Society.*

KEYWORDS: exatecan mesylate, camptothecin, clinical trials, Phase II, pharmacokinetics, breast neoplasms.

Breast carcinoma is the most common malignancy in women and is the second leading cause of cancer death in the United States. An estimated 203,500 women will be diagnosed with breast carcinoma, and approximately 40,000 women will die of breast carcinoma in 2002.[1] Cytotoxic chemotherapy plays an important role in the management of patients with hormone-insensitive or hormone-refractory, metastatic breast carcinoma. The most active agents are the anthracyclines and the taxanes, with response rates of 30–60%. Capecitabine induced responses in 20% of patients with disease that progressed after they received anthracycline-based and taxane-based

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chemotherapy.[2] The efficacy of other agents is limited. There is a need for developing novel treatment strategies for patients with advanced breast carcinoma that is refractory to conventional chemotherapy.

Exatecan mesylate (Daiichi Pharmaceutical Co., Ltd., Japan) is a totally synthetic camptothecin analog that is a more potent inhibitor of topoisomerase I than camptothecin, topotecan, and the active metabolite of irinotecan (CPT-11), SN-38.[3–5] In preclinical studies, exatecan mesylate demonstrated broad antitumor activity compared with available camptothecin analogs.[3–5] Furthermore, the antitumor activity observed in Phase I trials made exatecan mesylate an attractive compound for clinical development.[6–8] A Phase II trial was designed to further evaluate the activity and pharmacokinetic (PK) profile of exatecan mesylate and to determine its toxicity and tolerability in patients with metastatic breast carcinoma who were treated previously with anthracyclines and taxanes. In the current study, exatecan mesylate was administered as a 30-minute infusion daily for 5 days every 3 weeks. This schedule was selected based on preclinical models showing greater antitumor activity associated with cyclic dosing at lower doses compared with single-dose administration,[9] the documented antitumor activity of this regimen, and a favorable toxicity profile.[6–8]

MATERIALS AND METHODS

Patient Selection

Patients were eligible to participate if they had histologically confirmed, bidimensionally measurable, metastatic breast carcinoma and had disease that was resistant to or that showed progression after anthracycline-based and taxane-based chemotherapy administered either in the adjuvant setting or for metastatic disease. Patients had to age 18 years or older with a life expectancy of at least 12 weeks and were required to have an Eastern Cooperative Oncology Group (ECOG) performance status ≤ 2. Other eligibility criteria included adequate bone marrow, renal, and hepatic function, as evidenced by absolute neutrophil count (ANC) ≥ 1500 cells/mm^3, platelet count ≥ 100,000/mm^3, hemoglobin ≥ 9.0 g/dL, serum creatinine ≤ 1.5 mg/dL, total bilirubin ≤ 1.5 mg/dL, and serum alanine aminotransferase or aspartate aminotransferase scores ≤ 2.0 times the upper limit of normal or ≤ 5.0 times the upper limit of normal for patients with liver metastases. Informed consent was obtained in writing from all patients according to regulatory and institutional guidelines.

Patients were excluded from the study if they had a prior history of known allergy to camptothecin or its derivatives or if they had received prior radiation therapy to > 50% of bone marrow-bearing bone. Patients must have recovered fully from any previous surgery and from any toxic effects induced by previous chemotherapy or radiotherapy. Patients also were excluded if they had a history of other malignancy (except for nonmelanoma skin carcinoma or carcinoma in situ of the uterine cervix), unless their disease was in complete remission and they had not received any therapy for that disease for at least 5 years; if they had a known brain metastasis; or if they had a concurrent severe or uncontrolled medical disease. Because of the teratogenic potential of exatecan mesylate, women of child-bearing age who were not using reliable means of contraception or women who were pregnant or lactating also were excluded.

Treatment Plan

All treatments were given on an outpatient basis. Exatecan mesylate was provided by Daiichi Pharmaceutical Corporation. Vials containing 2 mg of exatecan mesylate (anhydrous free-base equivalent) were reconstituted with normal saline to obtain a stock of 0.5 mg/mL solution. Care was taken to ensure that exatecan mesylate did not mix with any dextrose solution in the infusion line, because this agent is incompatible with dextrose solutions.

Heavily pretreated patients (HP) included patients who had received > 6 courses of alkylating agent-containing chemotherapy (or > 4 courses of carboplatin), radiotherapy to > 25% of hematopoietic reserves, or > 2 courses of mitomycin C or a nitrosourea. All other patients were considered minimally pretreated (MP) patients. Exatecan mesylate was given initially at a dose of 0.5 mg/m^2 to MP patients. The first 5 patients developed prolonged neutropenia, and the starting dose was changed to 0.4 mg/m^2. Exatecan mesylate was given initially at a dose of 0.3 mg/m^2 to HP patients. Treatment was administered over 30 minutes into a peripheral vein or through a catheter using a programmed volumetric pump system every day for 5 days. This regimen was repeated every 21 days for at least 2 courses or until the patient experienced other disease progression or unmanageable toxicity. Patients were evaluated for response after every two courses of therapy. All lesions were measured bidimensionally. If Grade 2 or severe neutropenia, thrombocytopenia, and all associated nonhematologic toxicities (excluding alopecia) and recovery to Grade 0 or 1 did not occur by Day 36, then the patient was taken off the study. For patients who experienced Grade 3 nonhematologic toxicity, the dose was reduced by one dose level for subsequent courses.

No concurrent antitumor treatment was allowed while the patient was on the study. With the exception of antitumor treatment, patients were allowed to con-

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tinue any other medical treatment that was initiated > 30 days prior to study entry. Patients received full supportive care, including blood product transfusions, antibiotics, antiemetics, antidiarrheals, and analgesics, as appropriate. Prophylactic treatment with colony-stimulating factors to prevent neutropenia was not permitted.

Pretreatment and Follow-up Studies

A 12-lead electrocardiogram, a chest X-ray, and other radiologic studies, as indicated for tumor assessment, were performed within 28 days before the initiation of exatecan mesylate treatment. The location, type, and size of all measurable lesions were recorded, and at least one lesion representative of each involved organ site was identified. Medical history was recorded, and a physical examination was performed before the start of treatment and before each treatment course. All baseline laboratory values, including complete blood counts, clinical chemistries, and pregnancy test results for females of reproductive potential, were obtained 7 days or fewer before the initiation of treatment. Complete blood counts with blood cell differential were obtained weekly while patients were on the study. In addition, when the patient's ANC decreased to $< 1000/mm^3$, hematologic monitoring was performed twice weekly until ANC levels recovered. Serum chemistry values, including creatinine, total bilirubin, alkaline phosphatase, transaminases, albumin, amylase, lipase, and electrolytes, were obtained before treatment, weekly during the first course of treatment, and prior to each subsequent course. Values for tumor markers CA27.29 and carcinoembryonic antigen were measured serially only if baseline values were elevated. Abnormal values were monitored until they normalized. Urinalyses were done before treatment and prior to each course of therapy with study drug.

All toxic effects were graded according to National Cancer Institute Common Toxicity Criteria.[10] Tumor response was assessed according to modified Southwestern Oncology Group response criteria.[11] Tumor reassessment was performed by the same methods used to establish baseline measurements. For all patients who responded to treatment (complete responses [CRs] and partial responses [PRs]), response was confirmed 4–6 weeks after it was first documented. At the time of the conclusion of protocol participation, all patients underwent repeat history and physical examinations and repeat clinical laboratory assessments (as noted above), and clinical response was documented. Any ongoing adverse events that could be related to exatecan mesylate were followed until resolution.

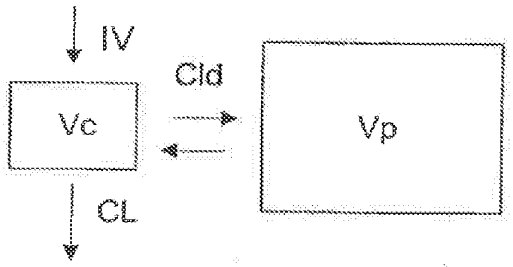


FIGURE 1. Graphic representation of the structural pharmacokinetic model. IV: intravenous; Vc: central volume of distribution (L/m^2); Vp: peripheral volume of distribution (L/m^2); Cld: distributional clearance ([L/hr]/m^2); CL: total clearance ([L/hr]/m^2).

Sampling Schedule and Analytical Assay

PK blood samples were collected from each patient during the first course of exatecan mesylate. Serial blood samples were collected over 24 hours on Day 1 and over 2 hours on Day 5. On Day 1, samples were obtained before the start of the infusion (time 0), at 0.48 hours (29 minutes into the infusion), and at the following sampling time windows: 0.5–2.0 hours, 4.0–8.0 hours, and 24.0 hours (prior to the start of infusion on Day 2) after the end of the infusion. On Day 5, samples were obtained before the start of the infusion (time 0), at 0.48 hours (29 minutes into the infusion), and after the end of the infusion within the sampling time window 0.5–2.0 hours. The blood draws taken during the sampling time windows 0.5–2.0 hours and 4.0–8.0 hours were obtained at specific times according to a randomization scheme (0.5 hours, 1.0 hours, 1.5 hours, 2.0 hours, 4.0 hours, 4.5 hours, 5.0 hours, 6.0 hours, 7.0 hours, and 8 hours, respectively).

The blood was collected in a heparinized tube and centrifuged at 3000 rpm for 15 minutes to separate out the plasma. The plasma was then transferred to a sample tube and frozen immediately at − 20 °C. All collected plasma was labeled and forwarded to MDS Pharma Services Inc. (Montreal, Canada) for PK analysis. Exatecan was analyzed in plasma using a validated high-performance liquid chromatography method.[12] The lower limit of quantitation was 0.201 ng/mL.

PK Analysis

Thus, linear PK models that included two and three compartments were investigated for their quality of fit using maximum likelihood analysis with ADAPT-II®.[13] The simplest model that fit the plasma concentrations of exatecan mesylate best was a two-compartment PK model (Fig. 1). A three-compartment PK model did not

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improve the fit significantly based on visual inspection of the graphs and evaluation of pertinent indicators of *goodness of fit*. The model discrimination process in our evaluation was based on the minimization of the values of the AKAIKE information criterion test, of the objective function, and of the residual variability. An additional criterion considered in the discrimination process was the maximization of the average coefficient of determination. PK parameters were calculated using a population PK method, the iterative two-stage method (ITS®).[14] This method was necessary due to the limited sampling of the study design and to ensure the robustness of the results. Parameters defined and fitted using the two-compartment PK model were Vc (central volume of distribution; L/m^2), Vp (peripheral volume of distribution; L/m^2), CLd (distributional clearance; L per hour per m^2), and CL (total clearance; L per hour per m^2). During the population analysis, the clearances (CL and CLd) and the volumes of distribution (Vc and Vp) were fit to the average, observed patient's body surface area of 1.82 m^2.

All plasma concentrations of exatecan mesylate were modeled simultaneously by using a weighting procedure of W_j (the weight [*W*] associated with each individual concentration time point *j*) = $1/S_j^2$. The variance S_j^2 (variance [*S*] calculated for each time point *j*) was calculated for each observation using the equation $S_j^2 = (a + b * Y)^2$, where *a* and *b* represent the additive and proportional error of this variance model, respectively.

Statistical Design

This was an open-label, Phase II study of exatecan mesylate in patients with metastatic breast carcinoma. The primary endpoint was overall response rate (CRs and PRs). Antitumor activity was evaluated in terms of the proportion of patients with responsive disease and by calculating the 95% confidence interval for response. Treatment toxicity and Kaplan–Meier estimates of time to progression also were determined. The optimal, two-stage design by Simon was employed to determine the sample size.[15] The null hypothesis was that the overall response rate would be ≤ 5% versus the alternative hypothesis that the overall response rate would be ≥ 20%. During the first stage, 12 evaluable patients were enrolled. Because at least a PR was observed, accrual continued to a total of 37 patients who were considered evaluable for response. This design yielded 0.90 probability of a positive result if the true response rate was ≥ 20%. It yielded a ≥ 0.90 probability of a negative result if the true response rate was ≤ 5%, with ≥ 0.54 probability that the study would be terminated early because of lack of efficacy.

TABLE 1
Patient Characteristics (*n* = 39)

Characteristic	No. of patients (%)
Age (yrs)	
Median	52
Range	29–69
Race	
Caucasian	25 (64)
African American	7 (18)
Hispanic	5 (13)
Other	2 (5)
ECOG performance status	
0	10 (28)
1	28 (72)
2	1 (3)
Metastatic sites[a]	
Liver	22 (56)
Lung	15 (38)
Lymph nodes	21 (54)
Bone	21 (54)
Pleura	5 (13)
Other	4 (10)
Prior chemotherapy	
Adjuvant only	8 (13)
Metastases only	16 (41)
Adjuvant and metastases	17 (43)

ECOG: Eastern Cooperative Oncology Group.
[a] Some patients had metastases at more than one site.

RESULTS

Patient Characteristics

Forty patients were enrolled in the study. Thirty-nine of 40 patients were evaluable for the assessment of safety and efficacy, because 1 patient withdrew consent before dosing. All evaluable patients had a histologically confirmed diagnosis of invasive breast carcinoma and clinical evidence of metastatic disease. The median age was 52 years (range, 29–69 years). The median ECOG performance status was 1. All evaluable patients had received prior anthracycline-based and taxane-based therapy either in the adjuvant setting or for metastatic breast carcinoma (Table 1).

Treatment Delivered and Toxicity

Overall, 172 courses of therapy with exatecan mesylate (median, 4 courses; range, 1–16 courses) were delivered during the study. Five MP patients received a total of 6 courses of exatecan mesylate at 0.5 mg/m^2 per day (median, 1 course; range, 1–2 courses). In 4 of those patients (80%), the dose of exatecan mesylate was reduced by 20% for subsequent courses. Twenty-three MP patients were treated initially with exatecan mesylate at 0.4 mg/m^2 per day. Of these, 5 patients (22%) patients required a 1-dose-level reduction on subsequent courses due to hematologic toxicity.

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TABLE 2
Treatment Summary (n = 39)

Exatecan mesylate dose level	No. of patients[a]	No. of courses	
		Total	Range
0.2 mg/m^2	2	2	N/A[b]
0.3 mg/m^2	16	66	1–8
0.4 mg/m^2	28	118	1–16
0.5 mg/m^2	5	6	1–2
Total	—	172	1–16

N/A: not applicable.
[a] Some patients were treated at more than one dose level because of dose reductions. The starting dose was 0.3 mg/m^2 in 11 patients, 0.4 mg/m^2 in 23 patients, and 0.5 mg/m^2 in 5 patients.
[b] Two patients were treated at the 0.2 mg/m^2 dose level, with each patient receiving one course.

Eleven HP patients received exatecan mesylate at a dose of 0.3 mg/m^2 per day. Two of those patients had disease progression after the first course. Two patients required a 1-dose-level reduction on subsequent courses of treatment with study drug because of Grade 3 hematologic toxicity. In one patient, the dose was escalated from 0.3 mg/m^2 to 0.4 mg/m^2 (Table 2).

The safety data are summarized in Table 3. Thirty-nine patients were evaluable for toxicity. Neutropenia was the dose-limiting toxicity, with Grade 3 and 4 neutropenia occurring in 46% and 33% of patients, respectively. Two patients developed infections associated with neutropenic fever. Other Grade 3–4 hematologic toxicities included leukopenia, anemia, and thrombocytopenia in 51%, 18%, and 18% of patients, respectively. No evidence of cumulative hematologic toxicity was observed in patients who received multiple courses of study drug. Nearly all nonhematologic toxic effects were mild (Grade 1 or 2). The most common nonhematologic toxicity was fatigue, which was reported by 69% of patients. Nausea was reported by approximately 66% of patients, and 51% of patients experienced emesis. Diarrhea was reported by approximately 30% of patients. However, no patient required therapy beyond the usual and customary medications, and no patient had associated dehydration that required either intravenous hydration or hospitalization. Stomatitis and alopecia were often mild, and each occurred in approximately 30% of patients. No deaths occurred in association with an adverse event.

Response

Thirty-nine patients were evaluable for efficacy, and 37 patients were evaluated for response. Two patients (5.1%) were not evaluated for tumor response due to patient refusal to continue treatment. No CRs were observed. Three patients (7.7%) had PRs, 4 patients (10.3%) had a minor response and remained stable for > 6 months, 16 patients (41.0%) had stable disease, and 14 patients (35.9%) had progressive disease. A summary of the response rates is presented in Table 4. All 3 patients who achieved a PR were MP patients who began treatment at the dose level of 0.4 mg/m^2. Three patients who achieved a minor response were MP patients who began treatment at the dose level of 0.4 mg/m^2, and 1 was an MP patient who began treatment at the dose level of 0.5 mg/m^2. All three patients who achieved PR had liver metastases. The median time to progression was 3 months, and the median survival was 14 months (Fig. 2).

PK Analysis

Of 39 patients enrolled in the study, 37 patients had their PK profile assessed. Eight patients presented unusually high concentrations at the end of the infusion time point (29 minutes into the infusion). They were deemed anomalous because they were approximately 21 times higher than the average peak concentration observed in the other patients (e.g., on Day 5, the anomalous exatecan mesylate concentration range was 401.3–2648.4 ng/mL, compared with the average of 25.5–200.9 ng/mL). In addition, although these exatecan mesylate concentrations were 21 times higher, the other concentrations seen in the rest of the profiles were similar to the other patients (e.g., on Day 5, the exatecan mesylate concentration range was 0.8–58.5 ng/mL, compared with 0.4–51.1 ng/mL). Therefore, two different population PK analyses were performed. In the first analysis (Analysis A), all concentration time points were kept; whereas in the second analysis (Analysis B), these anomalous concentrations simply were removed. The two analyses yielded similar results for the PK parameter point estimates (mean value results within 1 standard deviation of each other). The interindividual variability (CV) of the PK parameters was lower in Analysis B (CV% range, 11–49%) compared with Analysis A (CV% range, 41–101%; data retained on file). The reason for the presence of these abnormally high peak concentrations is unclear, but it is possible that these samples were taken from the arm where the infusion was running instead of the contralateral arm. We believe that the results from Analysis B are unbiased, and they are the results discussed in the current article. However, the results from both analyses are presented in Table 5 for informational purposes. Figure 3 shows a 'spaghetti plot' of all the fitted and observed concentration time points for each patient in the study.

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TABLE 3
Most Common Toxicities by Grade for All 172 Courses

Toxic effect	Grade of toxicity: no. of patients (%)					
	1	2	3	4	3–4	Total
Hematologic toxicity						
Anemia	10 (25)	21 (54)	7 (18)	0 (0)	7 (18)	38 (97)
Leukopenia	6 (15)	11 (28)	19 (48)	1 (3)	20 (51)	37 (94)
Neutropenia	1 (3)	4 (10)	18 (46)	13 (33)	31 (79)	36 (92)
Thrombocytopenia	12 (31)	6 (15)	6 (15)	1 (3)	7 (18)	25 (64)
Nonhematologic toxicity						
Fatigue	7 (18)	9 (23)	11 (28)	0 (0)	11 (28)	27 (69)
Nausea	11 (28)	11 (28)	4 (10)	0 (0)	4 (10)	26 (66)
Emesis	9 (23)	9 (23)	2 (5)	0 (0)	2 (5)	20 (51)
Myalgia	7 (18)	7 (18)	3 (8)	0 (0)	3 (8)	17 (43)
Constipation	6 (15)	6 (15)	3 (8)	0 (0)	3 (8)	15 (38)
Alopecia	13 (33)	3 (7)	N/A	N/A	N/A	13 (33)
Fever	8 (20)	3 (7)	0 (0)	1 (3)	1 (3)	12 (30)
Diarrhea	8 (20)	4 (10)	0 (0)	0 (0)	0 (0)	12 (30)
Stomatitis	6 (15)	5 (12)	1 (3)	0 (0)	1 (3)	12 (30)
Paresthesias	3 (7)	1 (3)	2 (5)	0 (0)	2 (5)	6 (15)
Headache	0 (0)	1 (3)	4 (10)	0 (0)	4 (10)	5 (13)

N/A: not applicable.

TABLE 4
Response Rates (n = 39 patients)

Response category	No. of patients (%)
Complete response	0 (0.0)
Partial response	3 (7.7)
Minor response	4 (10.3)
Stable disease	16 (41.0)
Progressive disease	14 (35.9)
Unknown	2 (5.1)

DISCUSSION

Phase I studies of exatecan mesylate showed antitumor activity in patients with a variety of epithelial malignancies, including breast carcinoma.[6–8] In the current study, the response rate to exatecan mesylate in patients with anthracycline-resistant and taxane-resistant, metastatic breast carcinoma was modest (8%). However, approximately 20% of patients achieved either an objective response or were stable for at least 6 months while they were receiving therapy.

The cytotoxic potency of camptothecin and camptothecin analogs is related to their ability to inhibit the catalytic activity of topoisomerase I, a nuclear enzyme that plays a critical role in DNA replication and cell division.[16–18] Compared with other camptothecins, exatecan mesylate is approximately 3 times more potent than SN-38, 10 times more potent than topotecan, and 20 times more potent than camptothecin as an inhibitor of topoisomerase I, and it is 5 times more potent than SN-38 as an inhibitor of DNA synthesis.[3,4] The antitumor activities of exatecan mesylate have been studied in a number of different model systems.[19] In cell-based cytotoxicity assays, exatecan mesylate showed impressive antiproliferative activity against a wide range of tumor cell lines, including breast carcinoma, lung carcinoma, gastric carcinoma, and colon carcinoma,[3,5,19–21] and it was effective in inhibiting the growth of clonogenic cells from human tumors.[22] In vivo, it was shown that exatecan mesylate had broad antitumor activity against a large panel of human tumor xenografts in nude mice, including SN-38-resistant and vincristine-resistant tumors.[3–5]

An integrated clinical development plan for exatecan mesylate led to parallel Phase I studies evaluating various dosing schedules. From these studies, it was determined that the dose-limiting toxicity of exatecan mesylate was hematologic toxicity for all dose schedules used.[6–8,23] Neutropenia was the dose-limiting toxic event for MP patients, whereas both neutropenia and thrombocytopenia were the dose-limiting toxic events for HP patients. The toxicity profile appeared to be dose-related and predictable, and it appeared to be consistent with preclinical toxicology results.[24] The toxicity profile of exatecan mesylate among patients in the current study was similar to that reported previously, with neutropenia the principal dose-limiting

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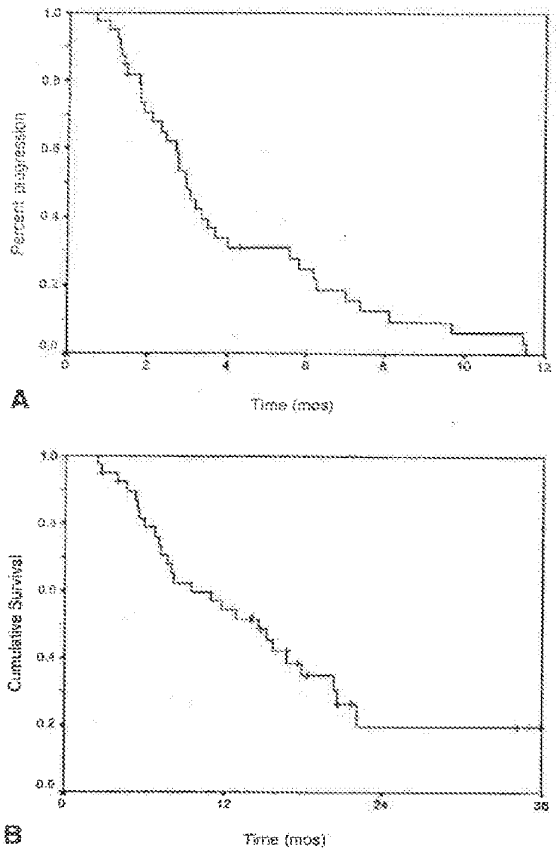


FIGURE 2. Kaplan–Meier curves showing (A) time to disease progression and (B) overall survival.

TABLE 5
Pharmacokinetic Parameters of DX-8951 (n = 37)[a]

	PK Analysis A		PK Analysis B	
Parameter	Average	CV%	Average	CV%
Cl (L/hr)/m^2)	1.3	38.5	1.4	48.5
Cl/f (L/hr)/m^2)	2.7	47.2	3.6	10.9
Vc (L/m^2)	4.5	39.4	5.1	33.5
Vp (L/m^2)	5.9	40.6	6.7	26.4
Vss (L/m^2)	10.4	40.6	11.8	21.2
λ_z HL (hr)	9.9	106.5	8.2	49.4

PK: pharmacokinetic; CV%: % interindividual variability; CL: total plasma clearance; Cld: distributional clearance; Vc: central volume of distribution; Vp: peripheral volume of distribution, λ_z-HL: terminal elimination half-life.

[a] For Analysis A, certain exposure opportunity index (EOI) plasma concentrations were deemed anomalous but they were kept in the analysis (this analysis was done for informative purposes; see Results); for Analysis B, certain EOI plasma concentration were deemed anomalous and were removed from the analysis (preferred analysis, see Results).

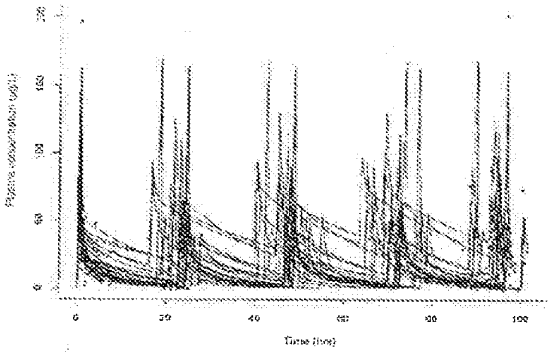


FIGURE 3. Observed (dots) and fitted (solid line) concentrations for each patient over the 5 days of administration ("spaghetti plot").

toxicity. Compared with CPT-11, in which the primary toxicity was diarrhea,[25] gastrointestinal toxicities, including diarrhea, nausea, and emesis, were mild to moderate when they occurred. Other nonhematologic toxicities, such as fatigue and alopecia, also were mild to moderate.

Previous studies showed that the PK behavior of exatecan mesylate is linear between the administered doses.[26] This means that the plasma clearance (CL) and the volume of distribution (Vss) did not change between the lowest dose group and the highest dose group. This linearity was observed again in the current patient population. In addition, exatecan mesylate was associated with a terminal elimination half-life of 8 hours, a total body clearance (CL) of 1.4 L per hour per m^2, and a total volume of distribution (Vss) of 12 L/m^2. These values are consistent with the values seen when exatecan mesylate was administered to patients with advanced solid malignancies or advanced leukemia.[27]

The efficacy of camptothecin analogs in patients with solid tumors appears to be schedule-dependent. Recently, Perez et al.[28] reported preliminary data from a randomized Phase II study of CPT-11 in patients with metastatic breast carcinoma. In that study, patients were randomized to receive CPT-11 100 mg/m^2 weekly × 4 and then had 2-week rest, or they were randomized to receive CPT-11 240 mg/m^2 every 3 weeks. Patients had to have previous exposure to either an anthracycline or a taxane. In that study, both schedules were tolerated well. The preliminary response rate was 29% for both groups. Although all patients in the current study had received both anthracyclines and taxanes, the possibility of schedule-

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dependent activity cannot be excluded. A minority of patients had excellent responses to exatecan mesylate therapy at the schedule used. However, it is possible that the efficacy of this novel agent may be greater in patients previously unexposed to chemotherapy or by using a different schedule. More studies will be required to determine the role of exatecan mesylate, either as a single agent or in combination, in patients with metastatic breast carcinoma.

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90 Long Acre
London WC2E 9RA
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Applicant/Proprietor	
Daiichi Sankyo Company, Limited	

Communication

The extended European search report is enclosed.

The extended European search report includes, pursuant to Rule 62 EPC, the supplementary European search report (Art. 153(7) EPC) and the European search opinion.

Copies of documents cited in the European search report are attached.

☑ 0 additional set(s) of copies of such documents is (are) enclosed as well.

Refund of the search fee
If applicable under Article 9 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.

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SUPPLEMENTARY EUROPEAN SEARCH REPORT

Application Number
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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
A	WO 2011/011474 A1 (ENZON PHARMACEUTICALS INC [US]; SAPRA PUJA [US]) 27 January 2011 (2011-01-27) * claims 1,3 *	1	INV. C07K16/28 A61K31/4745 A61K39/395 A61P35/00
A	PATRICK J. BURKE ET AL: "Design, Synthesis, and Biological Evaluation of Antibody-Drug Conjugates Comprised of Potent Camptothecin Analogues", BIOCONJUGATE CHEMISTRY, vol. 20, no. 6, 17 June 2009 (2009-06-17), pages 1242-1250, XP55079987, ISSN: 1043-1802, DOI: 10.1021/bc9001097 * Scheme 2 * * the whole document *	1	C07D491/052
A,D	EP 0 916 348 A1 (DAIICHI SEIYAKU CO [JP]; DRUG DELIVERY SYSTEM INST LTD [JP]) 19 May 1999 (1999-05-19) * page 11 *	1	
E	EP 2 907 824 A1 (DAIICHI SANKYO CO LTD [JP]) 19 August 2015 (2015-08-19) * the whole document *	1	TECHNICAL FIELDS SEARCHED (IPC) A61K A61P

The supplementary search report has been based on the last set of claims valid and available at the start of the search.

Place of search	Date of completion of the search	Examiner
Munich	2 August 2017	Skulj, Primoz

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
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O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C04)

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 15 74 3738

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
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02-08-2017

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011011474 A1	27-01-2011	AU 2010276261 A1	02-02-2012
		CA 2768494 A1	27-01-2011
		CN 102481364 A	30-05-2012
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		TW 201420117 A	01-06-2014
		US 2015297748 A1	22-10-2015
		US 2016279259 A1	29-09-2016

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 15 74 3738

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02-08-2017

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
		WO 2014057687 A1	17-04-2014

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

Information on Search Strategy - Pilot phase (see OJ 2015, A86)
The type of information contained in this sheet may change during the pilot for improving the usefulness of this new service.

Application Number
EP 15 74 3738

TITLE: ANTI-HER2 ANTIBODY-DRUG CONJUGATE

APPLICANT: Daiichi Sankyo Company, Limited

IPC CLASSIFICATION: C07K16/28, A61K31/4745, A61K39/395, A61P35/00, C07D491/052

EXAMINER: Skulj, Primoz

CONSULTED DATABASES: NPL, DOSYS, CA

CLASSIFICATION SYMBOLS DEFINING EXTENT OF THE SEARCH:

IPC:

CPC:

FI/F-TERMS:

KEYWORDS OR OTHER ELEMENTS FEATURING THE INVENTION:
tratuzumab exatecan conjugate

EPO FORM P04A42

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European Patent Office
Office européen des brevets

European Patent Office
80298 MUNICH
GERMANY
Tel: +49 89 2399 0
Fax: +49 89 2399 4465

Application No.: 15 743 738.5

The application and the invention to which it relates seem to meet the requirements of the EPC. Upon presence of a valid request for examination and consideration by the examining division, a communication under Rule 71(3) EPC may be issued.

The further procedure with respect to this application is dependent upon the result of the search for prior art under Article 54(3) EPC* which will be carried out at least 18 months from the date of filing of the application under consideration. If such prior art is found, a communication under Rule 71(1) EPC will be sent. The communication under Rule 71(3) EPC can be issued only after that search has been carried out and if no such prior art is found.

* It is to be noted that Article 54(4) EPC 1973 continues to apply to European patent applications still pending, filed before 13.12.2007

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Fairbairn, Angus Chisholm
Marks & Clerk LLP
15 Fetter Lane
London EC4A 1BW
ROYAUME UNI

Date
04.02.20

Reference	Application No./Patent No.
PN816532EPB	19206764.3 - 1108
Applicant/Proprietor	
Daiichi Sankyo Company, Limited	

Communication

The extended European search report is enclosed.

The extended European search report includes, pursuant to Rule 62 EPC, the European search report (R. 61 EPC) or the partial European search report/ declaration of no search (R. 63 EPC) and the European search opinion.

Copies of documents cited in the European search report are attached.

☑ 0 additional set(s) of copies of such documents is (are) enclosed as well.

The following have been approved:

☑ Abstract ☑ Title

☐ The Abstract was modified and the definitive text is attached to this communication.

The following figure(s) will be published together with the abstract:

Refund of search fee
If applicable under Article 9 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.

Should you wish to further prosecute this application in the examination phase, your attention is drawn to the provisions of Rule 70a EPC. An invitation to respond to the extended European search report will be issued once the date of publication of the European search report has been mentioned in the European Patent Bulletin (R. 69(1), R. 70(2) EPC).

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Information on Search Strategy - Pilot phase (see OJ 2015, A86)
The type of information contained in this sheet may change during the pilot for improving the usefulness of this new service.

Application Number
EP 19 20 6764

TITLE: ANTIBODY-DRUG CONJUGATE

APPLICANT: Daiichi Sankyo Company, Limited

IPC CLASSIFICATION: A61K47/68

EXAMINER: Bettio, Andrea

CONSULTED DATABASES: NPL, WPI

CLASSIFICATION SYMBOLS DEFINING EXTENT OF THE SEARCH:

IPC:

CPC: A61K47/6817, A61K47/6851, A61K47/6889

FI/F-TERMS:

KEYWORDS OR OTHER ELEMENTS FEATURING THE INVENTION:
anti-B7-H3 antibody-drug conjugates

EPO FORM P04A42

Datum / Date / Date	cf Form 1507	Blatt / Sheet / Feuille	1	Anmelde-Nr: / Application No: / Demande n°:	19 206 764.3

The examination is being carried out on the **following application documents**

Description, Pages

1-446 as originally filed

Sequence listings, SEQ ID NO

32 as originally filed

Claims, Numbers

1-24 as originally filed

Drawings, Sheets

1-22 as originally filed

Reference is made to the following documents; the numbering will be adhered to in the rest of the procedure.

D1 WO 2008/116219 A2 (SLOAN KETTERING INST CANCER [US]; CHEUNG NAI-KONG V [US]) 25 September 2008 (2008-09-25)

Art 76(1) EPC

The present application has been filed as a divisional application in the sense of Art 76(1) EPC from the earlier application EP18152381.

The subject matter of present claims 1-24 appears to have no basis in the above mentioned earlier application.

Thus the requirements of Art. 76(1) EPC are not fulfilled.

Art 54 and Art 56 EPC

The problem to be solved by the present application is the provision of an anti-tumor drug having an excellent therapeutic effect and safety (page 8, lines 2-6 of the present application).

Datum / Date / Date	cf Form 1507	Blatt / Sheet / Feuille	2	Anmelde-Nr: / Application No: / Demande n°:	19 206 764.3

D1 (claims 8, 10, 11) represents the closest prior art and discloses a method for improving the prognosis or prolonging the survival of a subject bearing a tumor , comprising administering to the subject a composition comprising a monoclonal antibody 8H9 recognizing a 4Ig-B7H3 antigen. Said antibody may be conjugated to a cytotoxic agent such as a radioisotope.

D1 does not teach neither the antitumor compound nor the linkers of present claim 1.

The objective problem is the provision of alternative antibody drug conjugates

The data disclosed in the examples of the present application show that antibody drug conjugates (ADCs) falling under the subject matter of claim 1 do have an in vivo antitumor activity

Nothing in the prior art suggests the use of a cytotoxic compound combined with one of the linker disclosed in claim 1 for the preparation of an ADC wherein the antibody is an anti-B7-H3 one; the subject matter of the present claims is therefore novel in the sense of Art. 54 EPC and inventive in the sense of Art. 56 EPC.

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EUROPEAN SEARCH REPORT

Application Number
EP 19 20 6764

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
A	WO 2008/116219 A2 (SLOAN KETTERING INST CANCER [US]; CHEUNG NAI-KONG V [US]) 25 September 2008 (2008-09-25) * claims 8, 10, 11; the whole document *	1-24	INV. A61K47/68
A	D. LOO ET AL: "Development of an Fc-Enhanced Anti-B7-H3 Monoclonal Antibody with Potent Antitumor Activity", CLINICAL CANCER RESEARCH, vol. 18, no. 14, 15 July 2012 (2012-07-15) , pages 3834-3845, XP055092714, ISSN: 1078-0432, DOI: 10.1158/1078-0432.CCR-12-0715 * the whole document *	1-24	
T	NAKADA TAKASHI ET AL: "Novel antibody drug conjugates containing exatecan derivative-based cytotoxic payloads", BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, PERGAMON, AMSTERDAM, NL, vol. 26, no. 6, 8 February 2016 (2016-02-08), pages 1542-1545, XP029436554, ISSN: 0960-894X, DOI: 10.1016/J.BMCL.2016.02.020 * the whole document *		TECHNICAL FIELDS SEARCHED (IPC) A61K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	27 January 2020	Bettio, Andrea

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
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ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 19 20 6764

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27-01-2020

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2008116219 A2	25-09-2008	CA 2680111 A1	25-09-2008
		CN 101687021 A	31-03-2010
		EP 2121008 A2	25-11-2009
		JP 2010523478 A	15-07-2010
		JP 2014088411 A	15-05-2014
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		KR 20100014527 A	10-02-2010
		US 2010143245 A1	10-06-2010
		WO 2008116219 A2	25-09-2008

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Fairbairn, Angus Chisholm
Marks & Clerk LLP
90 Long Acre
London WC2E 9RA
ROYAUME UNI

Date
10.05.17

Reference	Application No./Patent No.
PN821121EP	14874745.4 - 1466 / 3088419 PCT/JP2014006421
Applicant/Proprietor	
Daiichi Sankyo Company, Limited, et al	

Communication

The extended European search report is enclosed.

The extended European search report includes, pursuant to Rule 62 EPC, the supplementary European search report (Art. 153(7) EPC) and the European search opinion.

Copies of documents cited in the European search report are attached.

☑ 0 additional set(s) of copies of such documents is (are) enclosed as well.

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SUPPLEMENTARY EUROPEAN SEARCH REPORT

Application Number
EP 14 87 4745

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
Y	T. M. CARDILLO ET AL: "Humanized Anti-Trop-2 IgG-SN-38 Conjugate for Effective Treatment of Diverse Epithelial Cancers: Preclinical Studies in Human Cancer Xenograft Models and Monkeys", CLINICAL CANCER RESEARCH, vol. 17, no. 10, 3 March 2011 (2011-03-03), pages 3157-3169, XP055044596, ISSN: 1078-0432, DOI: 10.1158/1078-0432.CCR-10-2939 * Anti-TROP2 antibody conjugated to SN-38 for the treatment of cancer: see abstract results and discussion *	1-20	INV. C07K16/28 A61K31/4745 A61K39/395 A61K47/68 A61P35/00 C12N15/09
Y	WO 2011/068845 A1 (IMMUNOMEDICS INC [US]; GOVINDAN SERENGULAM V [US]; GOLDENBERG DAVID M) 9 June 2011 (2011-06-09) * Administering to a subject with cancer an Anti-Trop-2 antibody or antigen-binding fragment thereof conjugated to a therapeutic agent which can be camptothecin: see par. 9, 12, 16 (see "camptothecins") and par.205-218; claims *	1-20	TECHNICAL FIELDS SEARCHED (IPC) A61K
Y	US 2008/131363 A1 (GOVINDAN SERENGULAM [US] ET AL) 5 June 2008 (2008-06-05) * Conjugates of anti EPG-1 (anti TROP2) antibodies and antitumoral agents, including camptothecin, for use in the tretment of cancer *	1-20	

-/--

The supplementary search report has been based on the last set of claims valid and available at the start of the search.

Place of search	Date of completion of the search	Examiner
Munich	2 May 2017	Veronese, Andrea

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SUPPLEMENTARY EUROPEAN SEARCH REPORT

Application Number
EP 14 87 4745

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
Y	MORITZ N WENTE ET AL: "DE-310, a macromolecular prodrug of the topoisomerase-I-inhibitor exatecan (DX-8951), in patients with operable solid tumors", INVESTIGATIONAL NEW DRUGS ; THE JOURNAL OF NEW ANTICANCER AGENTS, KLUWER ACADEMIC PUBLISHERS, BO, vol. 23, no. 4, 1 August 2005 (2005-08-01) , pages 339-347, XP019206092, ISSN: 1573-0646, DOI: 10.1007/S10637-005-1442-2 * Conjugate of Exatecan (DX8951) and a polysaccharide via a spacer containing the -Gly-Gly-Phe-Gly- tetrapeptide moiety, and its use as antitumoral agent * -----	1-20	
Y	YUSUKE OCHI ET AL: "A possible mechanism for the long-lasting antitumor effect of the macromolecular conjugate DE-310: mediation by cellular uptake and drug release of its active camptothecin analog DX-8951", CANCER CHEMOTHERAPY AND PHARMACOLOGY, SPRINGER, BERLIN, DE, vol. 55, no. 4, 1 April 2005 (2005-04-01), pages 323-332, XP019334130, ISSN: 1432-0843, DOI: 10.1007/S00280-004-0911-1 * Conjugate of Exatecan (DX8951) and a polysaccharide via a spacer containing the -Gly-Gly-Phe-Gly- tetrapeptide moiety, and its use as antitumoral agent * ----- -/--	1-20	TECHNICAL FIELDS SEARCHED (IPC)

The supplementary search report has been based on the last set of claims valid and available at the start of the search.

Place of search	Date of completion of the search	Examiner
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P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
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SUPPLEMENTARY EUROPEAN SEARCH REPORT

Application Number
EP 14 87 4745

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
Y	YOSHINOBU SHIOSE ET AL: "Systematic Research of Peptide Spacers Controlling Drug Release from Macromolecular Prodrug System, Carboxymethyldextran Polyalcohol-Peptide-Drug Conjugates", BIOCONJUGATE CHEMISTRY, vol. 20, no. 1, 21 January 2009 (2009-01-21), pages 60-70, XP055333870, ISSN: 1043-1802, DOI: 10.1021/bc800238f * The Gly-Gly-Phe-Gly sequence for preparing drug conjugates capable of releasing the active agent in vivo: see abstract, results and discussion * -----	1-20	

TECHNICAL FIELDS SEARCHED (IPC)

The supplementary search report has been based on the last set of claims valid and available at the start of the search.

Place of search	Date of completion of the search	Examiner
Munich	2 May 2017	Veronese, Andrea

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D : document cited in the application
L : document cited for other reasons
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02-05-2017

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011068845 A1	09-06-2011	CA 2782194 A1	09-06-2011
		EP 2506881 A1	10-10-2012
		WO 2011068845 A1	09-06-2011
US 2008131363 A1	05-06-2008	NONE	

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

Information on Search Strategy - Pilot phase (see OJ 2015, A86)
The type of information contained in this sheet may change during the pilot for improving the usefulness of this new service.

Application Number
EP 14 87 4745

TITLE: ANTI-TROP2 ANTIBODY-DRUG CONJUGATE

APPLICANT: Daiichi Sankyo Company, Limited

IPC CLASSIFICATION: C07K16/28, A61K31/4745, A61K39/395, A61K47/68, A61P35/00, C12N15/09

EXAMINER: Veronese, Andrea

CONSULTED DATABASES: NPL, BIOSIS, MEDLINE, REAXYS

CLASSIFICATION SYMBOLS DEFINING EXTENT OF THE SEARCH:

IPC:

CPC:

FI/F-TERMS:

KEYWORDS OR OTHER ELEMENTS FEATURING THE INVENTION:

EPO FORM P04A42

Datum Date Date	cf Form 1507	Blatt Sheet Feuille	1	Anmelde-Nr: Application No: Demande n°:	14 874 745.4

The examination is being carried out on the **following application documents**

Description, Pages

1-252 as published

Claims, Numbers

1-20 filed with entry into the regional phase before the EPO

Drawings, Sheets

1-27 as published

Drawings, Figures

1-44 as published

The following documents were mentioned in the European and in the International search reports; the numbering will be adhered to in the rest of the procedure.

D1 T. M. CARDILLO ET AL: "Humanized Anti-Trop-2 IgG-SN-38 Conjugate for Effective Treatment of Diverse Epithelial Cancers: Preclinical Studies in Human Cancer Xenograft Models and Monkeys", CLINICAL CANCER RESEARCH, vol. 17, no. 10, 3 March 2011, pages 3157-3169, XP055044596

D2 WO 2011/068845 A1 (IMMUNOMEDICS INC [US]) 9 June 2011

D3 US 2008/131363 A1 (GOVINDAN SERENGULAM [US] ET AL) 5 June 2008

D4 MORITZ N WENTE ET AL: "DE-310, a macromolecular prodrug of the topoisomerase-I-inhibitor exatecan (DX-8951), in patients with operable solid tumors", INVESTIGATIONAL NEW DRUGS ; THE JOURNAL OF NEW ANTICANCER AGENTS, KLUWER ACADEMIC PUBLISHERS, BO, vol. 23, no. 4, 1 August 2005 (2005-08-01), pages 339-347, XP019206092, ISSN: 1573-0646, DOI: 10.1007/S10637-005-1442-2

Datum Date Date	cf Form 1507	Blatt Sheet Feuille	2	Anmelde-Nr: Application No: Demande n°:	14 874 745.4

D5 YUSUKE OCHI ET AL: "A possible mechanism for the long-lasting antitumor effect of the macromolecular conjugate DE-310: mediation by cellular uptake and drug release of its active camptothecin analog DX-8951", CANCER CHEMOTHERAPY AND PHARMACOLOGY, SPRINGER, BERLIN, DE, vol. 55, no. 4, 1 April 2005 (2005-04-01), pages 323-332, XP019334130, ISSN: 1432-0843, DOI: 10.1007/S00280-004-0911-1

D6 YOSHINOBU SHIOSE ET AL: "Systematic Research of Peptide Spacers Controlling Drug Release from Macromolecular Prodrug System, Carboxymethyldextran Polyalcohol-Peptide-Drug Conjugates", BIOCONJUGATE CHEMISTRY, vol. 20, no. 1, 21 January 2009, pages 60-70, XP055333870, ISSN: 1043-1802, DOI: 10.1021/bc800238f

D7 US 2011/293513 A1 (IMMUNOMEDICS, INC.) 1 December 2011

D8 IKUO MITSUI ET AL.: "A New Water-soluble Camptothecin Derivative, DX-8951f, Exhibits Potent Antitumor Activity against Human Tumors in vitro and in vivo", JPN. J. CANCER RES., vol. 86, 1995, pages 776-782, XP009059567,

D9 JP 2009 538629 A (GENENTECH, INC.) 12 November 2009

D10 JP 2008 521828 A (SEATTLE GENETICS, INC.) 26 June 2008

D11 JP 2005 511627 A (SEATTLE GENETICS, INC.) 28 April 2005

D12 WO 00/25825 A1 (DAIICHI PHARMACEUTICAL CO., LTD.) 11 May 2000

D13 JP 2012 100671 A (AMGEN INC.) 31 May 2012

D14 JP 2013 534906 A (INDIANA UNIVERSITY RESEARCH & TECHNOLOGY CORP.) 9 September 2013

D15 WO 2014/061277 A1 (DAIICHI SANKYO CO., LTD.) 24 April 2014

Datum Date Date	cf Form 1507	Blatt Sheet Feuille	3	Anmelde-Nr: Application No: Demande n°:	14 874 745.4

Amendments (Art.123 (2) EPC)

The claims were amended, but no basis for the amendments has been provided. The basis for all amendments cannot be found in the voluminous set of documents forming the application as filed. The applicant is required to provide such basis.

Novelty (Art.54 EPC)

None of the documents mentioned in the search report describes conjugates comprising exatecan and TROP2 antibodies. Thus, the claimed matter appears to be new.

Inventive step (Art.56 EPC)

The present application relates to the provision of conjugate compounds which can be used as anticancer agents. The claimed compounds comprise a residue of Exatecan (a camptothecin derivative), linked to a TROP2 antibody via a spacer containing a tetrapeptide having the sequence -Gly-Gly-Phe-Gly-. The TRPO2 antibody is used for targeting the cancer cells, whereas the tetrapeptide is chosen because it is cleaved by enzymes present in the targeted tissue. The following prior art documents leave no doubts that conjugates of camptothecin derivatives and TROP2 antibodies were already known before the relevant date.

Document D1 (from Cardillo) describes the anti-TROP2 antibody hRS7, which is the preferred antibody described in the present application, conjugated to SN-38, an analogous of camptotecin which is structurally very similar to the claimed compound exatecan. This conjugate incorporates some of the structural elements of the spacer present in the claimed agents, and also a cleavable peptide: see figure 1. The conjugate is proposed for treating cancer: see abstract results and discussion, Table 1; Figures. The authors indicate that, on the basis of the results, the conjugate has a significant effect against a range of human solid tumor types: see conclusions.

Document D2 (WO2011068845) discloses an Anti-Trop-2 antibody or antigen-binding fragment thereof, conjugated to a therapeutic agent, which can be camptothecin: see par. 9, 12, 16 (see "camptothecins") and see in particular par. 205-218 and the claims, describing a conjugate comprising the camptothecin derivative SN-38 and the TROP-2 antibody hRS7. The results reported in the example indicate that the conjugate comprising the TROP-2 antibody is more potent compared to other antibodies targeting cancer cells (see par. 298).

Document D3 (US20080131363), discloses conjugates of anti EPG-1 (anti TROP2) antibodies, including the preferred antibody hRS7, with antitumoral agents, including camptothecin, for use in the treatment of cancer: see par. 8, 9, 31-32, 37, 41, 148, 149; examples 4, 5, claims. See in particular par. 15, 92 claim 8 referring to camptothecin.

Some further conjugates of hRS7 and SN38 are mentioned in document D7 (US20110293513), cited in the international search report.

Starting from any of these documents (*e.g.* from D1 or D2) as closest prior art, the underlying technical problem can be regarded as that of providing a further drug conjugate suitable for treating cancer. When confronted with this problem, the skilled person would certainly consider replacing SN-28, mentioned in these documents, with other analogous camptothecin derivatives. When looking for such alternative compounds, he would take into account the teaching of the following documents:

D8 (from Ikuo), mentioned by the ISA, describes the preparation of the camptothecin Exatecan (DX-895-1) and its utility as an anti-cancer agent. This document also indicates, that this compound is 6 times more potent compared to SN-38. This teaching provides a clear incentive to replace SN-38 with exatecan.

Furthermore, document D5 (from Yusuke) describes DE-310, a conjugate between Exatecan (DX8951) and a polysaccharide polymer scaffold, the two moieties linked via a spacer containing the -Gly-Gly-Phe-Gly- tetrapeptide sequence and the use of such conjugate for providing a long lasting anti-cancer effect: see abstract, the introduction the restults and the discussion. Document D4 (from Wente) confirms that DE-8951 is an effective agent for treating solid tumors: see abstract and results.

Other camptotecin conjugates containing the relevant tetrapeptide sequence in the linking moiety are described in document WO0025825, cited by the ISA (D12, see also the corresponding European family member, EP1155702 and in particular claims 33-34, 37-38). Finally document D6 describes the rationale of using the claimed tetrapeptide sequence.

Thus, together, these documents teach that 1) there are good reasons for replacing SN-28 with exatecan in anticancer therapy 2) exatecan is suitable for conjugation to other larger macromolecular moieties 3) the -Gly-Gly-Phe-Gly- tetrapeptide sequence is suitable for cleavage and delivery of the active moiety at the target site.

The other features characterising the claims on file, *e.g.* the presence of a succinimid linkers or short chains of polyethylenglycol are commonly known in the art, as shown in the aforementioned prior art documents and in the other documents mentioned iby the ISA. Furthermore, the selection of these features or others, such as the specific sequences now indicated in the amended claims, is not associated to particular unexpected technical effects. Thus, even if new, the presently claimed conjugates, as well as the compositions comprising them and their use for the relevant therapeutic applications, do not involve an inventive step (Art.56 EPC).

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Fairbairn, Angus Chisholm
Marks & Clerk LLP
90 Long Acre
London WC2E 9RA
ROYAUME UNI

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Date
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Reference	Application No./Patent No.
PN817715EP	13847461.4 - 1453 / 2910573 PCT/JP2013006178
Applicant/Proprietor	
Daiichi Sankyo Company, Limited	

Communication

The extended European search report is enclosed.

The extended European search report includes, pursuant to Rule 62 EPC, the supplementary European search report (Art. 153(7) EPC) and the European search opinion.

Copies of documents cited in the European search report are attached.

☑ 0 additional set(s) of copies of such documents is (are) enclosed as well.

Refund of the search fee
If applicable under Article 9 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.

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SUPPLEMENTARY EUROPEAN SEARCH REPORT

Application Number
EP 13 84 7461

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
E	EP 2 907 824 A1 (DAIICHI SANKYO CO LTD [JP]) 19 August 2015 (2015-08-19) * claims; paragraph 180 * -----	1-54	INV. C07K16/28 A61K39/395 A61P35/00 A61K31/4745 A61K31/48 A61K47/48
T	NAKADA TAKASHI ET AL: "Novel antibody drug conjugates containing exatecan derivative-based cytotoxic payloads", BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, PERGAMON, AMSTERDAM, NL, vol. 26, no. 6, 8 February 2016 (2016-02-08), pages 1542-1545, XP029436554, ISSN: 0960-894X, DOI: 10.1016/J.BMCL.2016.02.020 * the whole document * -----		
A	WO 03/015826 A1 (TANABE SEIYAKU CO [JP]) 27 February 2003 (2003-02-27) * page 28 * -----	1-54	
			TECHNICAL FIELDS SEARCHED (IPC) A61K

The supplementary search report has been based on the last set of claims valid and available at the start of the search.

Place of search	Date of completion of the search	Examiner
The Hague	6 May 2016	Bettio, Andrea

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C04)

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 13 84 7461

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
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06-05-2016

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 2907824 A1	19-08-2015	AU 2013328111 A1	12-03-2015
		CA 2885800 A1	17-04-2014
		CN 104755494 A	01-07-2015
		EP 2907824 A1	19-08-2015
		HK 1210477 A1	22-04-2016
		KR 20150067149 A	17-06-2015
		PH 12015500667 A1	18-05-2015
		SG 11201502887W A	28-05-2015
		TW 201420117 A	01-06-2014
		US 2015297748 A1	22-10-2015
		WO 2014057687 A1	17-04-2014
WO 03015826 A1	27-02-2003	AR 035137 A1	14-04-2004
		AU 2002328093 B2	05-05-2005
		BR 0212036 A	17-08-2004
		CA 2457056 A1	27-02-2003
		CN 1545423 A	10-11-2004
		EP 1418947 A1	19-05-2004
		HU 0401351 A2	28-12-2004
		KR 20040027972 A	01-04-2004
		MX PA04001599 A	08-07-2004
		NO 20041194 A	19-03-2004
		NZ 530947 A	28-04-2006
		RU 2275913 C2	10-05-2006
		TW I313609 B	21-08-2009
		UA 75450 C2	17-05-2004
		WO 03015826 A1	27-02-2003
		ZA 200400917 A	25-08-2004

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

Information on Search Strategy - Pilot phase (see OJ 2015, A86)
The type of information contained in this sheet may change during the pilot for improving the usefulness of this new service.

Application Number
EP 13 84 7461

TITLE: ANTIBODY-DRUG CONJUGATE PRODUCED BY BINDING THROUGH LINKER HAVING HYDROPHILIC STRUCTURE

APPLICANT: Daiichi Sankyo Company, Limited

IPC CLASSIFICATION: C07K16/28, A61K39/395, A61P35/00, A61K31/4745, A61K31/48, A61K47/48

EXAMINER: Bettio, Andrea

CONSULTED DATABASES: BIOSIS, EMBASE, EPODOC, MEDLINE, NPL, WPI

CLASSIFICATION SYMBOLS DEFINING EXTENT OF THE SEARCH:

IPC:

CPC:

FI/F-TERMS:

KEYWORDS OR OTHER ELEMENTS FEATURING THE INVENTION:
conjugates according to present claim 1

Datum / Date / Date	cf Form 1507	Blatt / Sheet / Feuille	1	Anmelde-Nr: / Application No: / Demande n°:	13 847 461.4

The examination is being carried out on the **following application documents**

Description, Pages

1-5, 7-40, 42-89, 91-426	filed with entry into the regional phase before the EPO				
6, 41, 90	received on	07-12-2015	with letter of		07-12-2015

Claims, Numbers

1-54	filed with entry into the regional phase before the EPO

Drawings, Sheets

1/20-20/20	filed with entry into the regional phase before the EPO

Reference is made to the following document; the numbering will be adhered to in the rest of the procedure.

D1 EP 2 907 824 A1 (DAIICHI SANKYO CO LTD [JP]) 19 August 2015 (2015-08-19)

Art 84 EPC

The wording of present claims 50 and 52 does not allow the skilled person to understand whether the claimed subject matter is directed to a method or to a compound/composition, thus rendering the subject matter of said claims unclear in the sense of Art 84 EPC.

Art 53(c) EPC

Present claims 53 and 54 are directed to methods of treatment of the human/animal body, thereby contravening Art 53(c) EPC.

Art 54(3) EPC

D1 published on 19.08.2015 claims the priority date of 11.10.2012.

Its content as filed is therefore considered to be comprised in the state of the art relevant to the question of novelty, pursuant to Article 54(3) EPC.

Datum Date Date	cf Form 1507	Blatt Sheet Feuille	2	Anmelde-Nr: Application No: Demande n°:	13 847 461.4

This earlier application (claims; paragraphs 180, 314) shows: antibody drug conjugates according to the formula of present claim 1 wherein L^p is the tetrapeptide GGFG for the treatment of the same tumors disclosed in the present application. Alternative L^p sequences such as GGF, DGGF, DDGGF, EGGF, GGFG, SGGF,KGGF, DGGFG, GGFGG, DDGGFG, KDGGFG, GGFGGGF are also disclosed.

Thus, it is prejudicial to the novelty of the subject-matter of claims 1-54 of the present application.

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Marks & Clerk LLP
90 Long Acre
London WC2E 9RA
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Reference	Application No./Patent No.
PN816532EP	13845596.9 - 1453 / 2907824 PCT/JP2013006069

Applicant/Proprietor
Daiichi Sankyo Company, Limited

Communication

The extended European search report is enclosed.

The extended European search report includes, pursuant to Rule 62 EPC, the supplementary European search report (Art. 153(7) EPC) and the European search opinion.

Copies of documents cited in the European search report are attached.

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SUPPLEMENTARY EUROPEAN SEARCH REPORT

Application Number
EP 13 84 5596

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
T	NAKADA TAKASHI ET AL: "Novel antibody drug conjugates containing exatecan derivative-based cytotoxic payloads", BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, PERGAMON, AMSTERDAM, NL, vol. 26, no. 6, 8 February 2016 (2016-02-08), pages 1542-1545, XP029436554, ISSN: 0960-894X, DOI: 10.1016/J.BMCL.2016.02.020 * the whole document *		INV. C07K16/28 A61K39/395 A61P35/00 A61K31/4745 A61K31/48 A61K47/48
A	WO 03/015826 A1 (TANABE SEIYAKU CO [JP]) 27 February 2003 (2003-02-27) * Page 28 *	1-26	

TECHNICAL FIELDS SEARCHED (IPC)

A61K

The supplementary search report has been based on the last set of claims valid and available at the start of the search.

Place of search	Date of completion of the search	Examiner
The Hague	28 April 2016	Bettio, Andrea

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C04)

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 13 84 5596

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
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28-04-2016

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 03015826 A1	27-02-2003	AR 035137 A1	14-04-2004
		AU 2002328093 B2	05-05-2005
		BR 0212036 A	17-08-2004
		CA 2457056 A1	27-02-2003
		CN 1545423 A	10-11-2004
		EP 1418947 A1	19-05-2004
		HU 0401351 A2	28-12-2004
		KR 20040027972 A	01-04-2004
		MX PA04001599 A	08-07-2004
		NO 20041194 A	19-03-2004
		NZ 530947 A	28-04-2006
		RU 2275913 C2	10-05-2006
		TW I313609 B	21-08-2009
		UA 75450 C2	17-05-2004
		WO 03015826 A1	27-02-2003
		ZA 200400917 A	25-08-2004

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

Information on Search Strategy - Pilot phase (see OJ 2015, A86)
The type of information contained in this sheet may change during the pilot for improving the usefulness of this new service.

Application Number
EP 13 84 5596

TITLE: ANTIBODY-DRUG CONJUGATE

APPLICANT: Daiichi Sankyo Company, Limited

IPC CLASSIFICATION: C07K16/28, A61K39/395, A61P35/00, A61K31/4745, A61K31/48, A61K47/48

EXAMINER: Bettio, Andrea

CONSULTED DATABASES: NPL, BIOSIS, EMBASE, EPODOC, MEDLINE, WPI

CLASSIFICATION SYMBOLS DEFINING EXTENT OF THE SEARCH:

IPC:

CPC:

FI/F-TERMS:

KEYWORDS OR OTHER ELEMENTS FEATURING THE INVENTION:
formula of claim 1 and GGFG linker. compounds according to claim 26

Datum / Date / Date		Blatt / Sheet / Feuille		Anmelde-Nr: / Application No: / Demande n°:
	cf Form 1507		1	13 845 596.9

The examination is being carried out on the **following application documents**

Description, Pages

1-416 filed with entry into the regional phase before the EPO

Claims, Numbers

1-26 filed with entry into the regional phase before the EPO

The following document has been cited in the international search report; the numbering will be adhered to in the rest of the procedure.

D1 JP 2009 538629 A (GENENTECH, INC.) 12 November 2009 (2009-11-12)

Art 123(2) EPC

Present claim 21 discloses a tetrapeptide of sequence GGGF. Basis for such sequence cannot be found in the application as filed.

Consequently the subject matter of present claim 21 contravenes Art. 123(2) EPC.

Art 84 EPC

Present claim 16 discloses "the antitumor drug....which is applied to the lung cancer...".

The claim's wording does not allow to distinguish whether said wording is directed to a product claim or to a method claim, thereby rendering the subject matter of said claim unclear in the sense of Art 84 EPC.

Art 54 and Art 56 EPC

The problem to be solved by the present application is the provision of an antitumor drug having an excellent therapeutic effect and safety (page 8, lines 1-6 of the present application).

D1 (claim 217), representing the closest prior art discloses antibody drug conjugates wherein monomethylauristatine analogs are conjugated to an antibody via a linker comprising the dipeptide val-cit.

Datum / Date / Date: cf Form 1507
Blatt / Sheet / Feuille: 2
Anmelde-Nr: / Application No: 13 845 596.9 / Demande n°:

The use of antibody drug conjugates comprising the peptide linker GGFG and the exatecan analog of present claim 1 is neither disclosed nor suggested in the prior art.

Also the compounds of present claim 26 are novel and inventive because they represent intermediates useful for the synthesis of compounds according to claim 1.

Thus the subject matter of present claims 1-26 is novel in the snese of Art 54 EPC and inventive in the sense of Art 56 EPC.

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Fairbairn, Angus Chisholm
Marks & Clerk LLP
90 Long Acre
London WC2E 9RA
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Date
11.08.17

Reference	Application No./Patent No.
PN821970EP	15776810.2 - 1468 / 3130608 PCT/JP2015001922
Applicant/Proprietor	
Daiichi Sankyo Co., Ltd.	

Communication

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SUPPLEMENTARY EUROPEAN SEARCH REPORT

Application Number
EP 15 77 6810

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
E	EP 2 907 824 A1 (DAIICHI SANKYO CO LTD [JP]) 19 August 2015 (2015-08-19) * claims 35-49 * -----	32,34	INV. C07K16/32 A61K31/4745 A61K39/395
A	WO 2011/011474 A1 (ENZON PHARMACEUTICALS INC [US]; SAPRA PUJA [US]) 27 January 2011 (2011-01-27) * the whole document * -----	1	A61P35/00 C07K5/072 C07K5/083 C07K5/103 C07K7/06
Y	PATRICK J. BURKE ET AL: "Design, Synthesis, and Biological Evaluation of Antibody-Drug Conjugates Comprised of Potent Camptothecin Analogues", BIOCONJUGATE CHEMISTRY, vol. 20, no. 6, 17 June 2009 (2009-06-17), pages 1242-1250, XP55079987, ISSN: 1043-1802, DOI: 10.1021/bc9001097 * the whole document * * Scheme 2 * -----	1,6-8, 17,18	
Y	EP 0 916 348 A1 (DAIICHI SEIYAKU CO [JP]; DRUG DELIVERY SYSTEM INST LTD [JP]) 19 May 1999 (1999-05-19) * page 10 * -----	1,6-8, 17,18	TECHNICAL FIELDS SEARCHED (IPC) C07K A61P

The supplementary search report has been based on the last set of claims valid and available at the start of the search.

Place of search	Date of completion of the search	Examiner
Munich	4 August 2017	Skulj, Primoz

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C04)

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 15 77 6810

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
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04-08-2017

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 15 77 6810

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

Information on Search Strategy - Pilot phase (see OJ 2015, A86)
The type of information contained in this sheet may change during the pilot for improving the usefulness of this new service.

Application Number
EP 15 77 6810

TITLE: (ANTI-HER2 ANTIBODY)-DRUG CONJUGATE

APPLICANT: Daiichi Sankyo Co., Ltd.

IPC CLASSIFICATION: C07K16/32, A61K31/4745, A61K39/395, A61P35/00, C07K5/072, C07K5/083, C07K5/103, C07K7/06

EXAMINER: Skulj, Primoz

CONSULTED DATABASES: NPL

CLASSIFICATION SYMBOLS DEFINING EXTENT OF THE SEARCH:

IPC:

CPC:

FI/F-TERMS:

KEYWORDS OR OTHER ELEMENTS FEATURING THE INVENTION:
tratuzumab exatecan conjugate

EPO FORM P04A42

Datum Date Date	cf Form 1507	Blatt Sheet Feuille	1	Anmelde-Nr: Application No: Demande n°:	15 776 810.2

The examination is being carried out on the **following application documents**

Description, Pages

1-732	filed with entry into the regional phase before the EPO	

Claims, Numbers

1-34	filed in electronic form on	26-04-2017

Drawings, Sheets

1/5-5/5	filed with entry into the regional phase before the EPO	

D1 EP 2 907 824 A1 (DAIICHI SANKYO CO LTD [JP]) 19 August 2015 (2015-08-19)

D2 WO 2011/011474 A1 (ENZON PHARMACEUTICALS INC [US]; SAPRA PUJA [US]) 27 January 2011 (2011-01-27)

D3 PATRICK J. BURKE ET AL: "Design, Synthesis, and Biological Evaluation of Antibody-Drug Conjugates Comprised of Potent Camptothecin Analogues",
BIOCONJUGATE CHEMISTRY,
vol. 20, no. 6, 17 June 2009 (2009-06-17), pages 1242-1250,
XP55079987,
ISSN: 1043-1802, DOI: 10.1021/bc9001097

D4 EP 0 916 348 A1 (DAIICHI SEIYAKU CO [JP]; DRUG DELIVERY SYSTEM INST LTD [JP]) 19 May 1999 (1999-05-19)

The claimed subject matter

1 The present application relates to a bioconjugates of an anti-HER2 antibody (such as trustuzamab) and exatecan and the anticancer uses of these bioconjugates.

Datum Date Date	cf Form 1507	Blatt Sheet Feuille	2	Anmelde-Nr: Application No: Demande n°:	15 776 810.2

Novelty

2 The claimed subject matter is not novel in the sense of Art.54(3)

2.1 The intermediate compounds of claim 32 are not novel over D1. See claims 35-45 of D1. There is especially a novelty destroying overlap between claim 32 and the claim 35 of D1.

2.1.1 The specific intermediates of claim 33 seems novel over D1, however the following is noted: Because D1 was published as WO 2014057687 during the priority year, the applicant is requested to submit the translation of the priority document and indicate the basis for each of the structures in present claim 33 in said priority document.

2.2 Also claim 34 is not novel. It relates to a linker which is (at its simplest) L^1-L^2-L^P.

2.2.1 L^2-can be a bond, L^P is a simple dipeptide (such as diglycyl). L^1 can be a succinimidyl derivative, but can be also CH2-CO-NH-CH2-CO or CO-CH2-CO-. The structures thus cover the following structure CH2-CO-NH-CH2-CO- which is the diglycyl residue and the subject matter of claim 34 boils down to Gly-Gly-Gly-Gly. Such tetrateptide is not novel. For other novelty destroying compounds see claim 47 of D1.

2.3 The prior art does not disclose the bioconjugates of an anti-HER2 antibody (such as trustuzamab) and exatecan wherein the linker comprise the GGFG tetrapeptide linked to exatecan by at least a further -NH-CO- spacer. The subject matter of claims 1-31 is thus novel.

Inventive step

3 The problem to be solved by the present application is the provision of an antitumor drug having an excellent therapeutic effect, which is excellent in trems of antitumor effect and safety (paragraph 13 of the present application).

3.1.1 D2 discloses in claim 3 a bioconjugate comprising an anti-HER2 antibody and a drug similar to exatecan (an another camphotecin analogue): The linker comprises branched PEGylates structures.

3.1.2 D3 reviews the camphotecin analogue bioconjugate and e.g. on Scheme 2 discloses a similar maleimide stretcher on an oligopeptide linker connected to drugs similar to exatecan.

Datum Date Date	cf Form 1507	Blatt Sheet Feuille	3	Anmelde-Nr: Application No: Demande n°:	15 776 810.2

3.1.3 D4 discloses the exatecan functionalised with a GGFG tetrapeptide.

3.2 D2 or D3 relate to the same aim as the present application (i.e. treating breast cancer by bioconjugates of camphotecin analogue - see the introduction portion of both documents). D2 is specific to an anti-HER2 antibody but also D3 in the introduction implies such binding - see the reference to the citations 6 and 7 of D3. Because the present linker comprises an oligopeptide (L^P) and such oligopeptide can be found in D3 (Scheme 2) additionally linked to a maleimide linker, D3 is taken as the closest prior art.

3.3 The differences between the closest prior art and the subject matter of the claims are represented by:
-the linker used in the bioconjugate
-the cytotoxic agent.

3.4 No technical effect is derivable by said difference. The resulting objective technical problem may therefore be formulated as the provision of alternative bioconjugate.

3.5 The solution is provided with the structures of claim 1. These structures in the most simple embodiments boil down to L^1-L^2-L^P.This is however exactly the partial structure of the linker on Scheme 2 of D3 (L^1-is maleimide on a stretcher , L^2 is a bond and $L^P$is a dipeptide.

3.6 A skilled person however knows from D4 (see e.g. p. 10) that an olige-peptide can be linked to the exatecan directly when making conjugates through its amino side group and recognises that the specific structure (Ph-CH_2-O-CO-NH) depicted on the right to the dipeptide on Scheme 2 of D3 is not necessary because left col. on p. 1246 purports that such linkages are needed for the conjugates with aliphatic alcohols. In as far the structure boils down to dipeptide of Val-Cit (or its analogues Val-Lys), also such structure is obvious.

Clarity

4 The claims are unclear

4.1 The "drug" claims 25ff seem to relate to the "pharmaceutical compositions" or to medicinal use claims that should be reworded into proper "for use" format. It is not clear how e.g. claims 25 and 28 differ.

4.2 Method of use claims are not allowed at the present priority date.

Datum Date Date	cf Form 1507	Blatt Sheet Feuille	4	Anmelde-Nr: Application No: Demande n°:	15 776 810.2

4.3 The reasons for multiple independent claims in the same category are not given.

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XS CPRTENFRDE

Proc. Natl. Acad. Sci. USA
Vol. 75, No. 4, pp. 1947–1951, April 1978
Immunology

Antigens of human trophoblasts: A working hypothesis for their role in normal and abnormal pregnancies

(placentas/immunity/oncofetal/membranes)

W. PAGE FAULK*, ANNE TEMPLE†, R. E. LOVINS*, AND NANCY SMITH‡

* Department of Basic and Clinical Immunology and Microbiology, Medical University of South Carolina, Charleston, South Carolina, 29403; † Division of Cell Pathology, Clinical Research Centre, Northwick Park Hospital, Harrow, Middlesex, England; and ‡ Department of Gynecology, St. Thomas Hospital Medical School, London, England

Communicated by Philip Levine, January 11, 1978

ABSTRACT **This report describes the preparation and characterization of antisera to human trophoblast membranes. Rabbit antisera were raised to trophoblast microvilli prepared by differential ultracentrifugation. Antibodies to serum proteins were removed by solid-phase immunoabsorption with normal human serum, and indirect immunofluorescence experiments with cryostat sections of human placentas showed that the absorbed anti-trophoblast sera reacted with trophoblasts as well as with stromal cells and endothelium of chorionic villi. The antisera also produced membrane fluorescence when studied on viable lymphocytes and certain human cell lines. These anti-trophoblast sera were also lymphocytotoxic, and this reaction was abolished by prior absorption of the antisera with leukocytes. The leukocyte-absorbed anti-trophoblast sera retained their ability to react with trophoblasts and certain human cell lines, but no longer reacted with lymphocytes or placental stromal cells and endothelium. Two categories of trophoblast membrane antigens are thus defined: one present on trophoblasts and certain human cell lines (tentatively designated TA_1), and the other on trophoblasts and lymphocytes, villous fibroblasts, and endothelium (tentatively designated TA_2). A working hypothesis is proposed stating that normal pregnancy involves the generation of anti-TA_2 subsequent to blastocyst implantation and entrance of trophoblasts into the maternal circulation. This involves a mechanism similar to allogeneic cell stimulation and results in antibodies that block either the recognition or cytotoxicity of TA_1. Failure to mount this response allows TA_1 recognition and trophoblast immunopathology. Experimental and clinical studies in support of this working hypothesis, particularly involving abortion and toxemia, are cited from published reports.**

Several provocative ideas have been put forward suggesting that the mechanisms responsible for the integrity of the trophoblast homograft are essentially immunological in nature (1). In humans the functional interface between mother and fetus is the trophoblast (2), and immune reactions occurring on this membrane would seem to be central to the well being of the graft. Detailed investigation of trophoblast antigens is thus a necessary prerequisite to an understanding of the immunology of the materno-fetal relationship, yet current information is limited almost entirely to cell products of trophoblasts such as human chorionic gonadotropins and pregnancy-associated globulins (3). As a first step in more precisely defining antigens of the trophoblast membranes, we have studied the specificity of heterologous antisera to human trophoblasts. In this report we describe evidence suggesting that human trophoblast membranes contain two major groups of antigens: one that is shared between trophoblasts and normal peripheral white blood cells, and a second group that is shared between trophoblasts and certain human cell lines. These data, together with published reports from other laboratories, make it possible to construct a working hypothesis for the role of trophoblast membrane antigens in normal and abnormal pregnancies.

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MATERIALS AND METHODS

Trophoblast membranes were prepared from freshly collected, term, normal placentas by the differential ultracentrifugation technique of Smith *et al.* (4). Pellets contained large amounts of alkaline phosphatase [specific activity 0.89 with *p*-nitrophenyl phosphate as substrate (5)], indicating the presence of trophoblast membranes (6). Twelve outbred Old English rabbits were injected intramuscularly with 2 mg of the membrane preparation in 2.5 ml of Freund's complete adjuvant containing 1 mg of *Mycobacterium tuberculosis* per ml, boosted 6 weeks later with a further 2 mg interperitoneally, and bled after 12 days. Each rabbit received membrane preparations from a separate, normal placenta. The antisera were assayed for anti-β_2-microglobulin activity as an additional check for the purity of membranes used as antigens since we and others have previously reported (7–9) that, in contrast to cells of the villous stroma, human trophoblasts lack β_2-microglobulin. None of the antisera contained anti-β_2-microglobulin activity, as measured by radioimmunoassay.

The antisera raised against trophoblast pellets contained precipitating antibodies to several serum proteins, particularly IgG, transferrin, and α-2-macroglobulin, as measured by immunoelectrophoresis against pooled normal human serum and Ouchterlony gel diffusion against IgG, transferrin, and α-2-macroglobulin. To remove these activities we passed each antiserum through a column of Sepharose-bound normal human serum (10) and then heat-inactivated each one (56° for 30 min) and absorbed each with two volumes of pooled, washed, and packed human erythrocytes. After these procedures, antisera were negative by both immunoelectrophoresis and gel diffusion against normal human serum, IgG, transferrin, and α-2-macroglobulin and are hereafter referred to as anti-trophoblast sera. The fluorescein isothiocyanate (FITC) conjugate (Wellcome Reagents, Beckman, Kent, England) of sheep anti-rabbit Ig used for immunofluorescence microscopy was also chromatographed over an immunoabsorbent column of Sepharose-bound normal human serum to remove heterophilic antibodies, and both anti-trophoblast sera and FITC-conjugates were ultracentrifuged at 100,000 × *g* every week to remove IgG aggregates which are capable (11) of reacting with Fc receptors of fetal stem vessel endothelium as well as with mononuclear blood cells.

Abbreviations: FITC, fluorescein isothiocyanate; P_i/NaCl, phosphate-buffered saline.

Tissue and Cell Membrane Immunohistology. Central cotyledon tissues from fresh, normal human placentas were snap-frozen in 2-methylbutane that had been cooled with liquid nitrogen, and 4-μm sections were prepared with a cryostat. Sections were washed 1 hr in pH 7.2 phosphate-buffered saline (P_i/NaCl) at 5° and reacted with dilutions of normal rabbit sera and anti-trophoblast sera at room temperature for 20 min. The slides were washed twice in an excess of P_iNaCl for 15 min per wash, reacted for 20 min with the FITC conjugate sera of sheep anti-rabbit Ig, washed three times in P_i/NaCl and mounted in 50% glycerol in pH 8.6 P_iNaCl. Preliminary titration experiments according to Faulk and Johnson (12) indicated that anti-trophoblast sera could be used at 1:150 dilution and conjugate at 1:60. The preparations were then examined by transmitted light from an HBO-50 mercury arc lamp for immunofluorescence with a Zeiss Universal microscope fitted with an FITC interference primary filter, Tiyoda dark ground condenser with toric lens, a 520 barrier filter, and a 40× planapochromatic objective. Blocking and absorption controls were done according to Faulk and Hijmans (13). Photographs were made with Ektachrome film.

For experiments involving cell suspensions, 5×10^5 of human cell lines HeLa, AV_3, or Hep-2, or by the normal human peripheral blood mononuclear cells prepared by the Ficoll–Isopaque technique (14) were reacted for 20 min with increasing dilutions of normal rabbit control sera or anti-trophoblast sera in Eagle's minimal essential medium containing 3% fetal calf serum. The cells were then washed twice in medium and reacted for 20 min with serial dilutions of the FITC sheep anti-rabbit Ig. After the cells were washed three times they were suspended in 50% glycerol in P_i/NaCl and examined by epi-illumination for membrane immunofluorescence with a Leitz orthoplan microscope fitted with an FITC dichoric mirror and a 54× planapochromatic objective. Preliminary titration experiments indicated that membrane immunofluorescence experiments could be satisfactorily performed with anti-trophoblast sera at 1:100 dilution and its conjugate at 1:30.

Cytotoxicity Experiments. Ten microliters of human peripheral blood mononuclear cells at 2×10^6/ml was added to an equal volume of double serial dilutions of control or anti-trophoblast sera and incubated at room temperature for 1 hr, after which 20 μl of rabbit complement (Buxted Rabbit Co., Buxted, England) was added before incubation at 37° for 30 min. Cells were removed with a micropipette and cytotoxicity was measured by counting cells in a hemocytometer after addition of 0.1% trypan blue.

Absorption Experiments. Anti-trophoblast sera were diluted 1:10 in P_i/NaCl absorbed 6–11 times with packed leukocytes. Cells for absorption were prepared from human blood in citrate by mixing with an equal volume of 3% gelatin in P_i/NaCl and sedimenting at 37°. Supernatant cells were washed, counted, and packed at a concentration of 50×10^6 leukocytes per tube immediately before absorption. Absorptions were done at room temperature for 40 min with mixing. This procedure was repeated until the cells used for absorption were negative for rabbit Ig by membrane immunofluorescence. In other absorption experiments, ultracentrifuge-prepared trophoblast microvilli pellets were used. More recently, absorption-control experiments have been done with ultracentrifuge-prepared trophoblast pellets extracted with hypertonic salt and solubilized in nonionic detergent. For this, pellets were extracted for 12 hr in 3 M KCl to remove plasma membrane or plasma proteins, and the KCl-washed material was solubilized in 1.0% deoxycholate according to Faulk *et al.* (15). The KCl-extracted, detergent-solubilized trophoblast cell membranes were dialyzed against 0.15 M carbonate buffer (pH 7.8)/P_i/NaCl, containing 0.1% deoxycholate and 10 mM sodium azide, at room temperature and adjusted to a protein concentration of 0.5 mg/ml.

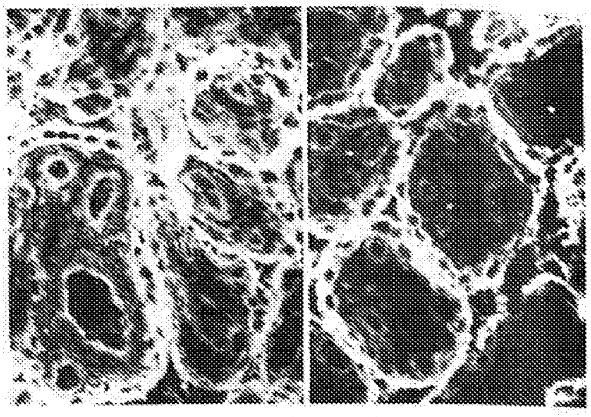


FIG. 1. Immunostaining of human placenta with (*Left*) anti-trophoblast serum and (*Right*) leukocyte-absorbed anti-trophoblast serum. Note staining of trophoblasts, placental vessel endothelium, and cells of the villous stroma with anti-trophoblast serum (*Left*) and staining reactions limited to trophoblasts with leukocyte-absorbed anti-trophoblast serum (*Right*). (×324.)

RESULTS

Specificity of Anti-Trophoblast Sera for Trophoblasts. Each of the 12 anti-trophoblast sera was studied for trophoblast specificity by indirect immunofluorescence of 25 normal human placentas. Brilliant fluorescence of trophoblasts was produced by all antisera (Fig. 1 *left*), and titration of individual antisera showed little variation of different placentas, suggesting that there was no type specificity. Staining of fetal stem vessel endothelium and some cells of the villous stroma was also observed (Fig. 1 *left*).

The specificity of trophoblast staining by anti-trophoblast sera was tested by the principle of antigen absorption control according to McCormick *et al.* (16), using KCl-extracted, detergent-solubilized trophoblast cell membranes. Volume-for-volume absorptions of these 12 antisera were done with solubilized trophoblast microvilli for 1 hr at 4° followed by ultracentrifugation for 1 hr at 100,000 × *g* in a Beckman model L5-75 ultracentrifuge to remove immune complexes. The absorbed antisera stained neither trophoblasts nor any of the cellular components seen in Fig. 1 *left*. In addition, the antisera absorbed with membrane preparations from 15 different placentas failed to stain any aspects of chorionic villi, suggesting no individual or type specificity.

Specificity and Cytotoxicity of Anti-Trophoblast Sera for Other Cells. The anti-trophoblast sera were studied for other membrane specificities by epi-illumination immunofluorescence using normal peripheral blood mononuclear cells and human amnion (AV_3), Hep-2, and HeLa cells. The human cell lines stained much more intensely than the peripheral blood mononuclear cells with anti-trophoblast sera (Fig. 2 *right*), and, in all experiments, greater than 95% of the cells were positive. As with the above results using trophoblasts, staining of mononuclear cells, AV_3, Hep-2, and HeLa cells was removed by absorption of anti-trophoblast sera with either intact ultracentrifuge-prepared trophoblast microvilli or with detergent-solubilized trophoblast cell membranes.

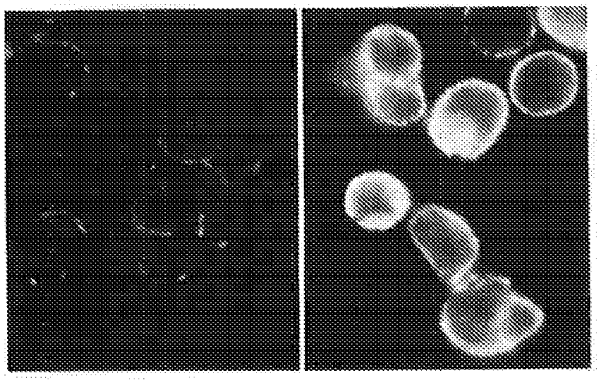


FIG. 2. Immunostaining of (*Left*) peripheral blood mononuclear cells and (*Right*) Hep-2 cells with anti-trophoblast serum. Note difference in intensity of staining of anti-trophoblast serum for blood mononuclear and Hep-2 cells. (×400.) HeLa and AV_3 cells were similarly stained, although HeLa cell staining was removed by leukocyte absorption (×20) of the antiserum.

Since the rabbit anti-human trophoblast membrane sera contained antibodies reacting with cell membranes of peripheral mononuclear cells, we also studied their cytotoxic effect. All antisera were cytotoxic for mononuclear cells from 10 different cell donors (Fig. 3). The least cytotoxic antiserum caused a loss of viability of 30% of the cells at 1:800 dilution; the most cytotoxic antiserum caused a 30% viability at 1:6000. The end-point for each was within two double serial dilutions for all 10 donors, suggesting that these antisera contained no specificities for individual HLA antigens.

In order to test the specificity of cytotoxicity of anti-trophoblast sera for human peripheral blood mononuclear cells, the anti-trophoblast sera were absorbed with human peripheral leukocytes. After 11 absorptions, none of the antitrophoblast sera were more cytotoxic than normal (nonimmune) rabbit serum, even at titers as low as 1:30 (Fig. 3). Indeed, some of the anti-trophoblast sera lost lymphocytotoxicity activity after as few as six absorptions with leukocytes (Fig. 3).

Immunohistological Specificity of Leukocyte-Absorbed Anti-Trophoblast Sera. Although leukocyte-absorbed anti-trophoblast sera were no longer cytotoxic for peripheral blood mononuclear cells, upon examination of cryostat sections of placentas it was found that as many as 20 absorptions with leukocytes did not noticeably diminish the intensity of trophoblast staining (Fig. 1 *right*), although staining of cells in the mesenchymal stroma and the endothelial staining of fetal stem vessels in chorionic villi was removed. This pattern of immunostaining was not affected either by absorption of the antiserum with human placental akaline phosphatase (Sigma Chemicals, St. Louis) or by blocking experiments using unlabeled antisera to human chorionic gonadotropin (National Institute of Biological Standards, Holly Hill, England) or to the placental glycoproteins SP_1 and PP_5 (kindly supplied by Behringwerke AG, Marburg, Germany). No other human tissues, including cryostat sections of liver, kidney, lung, spleen, brain, pituitary, ovary, testes, pancreas, stomach, and small and large bowel, were stained with the anti-trophoblast sera after leukocyte absorption. In contrast, the leukocyte-absorbed (×20) anti-trophoblast sera caused brilliant membrane fluorescence of a population (25–40%) of AV_3 and Hep-2 cells. Continued absorption of these reagents with leukocytes did not cause a further loss of AV_3 or Hep-2 cell staining, indicating that absorption was complete with 20 absorptions. Thus, in summary, trophoblast antigens, as studied by the specificity of heterologous anti-trophoblast sera, can be classified into two major

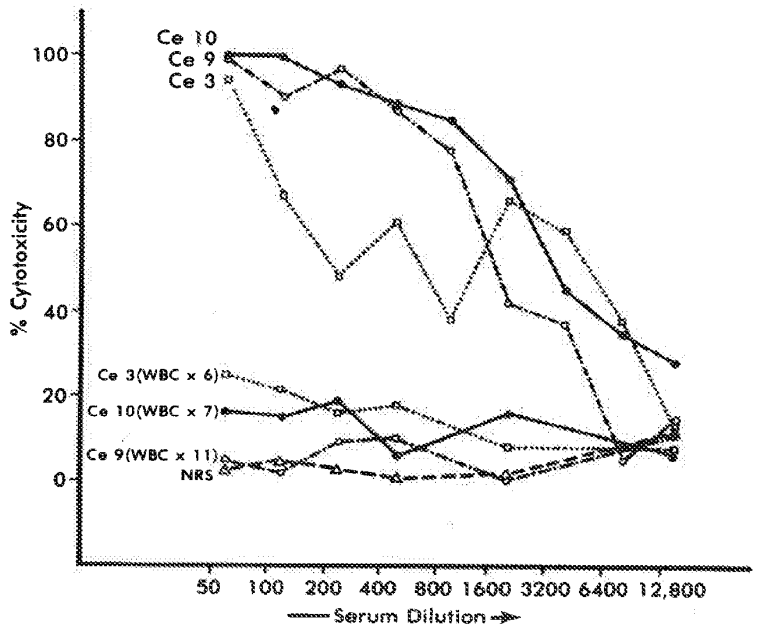


FIG. 3. Cytotoxicity and absorption experiments of anti-trophoblast serum for peripheral blood mononuclear cells. Different anti-trophoblast sera are designated by Ce. Cytotoxicity experiments are shown in the upper three lines. Lower three lines plus normal rabbit serum (NRS) are the same anti-trophoblast sera after leukocyte (WBC) absorption the designated number of times. Note that after leukocyte absorption the anti-trophoblast sera were no more cytotoxic for blood mononuclear cells than was normal rabbit serum.

groups: one that is shared between trophoblasts and certain human cell lines, and one that is shared between trophoblasts and normal human peripheral blood mononuclear cells.

DISCUSSION

We propose that the group of antigens that are sheared between trophoblasts (Fig. 1 *right*) and human cell lines (Fig. 2 *right*) be tentatively designated as TA_1 (trophoblast antigens, 1) and that antisera to these antigens be described as anti-TA_1. Similarly, we propose that the group of antigens responsible for the specificity of anti-trophoblast sera for peripheral blood mononuclear cells (Fig. 2 *left*), leukocytes (Fig. 3), and placental vessel endothelium (Fig. 1 *left*) be tentatively designated at TA_2 (trophoblast antigens, 2), and that antibodies to these antigens be described as anti-TA_2. This grouping of human trophoblast antigens as TA_1 and TA_2 can be used to interpret the result of an important previous investigation done in rats. Beer *et al.* (17) reported that heterologous antisera to rat trophoblasts cause abortion in pregnant rats as well as death in some of the pregnant mothers. However, when the antisera were absorbed with rat lymphocytes, the investigators observed only abortion without maternal death. According to our proposed nomenclature, it would appear that lymphocyte absorption removed anti-TA_2, thus eliminating the serum's ability to recognize maternal cells and leaving the oncofetal TA_1 specificity, which mediated immune damage to trophoblasts causing abortion without maternal death.

From our data we should like to suggest that normal human pregnancy involves a maternal immune response to the lymphocyte crossreactive trophoblast antigen, TA_2. Such antibodies would be expected to bind to TA_2 present in normal placentas. Support for this comes from two lines of evidence: First, antibodies of maternal origin that recognize and block certain lymphocyte functions can be eluted from placentas of both mice (18) and humans (19), and, second, similar yet more specific blocking antibodies of lymphocyte function have been identified in maternal sera (20). Even though these antibodies block certain lymphocyte responses *in vitro*, their function *in vivo* is unestablished. It is, however, interesting that mothers who lack *in vitro* evidence of serum blocking factors do tend to sustain repeated abortions (20). An absence of blocking function is thus suggestive of the absence of anti-TA_2.

In light of the above classification of trophoblast antigens, we wish to put forward a working hypothesis concerning the immunobiology of normal pregnancy that is relevant to the immunopathology of abnormal pregnancies. One traditionally assumes that mothers are "tolerant" to the placental homograft. We suggest that tolerance, if abortion is to be averted, is the lack of an effective response to TA_1. This can theoretically be accomplished by mounting an antibody response to TA_2 which inhibits either recognition of or cytotoxicity to TA_1. This could operationally be done in a hapten–carrier system where the lymphocyte crossreactive TA_2 (interpreted by mother as "self" antigen) is the hapten and the oncofetal trophoblast TA_1 (interpreted by mother as "foreign") antigen is the carrier. This would explain the presence of an antibody with crossreactive specificity for antigens on host tissues, but has the marked theoretical disadvantage of possibly generating cell-mediated immunity to TA_1. However, the need for cooperation of carrier T cells in a hapten–carrier system can be circumvented by allogeneic cell stimulation (21), providing that antigen-reactive B cells are present in the recipient (22, 23) and that a graft-versus-host reaction be induced (21, 22). Successful implantation and growth of the blastocyst is a type of graft-versus-host reaction, trophoblast tumors being the most extreme type of this example, and it is well documented that proliferation of the embryo and placenta are accompanied by the passage of trophoblasts (allogeneic cells) into the mother's circulation (24, 25).

There is ample information from normal subjects to support the presence of antigen-binding B lymphocytes for "self" antigens in conditions of low-zone tolerance, such as shown by Bankhurst *et al.* for thyroglobulin (26), and we have recently produced evidence for the presence of trophoblast-reactive lymphocytotoxic antibodies in the sera of pregnant and nulliparous subjects with systemic lupus erythematosus (27). The pregnant mother is thus endowed with antigen-reactive B lymphocytes for trophoblast antigens, and she receives allogeneic membranes (trophoblasts) subsequent to the implantation and proliferation of the blastocyst in her uterus. In light of these observations, we suggest that the production of anti-TA_2 in normal pregnancy results from allogeneic cell stimulation of maternal TA_2 antigen-reactive cells by trophoblast membranes. We presently envision that these antibodies function to block either the recognition or cytotoxicity of TA_1, but the role of helper and supressor cells in this reaction has yet to be established.

This mechanism for the immunobiology of normal pregnancy could fail if the allogeneic effect failed, resulting in recognition of TA_1 and abortion. Confirmation of this potentially immunopathological effect is drawn from a recent report by Komlos *et al.* (28) that abortions are more frequent in couples who share common HL-A antigens. Shared histocompatibility antigens would diminish the possibility of allogeneic cell stimulation of TA_2-reactive maternal B cells, thus allowing TA_1 recognition and abortion. This concept could also apply to other conditions of abnormal pregnancies. For example, Jenkins *et al.* (29) have reported that in toxemia there is a greater degree of HLA compatibility between couples and Feeney *et al.* have reported that toxemia is much less common in women who have received a blood transfusion before becoming pregnant (30). The observation of increased spontaneous abortion in HLA compatible couples is consistent with the above hypothesis of failed allogeneic helper effect, and, in the blood transfusion studies, we would suggest that TA_2 and histoincompatible leukocyte antigens in the blood transfusion primed the recipients for more effective allogeneic recognition during pregnancy. By the same token, toxemia without prior transfusion could serve to abrogate allogeneic recognition in subsequent pregnancies, accounting for the more frequent appearance of toxemia in the first pregnancy.

In closing, it should be emphasized that we do not suggest that toxemia is associated with particular HLA types [notwithstanding increased homozygosity as suggested by Redman and Bodmer (31), which is quite another matter], and such associations have not indeed been shown to exist (32), although the degree of parental HLA compatibility was not studied (32). Trophoblast antigens such as TA_1 and TA_2 are however coded by the embryo and, although most known antigens of the major histocompatibility complex are absent from trophoblasts (9), it is not unlikely that HLA compatibility between the mother and father will be associated with materno-fetal TA_2 compatibility, thus deminishing the possibility of maternal anti-TA_2, and thereby allowing TA_1 recognition and disease. These ideas will no doubt change as knowledge of trophoblast antigens expands, but in their present state they do seem to form a working hypothesis which draws support both from our own experimental work as well as from the published reports of others.

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Characterization of Murine Monoclonal Antibodies Reactive to Either the Human Epidermal Growth Factor Receptor or HER2/*neu* Gene Product

Brian M. Fendly,[1] Marcy Winget, Robert M. Hudziak, Michael T. Lipari, Mary Anna Napier, and Axel Ullrich

Departments of Medicinal and Analytical Chemistry [B. M. F., M. W.], Developmental Biology [R. M. H., A. U.], and Medicinal and Biomolecular Chemistry [M. T. L., M.A.N.], Genentech, Inc., South San Francisco, California 94080

ABSTRACT

High levels of expression of either the epidermal growth factor receptor or the receptor-like HER2/*neu* gene product p185^{HER2} have been observed in a variety of human malignancies. Because of the association of this high level expression with certain human tumors, we have generated a panel of monoclonal antibodies specific for either the epidermal growth factor receptor or p185^{HER2} to study their structure, function, and antigenic domains in the normal and neoplastic states. We used the epidermoid carcinoma line A431 to generate five monoclonal antibodies which immunoprecipitate the epidermal growth factor receptor. These monoclonal antibodies bind to the extracellular domain of the epidermal growth factor receptor and demonstrate variable effects on epidermal growth factor binding. We used a stably transfected NIH 3T3 cell line expressing the HER2/*neu* gene to produce and characterize 10 monoclonal antibodies which immunoprecipitate p185^{HER2}. These monoclonal antibodies bind to the extracellular domain of p185^{HER2} and do not cross-react with the epidermal growth factor receptor. The characteristics and potential applications of these monoclonal antibodies will be discussed.

INTRODUCTION

Alterations in growth factor receptor structure and level of expression have been implicated in the process of abnormal growth control, transformation, and oncogenesis. Some of these receptors share homology with retroviral oncogenes, have intrinsic tyrosine kinase activity, and are generally referred to as protooncogenes (for review see Ref. 1). Included in this group of receptors is the epidermal growth factor receptor which is the cellular homologue of the avian retrovirus oncogene v-*erb*B. The p185^{HER2} product[2] of the human EGFR related gene (HER2/*neu*) is a receptor-like tyrosine kinase without any oncogene homologue identified in a retrovirus. The EGFR and p185^{HER2} are similar but distinct glycoproteins encoded by genes located on human chromosomes 7 (2, 3) and 17 (4), respectively. The EGFR and p185^{HER2} have approximately 40% homology in their extracellular ligand binding domains and 78% homology in their intracellular ATP binding and tyrosine kinase regions as determined by their predicted primary amino acid sequences (4). The EGFR is a M_r 170,000 single chain glycoprotein of 1,186 amino acids with a 621-amino acid, extracellular, NH_2-terminal domain (5, 6). This extracellular domain of the EGFR binds the 53-amino acid, M_r 6,025 EGF (7) as well as the 50-amino acid, M_r 6,000 transforming growth factor α (8). In both cases binding of the growth factor results in activation of the receptor associated tyrosine kinase (9, 10). The p185^{HER2} receptor is also a single chain, M_r 185,000 glycoprotein of 1,255 amino acids with a 632-amino acid, extracellular domain which is thought to bind an unknown ligand (1, 4).

Amplification of expression of the EGFR is associated with human cervical and ovarian carcinomas (11), epidermal squamous cell carcinomas (12), and malignant gliomas (13). Amplification of the HER2/*neu* gene has been found in mammary gland carcinomas (14–16), and ovarian cancer (17) and is associated with the most aggressive forms of breast and ovarian tumors and poor prognosis from these cancers (17, 18). In addition, amplification of HER2/*neu* has also been shown in other human adenocarcinomas including salivary gland (19), stomach, and kidney (20). The MAbs to these cell surface glycoproteins and MAbs which are specific to phosphorylated tyrosines were generated to investigate expression of these protooncogenes in tumor cell lines and to study the structure/function relationships and antigenic domains of these receptors. We performed extensive specificity studies on the MAbs raised to either the EGFR or p185^{HER2} to evaluate shared antigenic sites on these related structures since these MAbs are candidates for the development of receptor based tumor therapies, *in vivo* and *in vitro* diagnostics, as well as important reagents for the development of assays to monitor the *in vitro* effects of receptor modulation.

MATERIALS AND METHODS

Cell Lines and Isolation of Receptors from Cells. The human epidermoid carcinoma A431 cell line (21) was used for isolation of the EGFR (22), since this cell line has been shown to express $2–3 \times 10^6$ copies/cell of the EGFR (21, 23–25). A431 cells were grown to confluence at 37°C and 10% CO_2 atmosphere in medium consisting of DMEM/Ham's F-12 (50/50, v/v), 5% fetal calf serum, 15 mM HEPES (pH 7.2), 50 units/ml penicillin, and 10 μg/ml streptomycin. The cells were solubilized in 1% Triton X-100 (26), and the EGFR was partially purified by WGA affinity chromatography. Elution of the receptor was achieved with 40 mM HEPES, pH 7.5, containing 0.3 M *N*-acetylglucosamine, 0.15 M NaCl, 0.2% Triton X-100, and 10% glycerol. Protein concentration was determined by the method of Bradford (27). The affinity (approximately 20 nM) of the solubilized receptor and the receptor concentration (approximately 5 pmol receptor/mg protein) were determined by Scatchard analysis of ^{125}I-EGF binding (28). EGF (Sigma, St. Louis, MO) was iodinated by the standard chloramine-T method (29). Tyrosine kinase activity was measured in the presence of 80 μCi/ml [γ-^{32}P]ATP, 10 μM ATP, 4 mM $MgCl_2$, in 2 mM $MnCl_2$ in 20 mM HEPES, pH 7.5, containing 0.1% Triton X-100, and 10% glycerol. HER2/*neu* transfected NIH 3T3 cells, NIH 3T3/HER2-3$_{400}$ (30), expressing approximately 1×10^5 p185^{HER2} molecules/cell (data not shown) were grown to confluency in the above media. The cells were extracted with 1% Triton X-100 and the membrane protein p185^{HER2} was partially purified by WGA affinity chromatography as described above. The tyrosine kinase activity was measured as above except the concentration of [γ-^{32}P]ATP was 100 μCi/ml and there was no unlabeled ATP in the kinase solution.

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[1] To whom requests for reprints should be addressed, at Department of Medicinal and Analytical Chemistry, Genentech, Inc., 460 Point San Bruno Blvd., San Francisco, CA 94080.

[2] The abbreviations used are: p185, M_r 185,000 protein; gp120, M_r 120,00 glycoprotein (other proteins and glycoproteins similarly designated are); EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; MAb, monoclonal antibody; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; WGA, wheat germ agglutinin-Sepharose; PBS, phosphate buffered saline, pH 7.2; ELISA, enzyme linked immunosorbent assay; PBST, PBS-0.05% Tween 20; HTG, 20 mM HEPES-0.1% Triton X-100-10% glycerol; RAM-SPA, protein A-Sepharose coated with rabbit anti-mouse IgG; SDS, sodium dodecyl sulfate; HNEG, 20 mM Hepes-150 mM NaCl-1 mM ethyleneglycol bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 10% glycerol; FITC, fluorescein isothiocyanate; FBS, fetal bovine serum; FACS, fluorescence activated cell sorter; RMF, relative mean fluorescence.

Generation of Monoclonal Antibodies Specific for the EGFR. BALB/c mice were immunized with 2–4 × 10^6 A431 cells in PBS, i.p. on weeks 0, 2, 4, and 6. The immunized mice were tested for an antibody response by immunoprecipitation of ^{32}P-labeled EGFR. The mice with the highest serum titers were given i.v. injections of a WGA purified A431 membrane extract during week 18. Three days later their splenocytes were fused with mouse myeloma line X63-Ag8.653 (31), using 50% polyethylene glycol 4000 by the procedure of Oi and Herzenberg (32). Fused cells were plated at a density of 2 × 10^5 cells/well in 96-well microtiter plates (day 0) and hybridoma selection was begun on day 1 with 10% fetal calf serum in DMEM media containing hypoxanthine/azaserine (33). Beginning on day 10 hybridoma supernatants were screened for the presence of EGFR specific antibodies as described below.

Generation of Monoclonal Antibodies Specific for p185^{HER2}. NIH 3T3/HER2-3$_{400}$ cells were harvested with PBS containing 25 mM EDTA and were used to immunize BALB/c mice. The mice were given injections i.p. of 10^7 cells in 0.5 ml PBS on weeks 0, 2, 5, and 7. The mice with antisera that immunoprecipitated ^{32}P-labeled p185^{HER2} were given i.p. injections of a WGA purified p185^{HER2} membrane extract on weeks 9 and 13. This was followed by an i.v. injection of 0.1 ml of the p185^{HER2} preparation and the splenocytes were fused as described for the anti-EGFR fusions. The hybridoma supernatants were screened by ELISA as described below.

Generation of Monoclonal Antibodies Specific for Phosphotyrosine. BALB/c mice were immunized s.c. with 200 μg of phosphotyramine coupled to keyhole limpet hemocyanin by using a bromoacetyl linker (34) in Freund's adjuvant on weeks 0, 2, 4, and 6. The mouse with positive antisera that immunoprecipitated ^{32}P-phosphorylated EGFR most efficiently was immunized i.v. with phosphotyramine keyhole limpet hemocyanin in PBS on week 8 and the splenocytes were fused as described above. The initial screen was an ELISA on phosphotyramine coupled to soybean trypsin inhibitor. ELISA positive parental culture supernatants were further assayed by immunoprecipitation with ^{32}P-phosphorylated EGFR as described below.

Enzyme-linked Immunosorbent Assays. ELISAs were performed as initial screens of fusions, to monitor the purification of the anti-p185^{HER2} and anti-EGFR MAbs, initial screens of the phosphotyramine fusions, and as one approach to determine the cross-reactivity between anti-EGFR and anti-p185^{HER2} MAbs. Immuno II (NUNC Roskilde, Denmark) microtiter plates were coated with 1 μg/ml of either the WGA affinity purified p185^{HER2}, WGA affinity purified EGFR membrane preparations, or phosphotyramine coupled to soybean trypsin inhibitor in PBS overnight at 4°C or for 2 h at room temperature. Antigen coated plates were washed with PBST and MAb samples were incubated for 1–2 h at room temperature on a shaker. Plates were washed with PBST and goat anti-mouse IgG conjugated with horseradish peroxidase (Tago, Burlingame, CA) was added and incubated for 1 h. The plates were washed with PBST and 100 μl/well of o-phenylenediamine dihydrochloride substrate (Sigma) were added at 0.5 mg/ml in 0.05 M citrate/phosphate buffer. The reaction was stopped after 15–30 min at room temperature with 100 μl/well of 2.5 M H_2SO_4 and absorbance was determined at 492 nm on a multiscan plate reader (Flow, McLean, VA).

Purification of MAb from Ascites. Selected ELISA positive hybridomas were cloned twice by limiting dilution and ascites fluid containing specific MAb was produced in pristane primed BALB/c mice (35). Antibodies were purified from the ascites fluid by protein A-Sepharose (Fermentech, Inc., Edinburgh, Scotland), using established procedures (36), stored sterile in PBS at 4°C, and further characterized in a variety of immunoassays.

Radioimmunoprecipitation of [γ-^{32}P]ATP Phosphorylated Receptors. Radioimmunoprecipitations were performed by first autophosphorylating the appropriate Triton X-100 cell lysates with [γ-^{32}P]ATP in kinase buffer containing 180 μCi/ml [γ^{32}P]ATP, 5 μM ATP, 2 mM $MnCl_2$, 4 mM $MgCl_2$, and the WGA purified extracts of either the p185^{HER2} (35 μg/ml) or the EGFR (17.5 μg/ml) in HTG buffer for 30 min at room temperature. The kinase mixture, 50 μl, was then mixed with 2 μg of the appropriate MAb, diluted to 50 μl with HTG buffer, and incubated for 1 h at room temperature. Protein A-Sepharose CL-4B, 50 μl, (Pharmacia, Uppsala Sweden), coated with 1 mg/ml rabbit anti-mouse IgG to enhance binding of all IgG subclasses, was added and incubated for 1 h at room temperature. Samples were washed twice with HTG and once with PBS containing 0.2% deoxycholate and 0.2% Tween 20. Reducing SDS sample buffer containing 30 μM β-mercaptoethanol-2% SDS in 14 mM Tris, pH 6.8, 50 μl, was added to the pelleted immunocomplexes and the samples were heated to 90°C for 5 min. The RAM-SPA was separated by centrifuging for 2 min at 10,600 × *g* and the supernatant was loaded on a 7.5% SDS polyacrylamide gel (37), electrophoresed at constant 30-mA current and followed by autoradiography to determine the relative molecular weights of the bound autophosphorylated proteins.

Radioimmunoprecipitation of [^{35}S]Cysteine Labeled Receptors. Subconfluent cell cultures in 100-mm plates were washed once with cysteine-free DMEM and incubated overnight in 2 ml of cysteine-free media containing 10% dialyzed fetal bovine serum and 0.5 mCi [^{35}S]-cysteine (Amersham, Arlington, IL). The labeling medium was aspirated and the cells were rinsed with HNEG buffer at pH 7.6. The cells were lysed with 0.5 ml of HNEG buffer with 1% Triton X-100 and 1.5 mM $MgCl_2$ (lysis buffer), diluted with 1 ml HNEG and 1% BSA (dilution buffer), and the cell membrane fragments were removed by centrifugation at 10,000 × *g*. The labeled cell extract was preincubated with RAM-SPA for 30 min at room temperature and centrifuged as above to remove labeled proteins which bound nonspecifically. This preabsorbed extract was divided into 4–6 samples and incubated with 2 μg of the appropriate MAb for 30–60 min. The washing, immunoprecipitation, and electrophoresis steps were performed as described above.

Radioimmunoprecipitation of Nonglycosylated Receptors. Cells were harvested by trypsinization, counted (Coulter Counter), and plated into 150-mm culture dishes. A431 and NIH 3T3/HER2-3$_{400}$ cells were plated at 2 × 10^6 or 6 × 10^6 cells/dish, respectively, and, after a 3-h attachment period, the antibiotic tunicamycin at 3 μg/ml was added to the experimental plates. Cell lysates were prepared after a further 6-h incubation and the anti-EGFR and the anti-p185^{HER2} MAb immunoprecipitations were [γ-^{32}P]ATP labeled by autophosphorylation at 4°C, washed, electrophoresed, and analyzed by autoradiography as described above. The polyclonal rabbit antibody, G-H2CT17, raised against the carboxy terminal 17 amino acids of p185^{HER2} (38), was used as a control antiserum to immunoprecipitate p185^{HER2} irrespective of glycosylation. Similarly, the polyclonal mouse antibody, R1/1080, raised to the EGFR, was used as the positive control for immunoprecipitation of the EGFR irrespective of glycosylation.

Isotyping of MAbs. Isotyping of the MAbs was performed by using the Pandex Screen Machine and anti-mouse IgG coated particles. Polystyrene Pandex assay particles, 1 ml of a 5% solution, were incubated with 1.0 mg of affinity purified rat anti-mouse IgG (PelFreez, Rogers, AZ) in carbonate buffer at pH 9.6 for 1 h at room temperature. The beads were washed twice with PBS and resuspended in 20 ml of PBS at 0.25% particles. Coated particles, 20 μl, were added to 20 μl of the appropriate MAb supernatants in wells of Pandex microtiter plates and incubated on a shaker at room temperature for 30 min. The plates were washed and FITC-conjugated rat monoclonal anti-mouse isotype specific reagents (generous gift of David Buck, Becton-Dickinson Monoclonal Center) were added at 1–2 μg/ml in PBST. Samples were incubated 15 min, washed, and the fluorescence was quantitated by the Pandex software.

Epitope Determination by MAb Competitive Binding Analysis. Cross-blocking studies were done on both panels of MAbs by direct fluorescence on intact cells by using the Pandex Screen Machine to quantitate fluorescence. Each MAb was conjugated with FITC, using established procedures (39). Confluent monolayers of A431 or NIH 3T3/HER2-3$_{400}$ cells were trypsinized, washed once, and resuspended at 1.75 × 10^6 cell/ml in cold PBS containing 0.5% BSA (BSA/PBS) and 0.1% NaN_3. A final concentration of 1% latex particles (IDC, Portland, OR) was added to reduce clogging of the Pandex plate membranes. Cells in suspension, 20 μl, and 20 μl of purified MAbs (100 μg/ml to 0.1 μg/ml) were added to the Pandex plate wells and incubated on ice for 30 min. A predetermined dilution of FITC-labeled MAbs in 20 μl was

added to each well, incubated for 30 min, washed, and the fluorescence was quantitated by the Pandex.

Blocking of EGF Binding by Anti-EGFR MAbs. To determine if any of the anti-EGFR MAbs blocked EGF binding to the EGFR on intact cells, 200 μl of A431 cells (5×10^5 cells/ml) were mixed with 200 μl of various concentrations of either purified MAbs or unlabeled murine EGF (Sigma), in triplicate, starting at 6 μM, 1.0 mg/ml or 100 μg/ml, respectively, and incubated 30 min on ice. Murine ^{125}I-EGF, 6×10^4 cpm in 100 μl, was added to each sample and incubated an additional 30 min on ice. Cells were washed once, counted on th gamma counter, and percentage of bound counts was calculated and the average of triplicates plotted.

Immunofluorescence Staining of Cells. The cell lines A431 and the breast adenocarcinoma cell line SK-BR-3, previously shown to express amplified levels of p185^{HER2} (40, 41), were used to characterize the EGFR and p185^{HER2} specific panels of MAbs by FACS techniques. Confluent monolayers of cells were harvested with 25 mM EDTA in PBS, washed, and resuspended at 1×10^6 cells/ml in PBS containing 1% FBS (1% FBS/PBS). An aliquot, 1 ml, of each cell line was added to 10 μg of the appropriate MAb and incubated for 1 h on ice. The cells were washed twice with 1% FBS/PBS and incubated with FITC conjugated F(ab')$_2$ fragment of goat anti-mouse IgG specific antibody (Zymed, South San Francisco, CA), for 30 min on ice. The cells were washed twice and resuspended in 0.5 ml of 1% FBS/PBS and analyzed on an EPICS 753 (Coulter, Hialeah, FL) FACS using 300 mW of 488 nm argon laser light for excitation and measuring emitted light with a 525 nm narrow band pass filter.

Inhibition of A431 Growth by Anti-EGFR MAbs. A431 cells were plated into 35-mm culture dishes at 1.0×10^4 cells/dish. Either an unrelated, isotype control MAb 9F6 (anti-gp120 of human immunodeficiency virus), the anti-EGFR MAbs, or the anti-185^{HER2} MAb 4D5 were added on day 0 at a final concentration of 2.5 μg/ml (16.7 nM). After 7 days the cells were removed from the plates by trypsinization and the total cell number was determined by Coulter counter. The difference in growth of cells treated with the experimental and control MAbs is expressed as the ratio of their respective cell numbers. Each MAb was assayed in duplicate and the cell counts were averaged. As an additional positive control, EGF at 40 ng/ml (8 nM) was included; previous work has shown this to be growth inhibitory for A431 cells (42, 43).

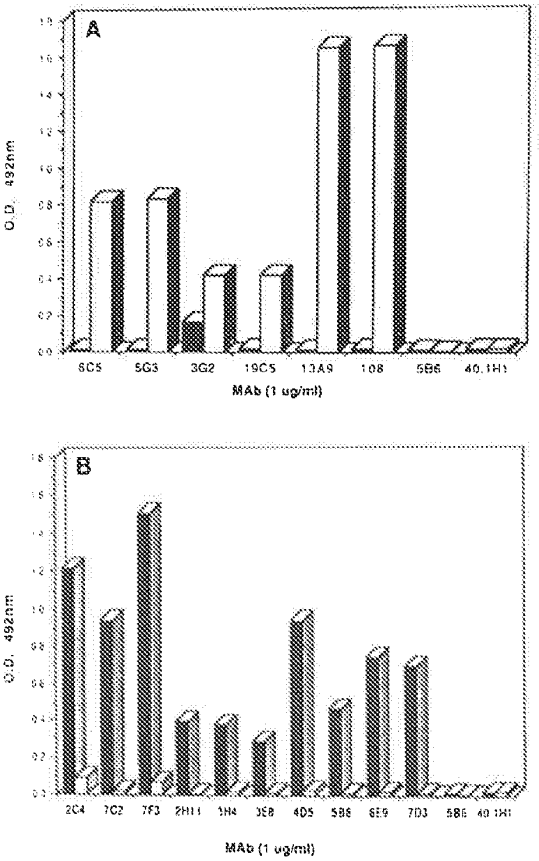


Fig. 1. Results from ELISA measuring the relative reactivities of either the purified anti-EGFR MAbs (*upper*) or the anti-p185^{HER2} MAbs (*lower*), at 1 μg/ml, with immobilized WGA affinity purified membrane extracts from A431 (□) and NIH 3T3/HER2-3$_{400}$ (■).

RESULTS

ELISA Characterization of MAbs. Since these anti-EGFR and anti-p185^{HER2} MAbs could be used to develop important diagnostic reagents, a variety of immunological techniques were used to determine their reactivity and specificity. The ability of the purified MAbs to bind to immobilized WGA purified EGFR or p185^{HER2} was determined by ELISA. Relative activities of the antibodies at various concentrations, measured as $A_{492\ nm}$, were determined and the results at 1-μg/ml MAb concentrations are presented (Fig. 1). Four of the five anti-EGFR MAbs (5G3, 6C5, 13A9, and 19C5) were specific for the EGFR, with their reactivity with p185^{HER2} at background levels. In contrast, MAb 3G2 appeared to bind to both the EGFR and p185^{HER2}. Our MAb 13A9 had reactivity to EGFR equivalent to the well characterized anti-EGFR MAb 108 (generous gift of Joseph Schlessinger, Rorer Biotechnology, Inc.) described earlier (44). The anti-p185^{HER2} MAbs exhibited positive but variable reactivity against immobilized p185^{HER2} in ELISA and none of these MAbs reacted with immobilized EGFR.

MAb Immunoprecipitation of ^{32}P Labeled Receptors. The specificity of the MAbs was also evaluated by utilizing radioimmunoprecipitation on ^{32}P labeled proteins. Purified MAbs were reacted with [γ-^{32}P]ATP autophosphorylated EGFR and p185^{HER2} receptor preparations (Fig. 2). All the MAbs raised to A431 cells immunoprecipitated EGFR, with MAbs 13A9 and 108 being the most efficient. MAb 3G2 also precipitated a strong p185^{HER2} band by comparison to the EGFR band precipitated by this antibody (Figs. 2*A* and 3). These precipitation results are consistent with the ELISA results showing cross-reactivity of MAb 3G2 with both EGFR and p185^{HER2} coated plates. The MAbs against p185^{HER2} precipitated p185^{HER2} and not the EGFR (Fig. 2). None of the anti-p185^{HER2} MAb precipitations of the EGFR were above background levels of the negative MAbs 5B6 (anti-gp120 of human immunodeficiency virus) or 40.1.H1 (anti-human B surface antigen of hepatitis B virus), which gave some weak background bands due to slight overexposure of the autoradiography film prior to photographing. The MAbs specific for phosphotyrosine were also generated to investigate growth factor receptor structure and function including the associated tyrosine kinase activity. The anti-phosphotyrosine MAb 5E2 was pursued since it efficiently immunoprecipitated both the EGFR and p185^{HER2} (Fig. 3). MAb 5E2 did not react with either phosphoserine or phosphothreonine (data not shown) and has also been useful in immunoblotting of phosphorylated EGFR receptors (45).

MAb Immunoprecipitation of [^{35}S]Cysteine Labeled Receptors. The antigen specificity of the MAbs was further confirmed by immunoprecipitation of the EGFR and p185^{HER2} from extracts of A431 and SK-BR-3 cells metabolically labeled with [^{35}S]-

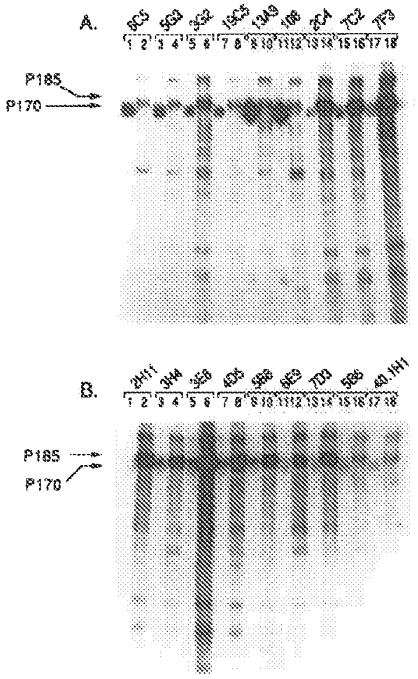


Fig. 2. Autoradiography of 7.5% SDS-polyacrylamide gel electrophoresis of [γ-^{32}P]ATP labeled proteins immunoprecipitated by either anti-EGFR MAbs (*A, Lanes 1–12*) or anti-p185^{HER2} MAbs (*A, Lanes 13–18*; *B, Lanes 1–14*) from either the A431 (*odd numbered lanes*) or the NIH 3T3/HER2-3$_{400}$ (*even numbered lanes*) WGA purified extracts. Negative control MAbs: 5B6 (anti-gp120 of human immunodeficiency virus, IgG$_1$; *Lanes 15* and *16*) and 40.1.H1 (anti-surface antigen B of hepatitis, IgG2a; *Lanes 17* and *18*) are included as isotype controls.

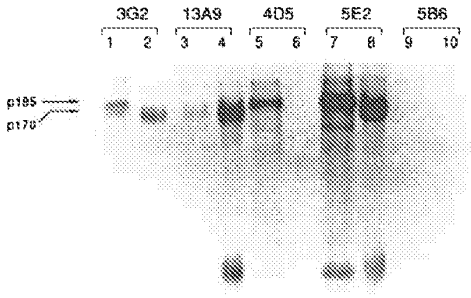


Fig. 3. Autoradiography of 7.5% SDS-polyacrylamide gel electrophoresis of [γ-^{32}P]ATP autophosphorylated, labeled EGFR and p185^{HER2} comparing anti-EGFR, anti-p185^{HER2}, and anti-phosphotyrosine MAbs. The p185^{HER2} extracts are run in the *odd numbered lanes* and the EGFR extracts are run in the *even numbered lanes*. Anti-EGFR MAbs (*Lanes 1–4*), anti-p185^{HER2} MAb (*Lanes 5* and *6*) anti-phosphotyrosine MAb (*Lanes 7* and *8*) and control MAb (*Lanes 9* and *10*).

cysteine (Fig. 4). All the anti-EGFR MAbs precipitated the EGFR from the extracts of ^{35}S labeled A431 cells with no or few additional bands being precipitated from this heterogeneous extract of labeled cellular proteins by comparison to the negative controls (Fig. 4*A*). The MAbs 13A9 and 108 were the most efficient at immunoprecipitation and this trend is consistent with the immunoprecipitations using γ-^{32}P-labeled EGFR (Fig. 2*A*). In addition, MAbs 13A9, 108, and 5G3 also precipitated

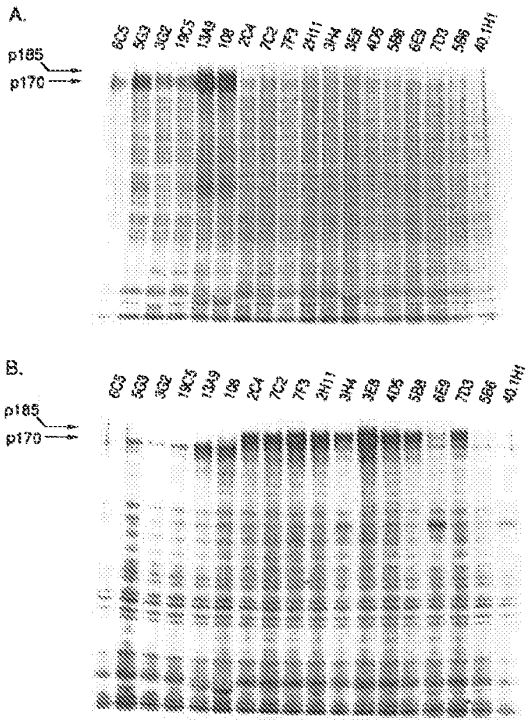


Fig. 4. Autoradiography of 7.5% SDS-polyacrylamide gel electrophoresis of [^{35}S]cysteine labeled proteins immunoprecipitated by anti-EGFR MAbs or anti-p185^{HER2} MAbs using (*A*) A431 cells and (*B*) SK-BR-3 cells.

the EGFR from the extract from SK-BR-3 cells (Fig. 4*B*). None of these EGFR MAbs, including MAb 3G2, precipitated p185^{HER2} from the [^{35}S]cysteine labeled SK-BR-3 extracts. The lack of detectable p185^{HER2} in the immunoprecipitation of [^{35}S]cysteine labeled p185^{HER2} by the anti-EGFR MAb 3G2 may be due to the relative lower specific activity of the ^{35}S *versus* ^{32}P labeled receptors.

The anti-p185^{HER2} MAbs 3E8 and 7F3 were the most efficient in precipitating [^{35}S]cysteine labeled p185^{HER2}; 2C4, 2H11, 4D5, 5B8, 7C2, and 7D3 were intermediate, while 3H4 and 6E9 were the least efficient (Fig. 4*B*). None of the p185^{HER2} MAbs immunoprecipitated the EGFR. These data are consistent with the rank order observed in the immunoprecipitations with autophosphorylated p185^{HER2}.

Mab Immunoprecipitation of Native and Deglycosylated Receptors. To determine whether individual MAb binding epitopes are protein or carbohydrate in composition, immunoprecipitations were performed on ^{32}P labeled, tunicamycin treated, and native EGFR from A431 cells and p185^{HER2} from NIH 3T3/HER2-3$_{400}$. The autoradiography bands from the MAb immunoprecipitations were compared with those from appropriate polyclonal antisera, following the rationale that those antisera bound both glycosylated and unglycosylated receptors. Anti-EGFR MAbs 3G2, 5G3, 6C5, 13A9, 19C5, and 108 immunoprecipitated both the native (*M*$_r$ 170,000) and the lower molecular weight (approximately *M*$_r$ 140,000), deglycosylated forms of the EGFR (Fig. 5*A*). The p140 band appears only in the tunicamycin treated A431 cells and not in the untreated cells prepared by the same method. The anti-hepatitis MAb 40.1H1 reproducibly bound to a deglycosylated determinant on the

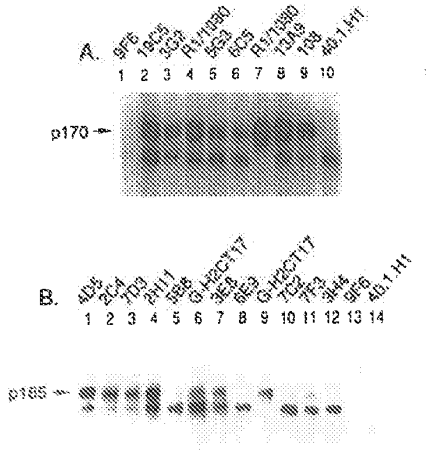


Fig. 5. Autoradiography of tunicamycin treated, partially nonglycosylated, [γ-^{32}P]ATP labeled A431 proteins immunoprecipitated by the anti-EGFR MAbs (*A*) or NIH 3T3/HER2-3$_{400}$ proteins immunoprecipitated by anti-p185^{HER2} MAbs (*B*). In *A*, *Lanes 2–3*, *5–6*, and *8–9* are immunoprecipitations with anti-EGFR MAbs; *Lanes 4* and *7* are immunoprecipitations with unglycosylated and native proteins, respectively, with mouse antiserum to the EGFR and *Lanes 1* and *10* are negative MAb immunoprecipitations with 9F6 (anti-gp120 of human immunodeficiency virus) and 48.1.H1 (anti-surface antigen B of hepatitis virus) respectively, with partially deglycosylated proteins. In *B*, *Lanes 1–5*, *7*, *8*, and *10–12* are immunoprecipitations with anti-p185^{HER2} MAbs; *Lanes 6* and *9* are immunoprecipitations with a mixture of deglycosylated and native proteins, respectively, with antiserum, G-H2CT17, to the COOH terminus of p185^{HER2} and *Lanes 13* and *14* are negative MAb immunoprecipitations as in *A*.

EGFR. This cross-reactivity was not seen with the anti-gp120 MAb 9F6. The anti-p185^{HER2} MAbs 3H4, 5B8, 6E9, 7C2, and 7F3 bound preferentially to the lower molecule weight, deglycosylated p185^{HER2} (approximately M_r 170,000), whereas 2C4, 2H11, 4D5, and 7D3 bound preferentially to the glycosylated form of p185^{HER2}, and 3E8 bound equally to the native and deglycosylated forms of p185^{HER2} by comparison to the polyclonal antisera G-H2C17 (Fig. 5*B*). As with the deglycosylated EGFR, the lower molecular weight band appears only in the tunicamycin treated NIH 3T3/HER2-3$_{400}$ cells and not in the untreated cells prepared by the same method. The immunological characteristics of the MAbs described including isotype, immunogen, ELISA reactivity, immunoprecipitation results, and epitope analysis and composition are summarized in Table 1.

Anti-EGFR MAbs Blocking of EGF Binding. The anti-EGFR MAbs were evaluated for their ability to block the ligand binding to the EGFR on A431 cells (Fig. 6). The anti-EGFR MAbs 5G3 and 6C5 inhibited ^{125}I-murine EGF ligand binding to receptor on A431 cells by 40–50% at an IgG concentration of 0.6 μM. MAb 19C5 showed 40% inhibition of ^{125}I-EGF binding at 6.0 μM, while MAb 108 inhibited ^{125}I-EGF binding by 25% to an IgG concentration of 0.06 μM. MAb 13A9 did not inhibit even at 6 μM, but instead appeared to enhance iodo-EGF binding. The unlabeled murine EGF control blocked ^{125}I-EGF binding by 95% at 6.0 and 0.6 μM.

FACS Analysis of MAbs Binding to Cells. To determine whether the MAbs could bind to and distinguish EGFR and p185^{HER2} on the surface of viable cells, FACS analysis was used with A431 and SK-BR-3 cells and the RMF of the molecules bound was calculated for each MAb (Fig. 7). The anti-EGFR MAbs bound to A431 cells with RMF values at least 100-fold greater than those measured on SK-BR-3 cells. This trend is consistent with the EGFR immunoprecipitation results from A431 and SK-BR-3 with the anti-EGFR MAbs (Fig. 4*B*). Although weak, all the EGFR MAbs, except 13A9, bound to the EGFR present on SK-BR-3 cells. The inconsistency with anti-EGFR MAb 13A9 in immunoprecipitation and FACS analysis may reflect an EGFR epitope which is sequestered on intact A431 cells. With the exception of MAbs 5B8 and 6E9, the anti-p185^{HER2} MAbs bound to SK-BR-3 cell with RMF values ranging 10- to 100-fold greater than the A431 cell values. These results are consistent with observed EGFR copy numbers of 1.2×10^6 and 8.7×10^4 for A431 and SK-BR-3 cells, respectively,[3] and a relative p185^{HER2} expression of approximately 20-fold lower on A431 and 10-fold higher on SK-BR-3 cells, by comparison to the EGFR expression on these cell lines.[4] The NIH 3T3/HER2-3$_{400}$ cells express 1.0×10^5 copies of p185^{HER2}. We have not determined the number of murine EGFR expressed by NIH 3T3/HER2-3$_{400}$ cells. Fig. 3 demonstrates that the anti-EGFR MAb 13A9 immunoprecipitates a weak p170 band from this cell line. Until we determine whether the entire anti-EGFR MAb panel binds to murine EGFR, the specificity of either the EGFR or p185^{HER2} specific MAbs should be concluded from the reactivity data generated with the human A431 and SK-BR-3 cell lines.

Growth Inhibitory Effects of Anti-EGFR MAbs on A431 Cells. We also investigated whether any of the anti-EGFR MAbs had growth inhibitory effects on A431 cells *in vitro*. Anti-EGFR MAbs 5G3 and 6C5, at final concentration of 16.7 nM, inhibited A431 cell proliferation by approximately 85% when compared to the A431 cell proliferation in the presence of anti-p185^{HER2} MAb 4D5 control (Fig. 8). This level of growth inhibition was equivalent to that of the EGF control at 8 nM. Anti-EGFR MAbs 13A9, 108, 3G2, and 19C5 had A431 growth inhibitory effects ranging from 50 to 30%, respectively. Hudziak *et al.* (38) performed similar *in vitro* growth inhibition experiments with the anti-p185^{HER2} MAbs and demonstrated that 4D5 was the most effective growth inhibitor of SK-BR-3.

DISCUSSION

We have used the established human epidermoid tumor line, A431, and a stably transfected murine cell line expressing the HER2/*neu* gene, NIH-3T3/HER2-3$_{400}$, as immunogens to produce MAbs which bind to the EGFR or p185^{HER2} on the surface of human cells. The resultant MAbs were used to investigate the expression and compare antigenic determinants on the extracellular domains of the EGFR and the related p185^{HER2}. Several groups have produced EGFR specific MAbs [(43, 46–49) partial list], some of which recognize noncarbohydrate determinants and react with the EGFR on a variety of cells. However, there has been little published antigenic characterization of these antibodies with respect to their cross-reactivity with p185^{HER2}. Schechter *et al.* (50) has shown that rabbit antisera raised to EGFR from A431 cells will immunoprecipitate p185^{neu} from NIH 3T3 cells transfected with the rat, ethylnitrosourea-induced *neu* oncogene. In contrast, the anti-rat p185^{neu} MAb 7.16.4 described by Drebin *et al.* (51) does not react with either the human p185^{HER2} or the human EGFR (50). The previous description of p185^{HER2} specific MAbs, reported by van de Vijver *et al.* (52), did not address the question of cross-reactive determinants present on both the EGFR and p185^{HER2}. Results from immunoprecipitation experiments with immune perfusion sera (data not shown) from our BALB/c

[3] M. Winget, personal communication.
[4] G. D. Lewis, personal communication.

Table 1 *Summary table of monoclonal antibodies described*

MAb	Isotype	EGFR ELISA[a]	p185^{HER2}ELISA	EGFR RIP	p185^{HER2}RIP	Epitope	FACS
3G2	IgG1κ	+	+	+	+	A(p)	+++
5G3	IgG1κ	++	–	++	–	B(p)	+++
6C5	IgG1κ	++	–	++	–	B(p)	+++
13A9	IgG1κ	+++	–	+++	–	C(p)	+++
19C5	IgG1κ	+	–	+	–	D(p)	+++
108[b]	IgG2aκ	+++	–	+++	–	E(p)	+++
2C4	IgG1κ	–	+++	–	++	F(c)	+++
2H11	IgG2aκ	–	+	–	++	H(c)	++
3E8	IgG2aκ	–	+	–	+++	H(p)	+++
3H4	IgG1κ	–	+	–	+	I(p)	+
4D5	IgG1κ	–	++	–	++	I(c)	+++
5B8	IgG1κ	–	+	–	++	nd(p)[c]	+
6E9	IgG1κ	–	++	–	+	nd(p)	–
7C2	IgG1κ	–	++	–	++	G(p)	+++
7D3	IgG1κ	–	++	–	++	F(c)	+++
7F3	IgG1κ	–	+++	–	+++	G/F(p)	+++
5E2	IgG1κ	nd	nd	+++	+++	nd	nd

[a] ELISA data columns (summary of $A_{492\ nm}$): <0.1 = –; 0.11–0.50 = +; 0.51–1.0 = ++; >1.0 = +++. RIP data columns (summary of autoradiography from immunoprecipitations): bands equal to negative control = –; weak bands but darker than negative control = +; moderately exposed bands = ++; strongly exposed bands = +++. Epitope data columns: letters assigned to represent individual epitopes A through I. MAbs were considered to share an epitope if each blocked binding of the other by 50% or greater in comparison to an irrelevant MAb control. The epitope composition recognized by immunoprecipitations with each MAb from tunicamycin treated cells is shown. A MAb is interpreted to bind predominantly to either a (p) protein determinant if that MAb binds preferentially to deglycosylated or equally to both the native and deglycosylated forms of the EGFR or p185^{HER2} or (c) carbohydrate determinant if that MAb binds preferentially to the native forms of the EGFR or p185^{HER2}. FACS data column: fluorescence staining of either A431 cells by the anti-EGFR MAbs or SK-BR-3 cells by the anti-p185^{HER2} MAbs equal to the negative control MAbs = –; 1–9-fold higher than the negative controls = +; 10–99-fold higher than the negative controls = ++; >100-fold higher than the negative controls = +++.

[b] Generous gift from J. Schlessinger.

[c] nd, not done.

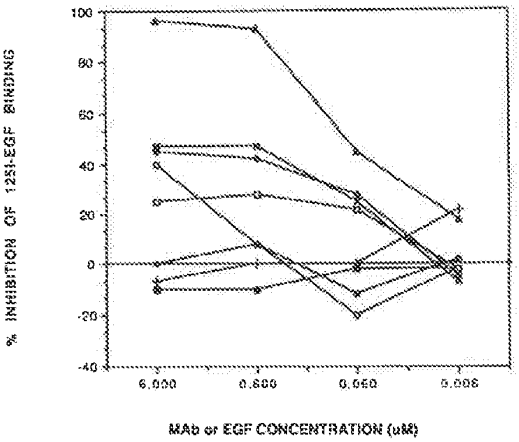


Fig. 6. MAb blocking of the ^{125}I-murine EGF binding to A431 cells. Presented are the dose-response curves with purified anti-EGFR MAbs 6C5 (×), 5G3 (✱), 3G2 (+), 19C5 (○), 13A9 (▩), 108 (□) compared to EGF (▲) and anti-phosphotyrosine MAb 5E2 (✱). The results are plotted as percentage of inhibition compared to maximal ^{125}I-EGF binding in buffer.

mice immunized with either A431 or NIH 3T3/HER2-3$_{400}$ cells showed no detectable antibodies which recognize shared, immunodominant epitopes on preparations of EGFR or p185^{HER2}. The resulting hybridomas produced from these mice were evaluated for antibody binding specificity to the EGFR or p185^{HER2} using either soluble receptors or intact cells. The specificity of these MAbs for Triton X-100 solubilized receptors was determined by ELISA on immobilized WGA purified membrane extracts and by immunoprecipitation using either extracts of [^{35}S]cysteine metabolically labeled cells or [γ-^{32}P]ATP autophosphorylated membrane extracts. Reactivity with extracellular domains of the receptors was determined by immunofluorescence by using the Pandex screen machine technology and FACS analysis on intact, viable cells. We have shown that our five anti-EGFR MAbs define at least three epitopes on the extracellular domain of the EGFR. One of these epitopes is shared by p185^{HER2} and is recognized by MAb 3G2. The p185^{HER2} MAbs recognize at least four epitopes on the extracellular domain of p185^{HER2} and none of these epitopes are shared with the EGFR. Experiments with the truncated, extracellular domain of the p185^{HER2} molecule (data not shown) indicates anti-p185^{HER2} MAbs 5B8 and 6E9 bind to epitopes very close to the transmembrane domain and may not be accessible to antibody binding on the surface of intact SK-BR-3 cells as compared to soluble p185^{HER2} receptors.

The binding site recognized by the individual MAbs was evaluated for its protein or carbohydrate composition by tunicamycin inhibition of glycosylation. The five EGFR specific MAbs bind preferrentially to the protein backbone of the EGFR. This is in contrast to many anti-EGFR MAbs reported that bind to carbohydrate blood group determinants found on the EGFR of A431 (47, 48) which are not expressed on the EGFR from other human cells. We have shown that our highest affinity anti-EGFR MAbs immunoprecipitate the EGFR from both A431 and SK-BR-3 cells. By comparison, 5 of the 10 anti-p185^{HER2} MAbs bind preferentially to the protein backbone while the remaining 5 MAbs bind either selectively to the glycosylated receptor or equally to both forms. The significance of the epitope composition recognized by the anti-p185^{HER2} MAbs may be elucidated as we continue to characterize the tissue distribution and epitope expression of p185^{HER2} in various tumors and normal tissues.

Hudziak *et al.* (38) has shown that several of the anti-p185^{HER2} MAbs inhibit breast tumor cell line proliferation *in vitro*. In an effort to perform similar studies on the EGFR specific MAbs, we determined the growth inhibitory effects of the anti-EGFR MAbs on the A431 cell line. MAbs 5G3 and 6C5 showed the greatest inhibition (85%) of cell proliferation. The cell proliferation data are consistent with the results obtained when the anti-EGFR MAbs were tested for blocking of murine ^{125}I-EGF binding to receptor on intact A431 cells. MAbs 5G3 and 6C5 demonstrated the strongest blocking of

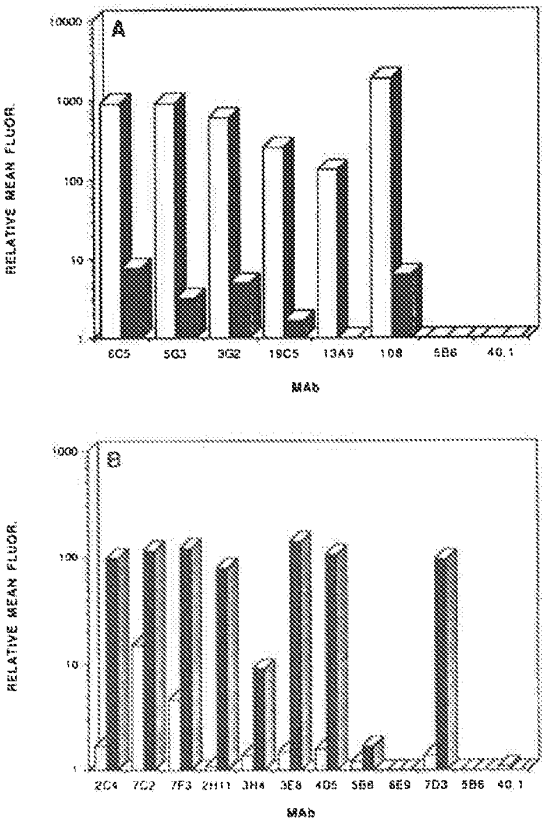


Fig. 7. RMF binding of purified anti-EGFR MAbs (*upper*) or the anti-p185^{HER2} MAbs (*lower*) to A431 (□) and SK-BR-3 (■). Included are the negative isotype controls 5B6 (IgG1) and 40.1.H1 (IgG2a). RMF was calculated as

$$N = e^{(0.009)xC}$$

where N is the "linearized fluorescence value", x is the log to linear transforming factor, and C is the channel number.

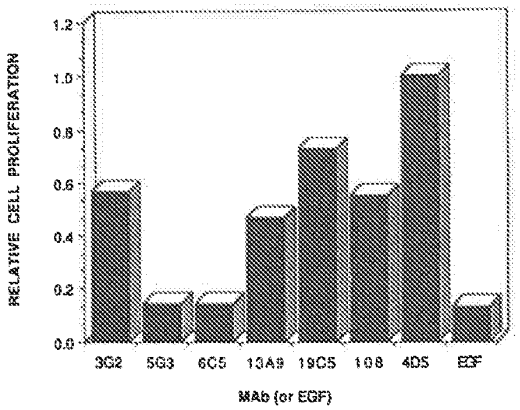


Fig. 8. The total number of A431 cells, after a 7 day growth period with either MAb at 2.5 μg/ml or EGF at 40 ng/ml, is compared using the anti-EGFR MAbs and anti-p185^{HER2} MAb 4D5. The relative proliferation is calculated by dividing the total A431 cells in the presence of anti-EGFR MAbs by the total A431 cells in the presence of anti-p185^{HER2} MAb 4D5.

the EGF binding (45% inhibition), while 19C5 and 108 show weaker EGF blocking (25–40% inhibition), and 13A9 and 3G2 did not block murine EGF binding to A431 cells. These results could reflect the mechanism by which MAbs to the EGFR inhibit A431 cell division *in vitro*. Similar receptor blocking experiments with the anti-p185^{HER2} MAbs await identification of the putative ligand.

It appears that in mice the major immunodominant determinants on the EGFR and p185^{HER2} are not in the homologous regions comprising 40% of the extracellular domains of these receptors. It has previously been shown that a membrane structure sharing homologous extracellular regions to the EGFR and p185^{HER2} exists in *Drosophila* (53, 54). A high degree of conservation throughout evolution, in certain extracellular regions of the EGFR and p185^{HER2}, could be accompanied by a lack of immunogenicity in these regions. Since two of the EGFR specific MAbs described here partially block EGF binding, it is likely that at least one of the immunogenic, nonhomologous epitopes on the EGFR is located in or near the ligand binding site. The possibility does exist that we have broken immunological tolerance to the murine EGFR, and future experiments, pending identification of murine cell line(s) which overexpress the EGFR, will include evaluation of these EGFR MAbs for species specificity. We have observed by FACS analysis (data not shown) that our p185^{HER2} MAbs do not bind to the endogenous *neu* on the surface of RAT-1 cells indicating that, like MAb 7.16.4, they bind to p185^{HER2} epitopes that may be species specific. This result might be predicted since the anti-p185^{HER2} MAbs resulted from fusions in the murine system and mice may be tolerant to conserved determinants on p185^{HER2}. Whether the immunogenic, nonhomologous regions on p185^{HER2} are in the putative ligand binding pocket will require further investigation.

Overexpression or altered forms of the EGFR and/or p185^{HER2} are found in many forms of human cancers including cervical, ovarian, squamous, adenocarcinomas, and primary breast tumors. In the case of breast and ovarian cancer, amplification of HER2/*neu* expression is associated with approximately 30% of the primary tumors tested and is correlated with metastatic disease and poor prognosis (17, 18). The location of these receptors on the surface of cells, prognostic significance and probable causal role makes them very attractive targets for production of monoclonal antibodies for immunodiagnostics and immunotherapy. Immunohistochemistry data on tumor biopsy tissue could be useful in determining the aggressiveness of therapy depending on the level of p185^{HER2} expression. These MAbs would also be candidates for *in vivo* radioimaging for detection of relevant primary tumors and micrometastases. The receptor nature of these antigens, including surface location, ligand binding, and receptor/ligand complex internalization, makes tumors expressing these receptors potential targets for immunotherapeutic intervention utilizing antibodies as radioconjugates, drug carriers, or immunotoxins. An alternate approach would be to exploit the ligand blocking characteristics of some receptor specific MAbs and deprive transformed cells of their intracellular signal transduction. This has been demonstrated with EGFR specific MAbs where EGF blocking is the mechanism for growth inhibition on EGF dependent cells (43, 55). The anti-p185^{HER2} MAbs should be useful reagents to investigate similar receptor/ligand studies on p185^{HER2} and may facilitate identification of the putative ligand.

We are optimistic that diagnostic and therapeutic applica-

tions of MAbs specific for receptor related molecules associated with human malignancy will allow exploitation of this probable causal relationship in transformation and the process of oncogenesis.

ACKNOWLEDGMENTS

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/221,851	07/28/2016	Hiroyuki NAITO	111119-0105	3964

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Foley & Lardner LLP
3000 K STREET N.W.
SUITE 600
WASHINGTON, DC 20007-5109

EXAMINER	
DUFFY, PATRICIA ANN	

ART UNIT	PAPER NUMBER
1645	

NOTIFICATION DATE	DELIVERY MODE
11/13/2017	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ipdocketing@foley.com

Office Action Summary	Application No. 15/221,851	Applicant(s) NAITO et al.	
	Examiner Patricia Duffy	Art Unit 1645	AIA Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 10-6-2017 .

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____ .

2a)☑ This action is **FINAL**. 2b) ☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☑ Claim(s) 1-4 and 6-11 is/are pending in the application.

5a) Of the above claim(s) 6 and 8 is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☑ Claim(s) 1-4,7 and 9-11 is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☑ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☑ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____ .

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____ .

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____ .

4) ☐ Other: _____ .

RESPONSE TO AMENDMENT

The amendment and response and translations filed 10-6-2017 has been entered into the record. Claim 5 has been cancelled. Claims 1-4 and 6-11 are pending. Claims 1-4, 7 and 9-11 are under examination.

Notice of Pre-AIA or AIA Status

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

The text of Title 35 of the U.S. Code not reiterated herein can be found in the previous office action.

Priority

It is noted that PCT/JP2015/000355, and Foreign priority applications filed 1/31/2014. 8, 22/2014 and 11/10/2014 are all in Japanese and no English translations have been filed. As such, the instant filing date 7-28-2016 is the priority date for prior art purposes.

Applicant has filed Certified Translations of Foreign Applications:

FOREIGN APPLICATIONS
JAPAN 2014-017777 01/31/2014
JAPAN 2014-168944 08/22/2014
JAPAN 2014-227886 11/10/2014

Applicant asserts that the instant filing is a "mirror image" of PCT/JP2015/000355. No certified translation of PCT/JP2015/000355. The instant application (USSN 15/221,851; filed 7-28-2106) is filed under 36 USC 111 as a continuation of PCT/JP2015/000355. The foreign priority documents are more than one year than the instant filing date and as such, filing of certified translations of the Foreign Priority documents only, is insufficient to establish an earlier priority of the instantly claimed invention.

A certified copy of the international application (and an English translation of the international application) may be required by the examiner to perfect the claim for benefit under **35 U.S.C. 120** and **365(c)** if the international application did not originate in the United States and such is necessary, for example, where an intervening reference is found and applied in a rejection of one or more claims. See MPEP 1895.01.

Applicant's assertion that the instant application is a "mirror image" of PCT/JP2015/000355 is insufficient to establish priority and a Certified translation is required to establish priority.

Election/Restrictions

This application contains claim 6 and 8 drawn to an invention nonelected and treated without traverse. A complete reply to the final rejection must include cancellation of nonelected claims or other appropriate action (37 CFR 1.144) See MPEP § 821.01.

Objections/Rejections Withdrawn

Duplicate Claim Warning

The advisory with respect to claim 5, under 37 CFR 1.75 as being a substantial duplicate thereof is withdrawn in view of the cancellation of claim 5.

Rejections Maintained

Please note that USSN 14/436,485, 14/435,114, 15/180,203, 15/187,179 have earlier effective filing dates.

Claim1-5, 7 and 9-11 stand provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claim 39, 40-44, 48-50 of copending Application No. **14/436,458** in view of Chamberlin et al (8,802,820 with an earlier filing date of October 31, 2007) and EP 1155702 A1 published 11-21-2001; of record for reasons made of Record in the Office Action Mailed 7-7-2017.

Applicant's request that the rejections be held in abeyance until allowable subject matter is identified. This is not persuasive, USSN 14/436,458 is now allowed, but not issued and has an earlier effective filing date.

Claims 1-5, 7 and 9-11 stand provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 13, 14, 20, 21, 22-26 and 30-33 of copending Application No. **14/435,114** in view of Chamberlin et al (8,802,820 with an earlier filing date of October 31, 2007) for reasons made of record in the Office Action mailed 7-7-2017.

Applicant's request that the rejections be held in abeyance until allowable subject matter is identified. This is not persuasive as the '114 application has an earlier effective filing date.

Claims 1-5, 7 and 9-11 stand provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-8 of copending **allowed but not yet issued**

Application No. 15/180,203 in view of Chamberlin et al (8,802,820 with an earlier filing date of October 31, 2007) for reasons made of record in the Office Action mailed 7-7-2017.

Applicant's request that the rejections be held in abeyance until allowable subject matter is identified. This is not persuasive as the '114 application has an earlier effective filing date.

Claims 1-5, 7 and 9-11 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-8 **now issued Application No. 15/187,179** published as US Patent 9,808,537 in view of Chamberlin et al (8,802,820 with an earlier filing date of October 31, 2007) for reasons made of record in the Office Action mailed 7-7-2017.

Applicant argues that the rejection over-reaches in that products used to treat different diseases are somehow not patentable distinct goes well beyond the law. This is not persuasive, as Applicant has not addressed the rejection as a whole. The rejection as stated provides for a different antibody and it is noted that both the antibody of the '179 application and the instant application are claimed to be useful for treating breast cancer. As such, since the drug was known to treat cancer and the antibodies both known and claimed for treatment of breast cancer the substitution is of one breast cancer tumor targeting antibody for another breast cancer tumor targeting antibody is prima facie obvious. Applicant was previously notified that **should the '203 application issue in the interim, the rejection will be converted to an actual double patenting rejection.**

The rejection is maintained.

Claims 1-5, 7 and 9-11 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-16 of copending 15/285,156 in view of Chamberlin et al (8,802,820 with an earlier filing date of October 31, 2007) for reasons made of record in the Office Action mailed 7-7-2017.

Applicant's request that the rejections be held in abeyance until allowable subject matter is identified. The argument that the '156 application is noted, however until the time as allowable matter is indicated, the rejection is maintained.

Claims 1-5, 7 and 9-11 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1, 2, 5, 18, 19 and 21-28 of copending 15/302,803 optionally in view of Chamberlin et al (8,802,820 with an earlier filing date of October 31, 2007) for reasons made of record in the Office Action mailed 7-7-2017.

Applicant's request that the rejections be held in abeyance until allowable subject matter is identified. The argument that the '156 application is noted, however until the time as allowable matter is indicated, the rejection is maintained.

Claim Rejections - 35 USC § 103

It is noted that a certified translation of PCT/JP2015/000355 has not been filed to establish priority. The filing of the certified translation of the Japanese applications alone is insufficient inasmuch as the instant application was filed more than one year after the filing of the Japanese applications. As such, the instant filing date 7-28-2016 is the priority date for prior art purposes is maintained.

In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

Claims 1-5, 7 and 9-11 are rejected under 35 U.S.C. 103 as being unpatentable over WO 2014/061277 published 4-2-2014 hereinafter references as '277; English language equivalent of specification and claims provided from European family member in view of Chamberlin et al (8,802,820 with an earlier filing date of October 31, 2007) is maintained for reasons made of record in the Office Action mailed 7-7-2017.

Applicant argues that the rejection is not proper because once the present application has been accorded its proper priority date, the WO 2014/061277 will become an exception under 35 USC 102(b)(2)(C). This is not persuasive as the rejection was made as a 103 based upon 102(a)(1) and the exception does not apply in this instance and priority has not been properly established. Should Applicant properly established priority then the rejection would be

applicable under 35 USC 102(a)(2). It is noted that Applicant has not provided the evidence and statement required to establish common ownership pursuant to MPEP 717.02(b) III and 37 CFR 1.104.

"The following statement is sufficient evidence to establish common ownership of, or an obligation for assignment to, the same person(s) or organizations(s):

Application [the application serial number] and reference(s) [the patent identifier of the commonly owned applied art] (whether U.S. patents, U.S. patent applications, U.S. patent application publications, or WIPO patent publication were, at the time the invention was effectively filed, owned by [the name of the person(s), organization(s), and/or business entity(ies) that own the application and the commonly owned applied art].

The applicant(s) or the representative(s) of record have the best knowledge of the ownership of their application(s) and reference(s), and their statement of such is sufficient evidence because of their paramount obligation of candor and good faith to the USPTO."

Status of Claims

Claims 1-5, 7 and 9-11 stand rejected. Claims 6 and 8 are withdrawn from consideration.

Conclusion

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Patricia Duffy whose telephone number is 571-272-0855. The examiner can generally be reached on 7:30 am - 4:00 pm. If attempts to reach the examiner by telephone are unsuccessful, the examiner's Supervisor Gary Nickol can be reached at 571-272-0835.

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//PATRICIA DUFFY/
Primary Examiner, Art Unit 1645

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High expression of TROP2 correlates with poor prognosis in pancreatic cancer

D Fong*[,1,2], P Moser[3], C Krammel[2], JM Gostner[2], R Margreiter[4], M Mitterer[5], G Gastl[1] and G Spizzo[2,5]

[1]Department of Hematology and Oncology, Innsbruck Medical University, Innsbruck 6020, Austria; [2]Tyrolean Cancer Research Institute, Innsbruck 6020, Austria; [3]Department of Pathology, Innsbruck Medical University, Innsbruck 6020, Austria; [4]Department of General and Transplant Surgery, Innsbruck Medical University, Innsbruck 6020, Austria; [5]Department of Oncology and Hematology, Paride Stefanini Hospital, Merano 39012, Italy

Pancreatic cancer is one of the most devastating human malignancies. Despite considerable research efforts, it remains resistant to almost all available treatment regimens. The human trophoblast cell-surface antigen, TROP2, was found to be strongly expressed in a variety of human epithelial cancers, correlating with aggressiveness and poor prognosis. TROP2 antigen expression was investigated retrospectively by immunohistochemistry in paraffin-embedded primary tumour tissue samples from a series ($n = 197$) of consecutive patients with pancreatic adenocarcinoma. Survival was calculated using Kaplan–Meier curves. Parameters found to be of prognostic significance in univariate analysis were verified in a multivariate Cox regression model. TROP2 overexpression was observed in 109 (55%) of 197 pancreatic cancer patients and was significantly associated with decreased overall survival ($P < 0.01$). By univariate analysis, TROP2 overexpression was found to correlate with the presence of lymph node metastasis ($P = 0.04$) and tumour grade ($P = 0.01$). Furthermore, in the subgroup of patients treated surgically with curative intent, TROP2 overexpression significantly correlated with poor progression-free survival ($P < 0.01$). Multivariate analyses revealed TROP2 to be an independent prognosticator. These findings suggest for the first time that TROP2 could be a novel prognostic biomarker for pancreatic cancer. Targeting TROP2 might be a useful treatment approach for patients with pancreatic cancer overexpressing this cell-surface marker.
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Molecular Diagnostics

Pancreatic cancer is a fatal disorder. At the time of diagnosis, most patients already have locally advanced or metastatic disease excluding surgical resection (Yeo *et al*, 1997). Treatment regimens followed at present are ineffective and do not prolong patient survival significantly. Survival rates for the 15–20% of patients who have resectable disease at first presentation are disappointing, depending on the status of resection margins and surrounding lymph nodes. Moreover, the prognosis is poor even for those who undergo complete (R0) resection. The 5-year overall survival rate for all stages is approximately 5%, whereas after radical pancreaticoduodenectomy it has been reported to range from 25 to 30% (Sohn *et al*, 2000; Jemal *et al*, 2005). To date, there are no validated prognostic indicators for identifying patients with non-metastatic disease and good or poor prognosis. As a consequence, there is an urgent need for novel prognostic biomarkers as well as for therapeutic target structures in pancreatic cancer.

In the past, we found GA733-encoded proteins (EpCAM, TROP2) to be prognostic for a variety of epithelial cancers (Gastl *et al*, 2000; Varga *et al*, 2004; Fong *et al*, 2008). GA733 is a member of a family of at least two closely related genes (*GA733-1*; *TACSTD2* and *GA733-2*; *TACSTD1*) that share sequence homology with both thyroglobulin type I and interleukin-2 receptors. The integral glycoprotein EpCAM is encoded by the *GA733-2* gene functions as a homotypic intercellular adhesion molecule (Litvinov *et al*, 1994), and has been targeted by antibody-mediated immunotherapy (Riethmuller *et al*, 1998; Ruan *et al*, 2003).

The human trophoblast cell-surface antigen, TROP2 (also termed GA733-1), is a transmembrane glycoprotein found to be expressed at high levels by various types of human carcinomas (Fradet *et al*, 1984; Stein *et al*, 1993a; Nakashima *et al*, 2004). In contrast, normal epithelial tissues show little or no TROP2 expression (Stein *et al*, 1993a; Zhang *et al*, 1997). TROP2 is encoded by the *GA733-1* gene, which is intronless, formed by exon-shuffling and retroposition of the *GA733-2* gene through mRNA intermediate (Linnenbach *et al*, 1993). Recently, TROP2 antigen expression in colorectal and squamous cell carcinomas of the oral cavity was shown to correlate with tumour aggressiveness and poor prognosis (Ohmachi *et al*, 2006; Fong *et al*, 2008). Moreover, Wang *et al* (2008) were able to identify TROP2 as an oncogene and an attractive therapeutic target in colon cancer. These findings indicate a potential role for TROP2 in tumour development and progression. In this retrospective study, we evaluated TROP2 antigen expression and its correlation with clinicopathologic features in pancreatic cancer.

MATERIALS AND METHODS

Patients and tissue samples

This study was conducted according to the regulations of the local Ethics Committee and Austrian law. A total number of 197 consecutive patients with pancreatic ductal adenocarcinoma, from

*Correspondence: Dr D Fong; E-mail: dominic.fong@i-med.ac.at
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whom formalin-fixed, paraffin-embedded tissue samples were available, diagnosed between 1990 and 2006 at the Department of Pathology, Innsbruck Medical University, were included in this retrospective study. Patients with other pancreatic malignancies, such as intraductal papillary mucinous adenocarcinoma, acinar cell carcinoma and malignant endocrine tumours, were excluded. All tumour specimens were reclassified on hematoxylin-and-eosin-stained slides, and histological type and tumour grade were reassessed by a pathologist (PM) using standard diagnostic criteria. Clinical data were obtained by reviewing the charts and contacting the physicians in charge. Tumours were histologically classified according to the WHO classification and staged according to the tumour node metastasis classification (Sobin and Fleming, 1997). Overall survival was defined as the period of time from initial diagnosis to death or last contact, that is, date of last follow-up visit. Progression-free survival was defined as the time from surgery with curative intent to appearance of disease recurrence or evidence of new lesions detected by computed tomography.

Immunohistochemistry

TROP2 expression was determined by immunohistochemistry using a purified goat polyclonal antibody detecting the recombinant human TROP2 extracellular domain at a dilution of 1 : 50 (AF650, R&D Systems, Inc., Minneapolis, MN, USA) as described earlier (Fong *et al*, 2008). Colon carcinoma samples with various TROP2 expression levels (no, low, moderate, or high expression) were used as positive and negative controls. In addition, 10 samples of normal pancreatic tissue were collected from patients undergoing pancreatic surgery at Innsbruck Medical University. TROP2 antigen overexpression was evaluated by two independent observers (PM and DF) using light microscopy in a blinded manner. Discordant cases were reevaluated on a double-headed microscope to achieve a consensus. Antigen expression was defined as the presence of specific staining on surface membranes of tumour cells. TROP2 overexpression was evaluated for each tissue sample by calculating a total immunostaining score as the product of a proportion and intensity score. The proportion score described the estimated fraction of positive-stained tumour cells (0, none; 1, <10%; 2, 10–50%; 3, 51–80%; 4, >80%). The intensity score represented the estimated staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong). The total score ranged from 0 to 12. As described earlier, TROP2 'overexpression' was defined as a total score of more than 4 (Fong *et al*, 2008). Representative micrographs of tumours with predominant membranous staining of TROP2 are shown in Figure 1.

Statistical analysis

Statistical analysis was performed using the Statistical Package of Social Sciences (SPSS, version 10.0, Chicago, IL, USA). Correlations between TROP2 expression and clinicopathological variables were assessed with the χ^2-test. Survival rates were calculated using the Kaplan–Meier method and compared by means of the log-rank test. Follow-up time was censored if the patient was lost during follow-up. Factors with prognostic significance in the univariate models were further evaluated in a multivariate Cox regression model. For all analyses, a P-value of less than 0.05 was considered statistically significant.

RESULTS

Patient characteristics

Demographic data and tumour characteristics are summarised in Table 1. At the time of last clinical follow-up (February 2008), 155 (79%) patients out of the total group had died. Median overall survival was 9 months (range, 1–68). Most patients (68%) had undergone primary surgical intervention, whereas 54 (27%) patients were considered inoperable (9 cases unknown). Palliative surgery included palliative bypass or endoscopic bile duct stenting. If indicated, patients received standard gemcitabine-based chemotherapy according to their clinical stage and performance status. In 17 patients, exact staging according to UICC was not feasible.

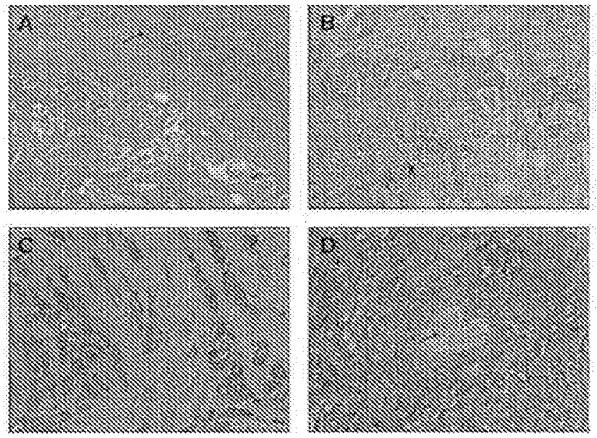


Figure 1 TROP2 immunostaining in pancreatic tissue, original magnification ×100. (**A**) TROP2-negative tumour sample (score 0), normal pancreatic tissue (arrow) showing weak (score 2) immunostaining. Pancreatic ductal adenocarcinoma with moderate (score 6) (**B**) and strong (score 12) (**C** and **D**) TROP2 expression, note the TROP2-negative islets of Langerhans (arrow).

Immunohistochemistry

As reported earlier, islets of Langerhans displayed no TROP2 staining (Stein *et al*, 1993a), whereas in normal pancreatic epithelium TROP2 expression was weak to moderate (Figure 1). In contrast, moderate-to-strong homogeneous membranous expression of TROP2 was detected in 109 (55%) of 197 carcinoma specimens. Strong TROP2 expression (score 9–12) was found in 57 (29%) of 197 cases, moderate expression (score 6–8) in 52 (26%) cases, weak expression (score 1–4) in 76 (39%) cases and absent expression (score 0) in 12 (6%) of 197 cases. Thus, according to our earlier defined criteria (Fong *et al*, 2008), TROP2 was found to be overexpressed in 109 (55%) of 197 cases.

Clinicopathological variables and patient survival

Univariate analysis showed TROP2 overexpression to be significantly correlated with histologic grading ($P = 0.01$) and the presence of lymph node metastases ($P = 0.04$), but not with sex, age, T stage, presence of distant metastasis, or involvement of the resection margin (Table 2). To assess the impact of clinicopathological features and TROP2 overexpression on survival, we used Kaplan–Meier analysis and the log-rank test for censored survival data. Consistent with earlier findings, early tumour stage and a negative resection margin were associated with longer overall survival (Table 1). It should be noted that TROP2 overexpression was significantly correlated with poor overall survival ($P < 0.01$; Figure 2). Median overall survival time for patients with tumour presenting with and without TROP2 overexpression was 8 and 14 months, respectively. Overall survival gradually declined with

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Table 1 Patient characteristics

Variable	Pancreatic cancer (n) (%)	P-value (log-rank test)
Sex (n = 197)		
Male	111 (56)	0.80
Female	86 (44)	
Age (n = 197)		
Mean	65	
Median	67	
Range	37–91	
T stage (n = 176)		
pT1	13 (7)	<0.01
pT2	52 (29)	
pT3	80 (46)	
pT4	31 (18)	
Lymph node status (n = 176)		
Negative	65 (37)	<0.01
Positive	111 (63)	
Distant metastasis (n = 142)		
No	117 (82)	<0.01
Yes	25 (18)	
Surgical margin (n = 128)		
Negative	86 (67)	<0.01
Positive	42 (33)	
Stage (UICC) (n = 180)		
IA	6 (3)	<0.01
IB	24 (13)	
IIA	34 (19)	
IIB	70 (39)	
III	20 (11)	
IV	26 (15)	
Differentiation (n = 181)		
Well	24 (13)	0.07
Moderate	84 (47)	
Poor	73 (40)	
TROP2 overexpression (n = 197)		
Yes	109 (55)	<0.01
No	88 (45)	

Table 2 Correlation of TROP2 overexpression with conventional clinicopathological parameters

	Total patients (n)	TROP2 overexpression: No n (%)	TROP2 overexpression: Yes n (%)	P-value
Sex (n = 197)				
Male	111	51 (46)	60 (54)	0.68
Female	86	37 (43)	49 (57)	
Age at diagnosis (n = 197)				
<65 years	84	37 (44)	47 (56)	0.88
≥65 years	113	51 (45)	62 (55)	
Differentiation (n = 181)				
Well	24	17 (71)	7 (29)	0.01
Moderate	84	38 (45)	46 (55)	
Poor	73	26 (36)	47 (64)	
T stage (n = 176)				
pT1	13	5 (38)	8 (62)	0.06
pT2	52	27 (52)	25 (48)	
pT3	80	36 (45)	44 (55)	
pT4	31	7 (23)	24 (77)	
Lymph node status (n = 176)				
No	65	34 (52)	31 (48)	0.04
Yes	111	41 (37)	70 (63)	
Distant metastasis (n = 142)				
No	117	56 (48)	61 (52)	0.14
Yes	25	8 (32)	17 (68)	
Surgical margin (n = 128)				
Negative	86	45 (52)	41 (48)	0.10
Positive	42	16 (38)	26 (62)	
Stage (UICC) (n = 180)				
IA	6	4 (67)	2 (33)	0.03
IB	24	15 (63)	9 (37)	
IIA	34	15 (44)	19 (56)	
IIB	70	32 (46)	38 (54)	
III	20	3 (15)	17 (85)	
IV	26	9 (35)	17 (64)	

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increasing TROP2 scores. In the subset of patients with tumours lacking TROP2 overexpression (score 0), median overall survival time was 15 months and decreased to 14 months (score 1–4), 10 months (score 5–8) and 7 months (score 9–12) with increasing staining scores. Multivariate analysis identified TROP2 overexpression together with a negative resection margin as an independent prognostic factor for poor overall survival (Table 3a). Subsequently, a detailed analysis for the subgroup of patients who underwent surgery with curative intent ($n = 134$) was performed.

Resected cohort with curative intent

Data on relapse or disease progression were available in 78% ($n = 105$) of the patients. Median overall and progression-free survival was 13 (range, 1–68) months and 6 (range, 1–56) months, respectively. Survival analyses revealed that TROP2 overexpression was significantly correlated with poor overall survival ($P < 0.01$; Figure 3A) as well as a shortened progression-free interval ($P < 0.01$; Figure 3B). Table 3b shows the multivariate analyses for patients treated surgically with curative intent. This model was refined by stepwise removal of variables. Regarding overall survival, TROP2 overexpression together with a negative resection margin has been identified to be an independent prognostic marker. Concerning progression-free survival, TROP2 overexpression was independent of nodal status and margin involvement by the tumour.

DISCUSSION

This study describes the prognostic value of TROP2 expression in pancreatic cancer. Overexpression of TROP2 was detectable in 109 (55%) of 197 tumour samples and was significantly correlated with decreased overall survival. In multivariate analyses, TROP2 overexpression was shown to be an independent prognostic biomarker for poor overall survival. Furthermore, TROP2 overexpression was significantly associated with a shortened progression-free survival in the subgroup of patients who underwent surgery with curative intent.

Both cell-surface glycoproteins, TROP2 and EpCAM, are encoded by the GA733 gene family and have been efficiently

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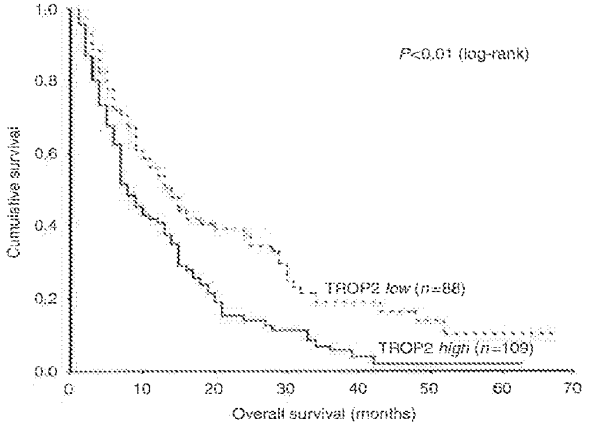


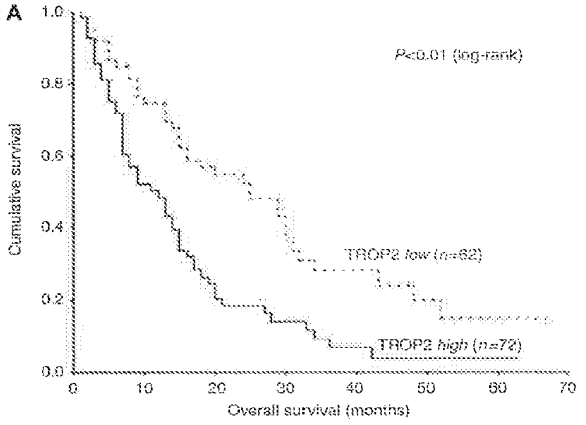
Figure 2 Prognostic significance of TROP2 antigen expression in 197 patients with pancreatic ductal adenocarcinomas regarding overall survival as calculated by Kaplan–Meier analysis. Low, patients with tumour ($n = 88$) without TROP2 overexpression; high, patients with tumour ($n = 109$) with TROP2 overexpression. Patients with low TROP2 expression had significantly better overall survival than patients with high TROP2 expression as defined by the log-rank test ($P < 0.01$).

Table 3 Multivariate analyses (Cox regression) of various prognostic parameters in the total cohort and subgroup of patients with curative intent

	Overall survival		
	P-value	RR	95% CI
(a) Total group of patients (n = 197)			
T stage	0.81	1.0	0.7–1.4
Nodal status	0.94	0.9	0.5–1.6
Distant metastasis	0.71	0.7	0.1–5.2
Surgical margin	0.02	1.9	1.1–3.5
TROP2 overexpression	0.01	1.8	1.1–3.1

	Progression-free survival			Overall survival		
	P-value	RR	95% CI	*P*-value	RR	95% CI
(b) Subgroup of patients treated surgically with curative intent (n = 134)						
T stage	0.720	0.9	0.6–1.3	0.721	0.9	0.6–1.3
Nodal status	0.545	1.1	0.6–2.1	0.761	0.9	0.5–1.5
Surgical margin	0.276	1.4	0.7–2.7	0.012	2.0	1.1–3.5
TROP2 overexpression	0.070	1.6	0.9–2.8	0.009	1.8	1.1–3.0
Nodal status	0.562	1.1	0.6–2.1	0.772	0.9	0.5–1.5
Surgical margin	0.301	1.3	0.7–2.6	0.012	1.9	1.1–3.3
TROP2 overexpression	0.073	1.6	0.9–2.7	0.009	1.8	1.1–3.0
Surgical margin	0.185	1.4	0.8–2.7	0.009	1.9	1.1–3.1
TROP2 overexpression	0.053	1.6	0.9–2.8	0.009	1.8	1.1–2.9
Nodal status	0.193	1.5	0.8–2.2	0.268	1.2	0.8–1.9
TROP2 overexpression	0.014	1.8	1.1–2.9	0.000	2.0	1.3–3.1

Abbreviations: CI = confidence interval; RR = relative risk.



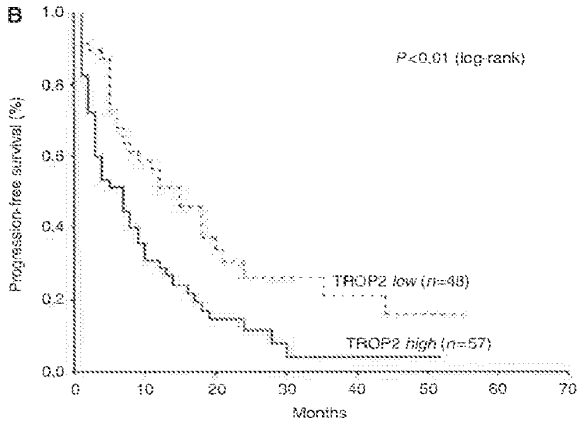


Figure 3 Prognostic significance of TROP2 antigen expression in the subgroup of patients who underwent surgery with curative intent regarding overall ($n = 134$) and progression-free ($n = 105$) survival as calculated by Kaplan–Meier analysis. Patients with tumour tissue presenting TROP2 overexpression (TROP2 high) had a significant shortened overall survival (**A**) and progression-free (**B**) interval as compared with patients with tumours lacking TROP2 overexpression (TROP2 low).

targeted by monoclonal antibodies *in vitro* as well as *in vivo* (Velders *et al*, 1998; Ruan *et al*, 2003; Wang *et al*, 2008). Similarly TROP2, EpCAM overexpression was also found to be a strong prognostic marker for poor survival in various epithelial cancer entities (Gastl *et al*, 2000; Varga *et al*, 2004; Spoecklein *et al*, 2006). Owing to its immunogenic properties in cancer patients, active and passive immunotherapeutic agents against the EpCAM antigen have been developed (Riethmuller *et al*, 1998; de Bono *et al*, 2004).

The functional role of TROP2 in carcinogenesis and tumour progression is only poorly understood. Fornaro *et al* (1995), who first cloned the human TROP2 gene, speculated that TROP2 might be a cell-surface signal transducer and might regulate tumour cell proliferation. This assumption is substantiated by the finding that the cytoplasmic domain of TROP2 contains several potential phosphorylation sites and TROP2 cross-linking antibodies can cause a transient increase in intracellular calcium levels (Basu *et al*, 1995; Ripani *et al*, 1998). Moreover, it has been shown that human cancer cells can express an oncogenic hybrid mRNA between TROP2 and cyclin D1, a master regulator of the cell cycle (Tertinoni *et al*, 2001). Interestingly, TROP2 seems to be involved

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in regulating the shift between cell migration and differentiation in primordium cells (Villablanca *et al*, 2006). However, its physiological ligand is still unknown. Schon *et al* (1993) were able to demonstrate that EpCAM can undergo proteolytic processing and that cleavage may activate EpCAM signalling. Similar mechanisms have been hypothesised for TROP2 (Fornaro *et al*, 1995). Recently, TROP2 expression was shown to correlate with invasive tumour phenotypes and poor prognosis (Ohmachi *et al*, 2006; Fong *et al*, 2008). In pancreatic cancer, TROP2 was previously reported to be highly expressed (Iacobuzio-Donahue *et al*, 2002), whereas Wang *et al* (2008) identified TROP2 as an oncogene and a potential therapeutic target in colon cancer. In fact, blocking experiments with anti-TROP2 antibodies revealed that TROP2 can contribute to tumour cell migration and invasion. Moreover, increasing TROP2 expression prompted an anchorage-independent growth of colon cancer cells.

The murine monoclonal antibody (mAb) RS7 reacts specifically with TROP2 and exhibits functional properties that make it an attractive tool for clinical diagnostic and therapeutic applications (Stein *et al*, 2003b). Indeed, radiolabelled RS7 has been applied for tumour imaging and revealed significant antitumour activity in several animal models (Shih *et al*, 1995; Stein *et al*, 1999c). Meanwhile, RS7 was successfully humanised and showed promising antitumour effects in an *in vivo* breast cancer model (Govindan *et al*, 2004).

In conclusion, we have demonstrated that TROP2 is a novel prognostic biomarker for pancreatic adenocarcinoma. These data must be confirmed and validated prospectively. Furthermore, TROP2 harbours the potential to become an attractive target for antibody-based therapies for the subset of patients with TROP2-overexpressing pancreatic cancer.

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ORIGINAL RESEARCH

Validation study of a method for assaying DE-310, a macromolecular carrier conjugate containing an anti-tumor camptothecin derivative, and the free drug in tumor tissue by high performance liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry

Toshihiro Oguma,[1]* Hiroshi Morikawa,[2] Daisuke Iwasaki[2] and Ryo Atsumi[1]

[1]Drug Metabolism and Physicochemical Property Research Laboratory, Daiichi Pharmaceutical Co. Ltd, Tokyo 134-8630, Japan
[2]Pharmacokinetics and Bioanalysis Center, Shin Nippon Biomedical Laboratories Ltd, Wakayama 642-0017, Japan

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ABSTRACT: DE-310 is a macromolecular carrier conjugate containing an anti-tumor camptothecin derivative, DX-8951, which is conjugated to a water-soluble polymer via a peptide spacer. Assay methods have been developed for the determination of a polymer-bonded DX-8951 conjugate, DX-8951, and Glycyl-DX-8951 concentrations in murine Meth A tumor tissue. Free DX-8951 and Glycyl-DX-8951 were extracted from tumor tissue homogenates by protein precipitation and analyzed by LC/MS/MS (method I). Conjugated DX-8951 was isolated by solid-phase extraction after digestion with a thermolysin. The productive phenylalanyl-glycyl-DX-8951 was analyzed by LC/MS/MS (method II). The lower limits of quantitation of DX-8951, Glycyl-DX-8951, and conjugated DX-8951 were 1.36, 1.34 and 73.7 ng/g (as DX-8951 equivalent). These two methods showed satisfactory sensitivity, precision and accuracy. To study the pharmacokinetics of DE-310, it would be of great help to assay the polymer-bonded DX-8951 and its released drugs in tumor tissue. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: LC/MS/MS; DE-310; camptothecin; tumor; tissue

INTRODUCTION

DE-310 is a macromolecular carrier conjugate containing DX-8951, which is conjugated to a carboxymethyldextran polyalcohol via a glycyl-glycyl-phenylalanyl-glycyl-peptide spacer (Fig. 1). DX-8951 is a free form of DX-8951f [exatecan; monomethanesulfonate salt of (1*S*,9*S*)-1-amino-9-ethyl-5-fluoro-1,2,3,9,12,15-hexahydro-9-hydroxy-4-methyl-10*H*,13*H* benzo[*de*]pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinoline-10,13-dione]. Several different drug delivery systems have been developed for camptothecin analogs to reduce their severe systemic toxicities and to enhance their antitumor effects. DE-310 is designed to permit specific distribution to tumor tissue and intra-tumoral cleavage by lysosomal enzymes based on the EPR (enhanced permeability and retention) effect named by Maeda *et al.* (Matsumura and Maeda, 1986; Maeda *et al.*, 2000; Maeda, 2001). This enzymatic cleavage is supposed to provide a desired active product. From a pre-clinical study, it was suggested that cleavage of this linkage releases free DX-8951 and Glycyl-DX-8951 (G-DX-8951) (Fig. 2) into the blood stream (Inoue *et al.*, 2003). To study the pharmacokinetics of DE-310, it would be of great help to assay the polymer-bonded DX-8951 (conjugated DX-8951) and its released drugs (DX-8951 and G-DX-8951) in tumor tissue.

Several analytical methods have been developed for studying polymer-bounded anticancer drugs. Previous work on a polymer-bounded camptothecin derivative (MAG-CPT) has demonstrated that the total levels of CPT (free CPT plus polymer-bounded CPT) are determined after alkaline-mediated hydrolysis of MAG-CPT (Fraier *et al.*, 2000; Schoemaker *et al.*, 2001). The lower limits of quantitation (LLOQs) of this method were 100 ng/mL of total and 1 ng/mL of free CPT for 50 and 250 μL of human plasma. A sensitive HPLC method for a different water-soluble polymer-bounded compound, doxorubicin (PK1), has also been developed (Fraier *et al.*, 1995). Total (polymer-bound plus free) doxorubicin was determined after acid hydrolysis to release doxorubicinone as aglycones from free or polymer-bounded doxorubicin. The LLOQs of this method were 5.1 ng/mL of bounded doxorubicin

*Correspondence to: T. Oguma, Drug Metabolism and Physicochemical Property Research Laboratory, Daiichi Pharmaceutical Co. Ltd, 1-16-13 Kitakasai, Edogawa-ku, Tokyo 134-8630, Japan.
E-mail: ogumab0g@daiichipharm.co.jp

Abbreviations used: APCI, atmospheric pressure chemical ionization; EPR, enhanced permeability and retention; MRM, multiple reaction monitoring; MTD, maximum tolerated dose; QC, quality control.

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