



Review

Antibody–drug conjugates: Basic concepts, examples and future perspectives

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ARTICLE INFO

Article history:

Received 30 October 2011

Accepted 19 January 2012

Available online 28 January 2012

Keywords:

Antibody–drug conjugates

Non-internalizing monoclonal antibodies

Traceless linkers

ABSTRACT

Conventional anticancer therapeutics often suffer from lack of specificity, resulting in poor therapeutic indexes and substantial toxicities to normal healthy tissues. Monoclonal antibodies have demonstrated considerable utility in cancer medicine, but their curative potential is often limited. Antibody–drug conjugates represent an innovative therapeutic approach that combines the desirable properties of monoclonal antibodies, with the cell killing activity of cytotoxic drugs, reducing systemic toxicity and increasing the therapeutic benefit for patients.

In this review, we outline prominent examples of early and recent antibody–drug conjugates, discussing drugs, linker chemistries and classes of targets for product development.

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1. Antibody–drug conjugates (ADCs): general considerations and historical perspectives

Monoclonal antibodies (mAbs) have led to the development of approved biopharmaceutical products in many therapeutic areas, including cardiovascular diseases, infection, immune disorders and cancer [1]. At present, the last two therapeutic fields dominate both in terms of sales and number of antibody products in industrial development. However, with few exceptions, the curative potential of “naked” immunoglobulins is limited and it is not surprising that large research efforts are being devoted to the “arming” of monoclonal antibodies with bioactive payloads (e.g., drugs, cytokines, radionuclides) [2].

Cytotoxic agents often display little selectivity and substantial toxicity to normal tissues. Considerable efforts have been invested for combining the desirable properties of monoclonal antibodies with the cell killing activity of cytotoxic drugs, reducing systemic toxicity and increasing the therapeutic benefit for patients [2–5].

Although the concept of antibody–drug conjugates (ADCs) appears simple, the development of efficacious products often represents a considerable challenge. Many parameters have to be optimized for a successful ADC product, some of which are specific to individual tumor types. These include ADC stability (the drug should not be released before the ADC reaches its target *in vivo*), pharmacokinetics (the drug should not confer an undesired accumulation in a non-target organ), retention of immunoreactivity, and efficient release of the active form of the drug at site of disease.

Early work on ADCs relied on clinically approved chemotherapeutic drugs with the aim to increase tumor specificity. Initially this strategy took advantage of readily available drugs with known toxicity profiles. However, many other parameters related to ADC development, such as the choice of mAb, drug loading and potency, as well as release mechanisms were not immediately considered as crucial determinants, for the success of ADC strategies.

A prominent example of this first-generation ADCs is represented by BR96–doxorubicin [6]. This immunoconjugate consists of a chimeric mAb directed against the Lewis^Y tetra-saccharide commonly expressed on human carcinomas, modified with eight molecules of doxorubicin conjugated to the hinge cysteines. Doxorubicin is a DNA intercalator

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approved for the treatment of a broad range of tumors [7]. Drug release was engineered through an acid-labile hydrazone linker. Upon binding of the ADC to the cell surface antigens, the conjugate would be internalized inside cells, and doxorubicin released within the acidic endosomal and lysosomal vesicles. Treatment of tumor-bearing mice and rats with BR96-doxorubicin led to cures in tumors, which displayed the cognate antigen. Doses in excess of 100 mg/kg were used, reflecting the low potency of the targeted drug [6]. A phase I clinical trial confirmed the ability of the immunoconjugate to deliver doxorubicin to the tumor cells. The maximum tolerated dose (MTD) of BR96-doxorubicin was found to be approximately 700 mg/m². Despite higher total serum levels of doxorubicin (i.e. free doxorubicin plus doxorubicin bound to the antibody) were achieved compared to an intravenous administration of the unmodified drug, dose-limiting toxicities were not the same as conventional doxorubicin-related adverse events. Indeed, serum levels of free doxorubicin were below the detection limit in all patients assessed [8]. A subsequent randomized phase II trial on a population with confirmed sensitivity to doxorubicin revealed that the toxicities might have been of gastrointestinal origin, due to normal gut expression of Lewis^y [9]. In both trials little antitumor activity was observed. Therefore, the normal tissue cross-reactivity of BR96 and the low molar potency of doxorubicin represented clear limitations for the industrial development of BR96-doxorubicin. In addition, the discrepancy between hydrazone stability ($t_{1/2} \approx 43\text{h}$) and pharmacokinetic terminal half-life of the BR96 antibody (12 days, roughly 7 fold longer than the hydrazone half-life) represented a cause of concern. The development of doxorubicin-based ADC may still continue in the future, since a second ADC loaded with the same drug successfully cured SCID mice at single low doses of immunoconjugate in non-Hodgkin lymphoma tumor model [10]. Moreover novel classes of ADCs produced with hydrazone technology are currently in advanced stages of clinical trials for non-Hodgkin lymphoma [11], confirming that the combination antibody–drug–linker has to be properly investigated on a case-to-case basis.

Today ADCs are developed starting from mAbs that selectively bind to tumors and display little cross-reactivity with healthy tissues. Moreover, the appropriate mAb format may have to be selected in order to tune ADC tumor uptake and blood clearance properties with linker stability and drug release, thus minimizing the exposure of healthy organs to toxic agents [12]. Since the total amount of ADC which can be realistically administered to patients is limited by cost-of-goods, and since coupling of too many drug molecules may lead to a loss of potency [13], (i.e., compromised binding ability and pharmacokinetic properties) more effective drugs became necessary for the industrial development of next-generation ADC. The requirement for potent bioactive molecules is also a consequence of the inefficient localization of mAbs at the tumor site. Typically, a maximum of 0.08% injected dose per gram of tumor accumulates in human malignancies. This is due to the increased interstitial fluid pressure (IFP) in the tumor environment, which represents a barrier to the extravasation and diffusion of antibodies [14]. The use of different signal transduction antagonists (e.g. PGE₁, VEGF and PGDF, TGF β) or of pro-inflammatory cytokines that lower tumor IFP has, in some cases led to an improved anticancer activity [15].

Recognizing the value of drug choice in ADC development, several programs were started in parallel in the late 1990s, with the aim to identify more potent drugs for ADC application. Here it is worth mentioning calicheamicins, [16] maytansinoids, [3] auristatins, [2] and CC1065 analogs [17], since these classes of potent cytotoxics represent the “backbone” of current industrial development programs.

Finally, the choice of cleavable linkers is crucial for the performance of ADC strategies. Depending on the drug delivery mechanism selected, the active form of the cytotoxic agent may be released more or less efficiently inside the target cell.

2. Internalizing vs. non-internalizing ADCs

Targeted antitumor therapy takes advantage of specific molecular differences between healthy and diseased tissues. So far, most efforts have been devoted to the targeting of tumor-associated antigens expressed on the surface of cancer cells [4] (Fig. 1, right) (Table 1). This class of ADC products typically requires mAbs that are internalized inside cells by receptor-mediated endocytosis. Different chemical mechanisms may allow the subsequent drug release, as described in the next section. The use of internalizing ADC is at the basis of products that are either approved or in advanced stages of clinical trials. However, targeting antigens on tumor cells is a complex task, due to a number of physical and kinetic barriers associated with large solid tumors, and to the potential down-regulation of tumor-associated antigens targeted by the immunoconjugate, as each cell has to express the target antigen in order to be reached by the cytotoxic agent.

An alternative approach has been developed that targets markers expressed on tumor neo-vasculature (Fig. 1, left). This strategy is compatible with the use of non-internalizing antibodies [18,19]. Vascular targets are more easily accessible from the blood stream compared to systemically administered agents. Tumors require blood supply for their growth and neoplastic capillaries are significantly different at the molecular level from normal healthy vasculature. Remarkably, since angiogenesis is a common feature of all malignancies and tumors express common markers of angiogenesis, a single agent should in principle be applicable to a wide range of tumor entities [18,19]. In particular, markers of angiogenesis, found in the subendothelial extracellular matrix (ECM), are more stable compared to cellular antigens and facilitate an antibody residence time of days/weeks. The potential of targeting antigens expressed on tumor neo-vasculature has been confirmed in pre-clinical and clinical applications [20].

Endothelial cells (EC) are readily accessible from the blood stream. Anti-VEGF mAbs have been shown to have the potential to localize at tumor sites *in vivo* [21]. However, this strategy is hardly compatible with non-internalizing antibodies, as the released drug would enter the bloodstream. Alternatively, subendothelial modified ECM components may serve as target for ADCs, which do not require internalization for their activity. Upon extravasation, a specific mAb product comes into contact with more abundant and stable antigens, thus ensuring a long residence time of ADC at the tumor site. Work in this area has been performed with the human mAb (L19), specific to the extra-domain B (EDB) of fibronectin, a marker of angiogenesis. EDB is normally not present in the healthy protein, but is inserted during active tissue remodeling by alternative splicing, and is highly abundant in many aggressive solid tumors. L19 fused to different effector molecules is currently in multiple stages of clinical development [22]. In addition, we have recently started the use of oncofetal fibronectin as a target for ADC development [23].

3. Drug release strategies for ADCs

Specific linkers are designed to append toxic payloads to mAbs and to allow a selective drug release at the tumor site. Linkers differ in terms of plasma stability and in the mechanism of release. Acid-labile hydrazone linkers have been employed in the first class of ADCs developed (Table 2, entries 1, 4) [6,31–33]. Hydrazones are preferentially cleaved within intracellular lysosomes and endosomes, as a consequence of the lower pH found in these environments (pH $\approx$ 5).

Disulfides (Table 2, entry 2) have been extensively exploited as ADC linkers since they are stable at physiological pH and release their payload upon internalization inside cells, where the cytosol provides a significantly more reducing environment compared to the extracellular milieu [34]. Several *in vitro* and *in vivo* studies have been performed to establish the effect of increasing substitution at disulfides in the pharmacokinetic and toxicity profiles of different ADCs [3,25]. Two classes of calicheamicin–antibody conjugates containing

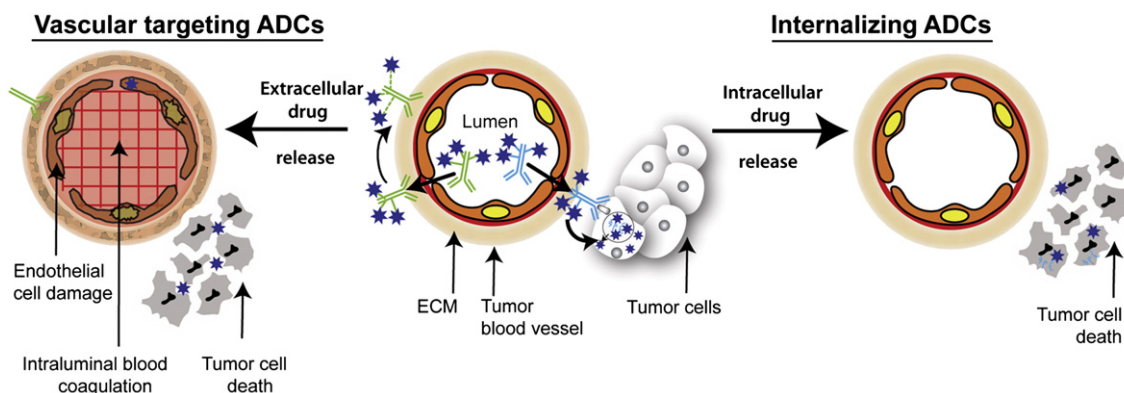


Fig. 1. Antibody–drug conjugates (ADC) and their effect. (Right) Tumor cell death induced by internalizing ADCs (blue). Upon ADC internalization inside tumor cells, the active drug is released and leads to tumor cell death. (Left) Effect of tumor targeting ADCs specific to markers of angiogenesis expressed on the endothelial extracellular matrix (green). The long residence time of vascular targeting ADCs allows the localized drug release, leading to intravascular blood coagulation, and to an avalanche of tumor cell death.

both disulfide linkers were tested in different tumor models. An additional hydrolyzable hydrazone group was present in a subgroup of the ADCs, and allowed direct comparison between the two linkers.

In an ADC based on the CTM01 antibody (where CTM01 is a murine IgG1 that recognizes MUC1 antigen present on a broad spectrum of solid tumors), the additional hydrazone was not necessary for activity [35]. Surprisingly, when the Anti-CD33 P67.6 antibody was used for ADC development, the disulfide moiety in the linker was insufficient for the proper release of calicheamicin, while the hydrazone moiety was essential [33].

Alternative linker strategies rely on “non-cleavable” linkers and take advantage of the catabolic degradation of internalized antibodies in the lysosomal and endosomal compartments. ADCs that consist of either peptide bonds, or of chemical bonds normally stable under physiologic conditions, rely in the statistical proteolytic degradation of the conjugate [25,36] (Table 2, entries 3, 6). In a series of disulfide-linked ADCs for the intracellular release of cytotoxic drugs, it was surprisingly found that a Trastuzumab-DM1 control conjugate in which the disulfide was replaced with a non-cleavable thioether bond, was the most effective ADC variant in *in vivo* therapy tests [25]. Thus, while cleavable disulfide linkers are often associated with ADC activity, the precise choice of the most active linker chemistry requires the screening of several candidates, including non-cleavable linkers which may release drug only after a proteolytic digestion of the antibody molecule [37].

Alternatively protease-specific sequences introduced in the linker control the hydrolytic process, as reported for the lysosome abundant protease cathepsin B [24,38] (Table 2, entry 5). This approach was confirmed to be superior to the hydrolytically labile hydrazone linker both in terms of specificity and toxicity in a series of monomethyl-austatins (MMAE) conjugated to cBR96 mAb [24].

Most ADC manufacturing processes exploit the chemical modification of the primary amino group of lysine residues or of the thiol group

of cysteines. The different abundance of the two residues is reflected also in the complexity of the heterogeneous mixture generated [39]. When the release technology relies on statistical proteolysis, traces of the modified amino acids might be found in the active released form of the drug (Table 2, entries 3, 6).

In the examples described so far, bifunctional linkers feature one moiety for conjugation of the drug to the mAb, and one moiety for drug release.

Traceless linkers eliminate this distinction since coupling and release chemistry are identical (Table 2). We have recently described an innovative linker technology that relies on the site-specific coupling and release of cytotoxic drugs from thiazolidines. The linker, generated upon reaction of aldehydes with 1,2-aminothiol moieties on the antibody, is stable for synthesis, and allows the hydrolytic drug release under physiological conditions. The release process is traceless as it restores the free drug, and the intact antibody (Table 3, entry 1) [40]. Alternatively cysteines introduced in mAb sequences by site directed mutagenesis afford site-specific disulfide linkages with thiol-drugs (Table 3, entry 2) [23].

Disulfides based linkers may find ADC applications also with non-internalizing antibodies. Indeed, the localization of these ADCs at the tumor ECM could expose the disulfide linkage to reducing agents (cysteine, glutathione) initially released by dying cells and progressively amplified by drug-mediated cell killing.

Remarkably, in these two last examples, the release process restores the original antibody and the active drug, which simplifies drug manufacturing and should diminish ADC immunogenicity.

4. Representative ADCs at different stages of clinical development

Mylotarg was the first ADC ever approved in the US. The product had an indication for the treatment of acute myeloid leukemia (AML) in patients older than 60 not eligible for other chemotherapies. The ADC consisted of a humanized anti-CD33 mAb (hP67.6) attached to the calicheamicin drug through an acid labile hydrazone linker [41,42] (Fig. 2A).

Calicheamicin derives from the family of enediyne antibiotics extracted from the soil microorganism *Micromonospora echinospora*, and binds in the minor groove of DNA. The highly reactive 1,4-benzenoyl diradical generated by intracellular reduction, caused extensive DNA damage and subsequent cell apoptosis [43,16]. The acid labile hydrazone linker [33] held the two components together and supposedly was cleaved before the sterically hindered disulfide, and thioester moieties. Mylotarg was administered as a 1:1 mixture of unconjugated and conjugated mAb to 4–6 equivalents of drug, with an average drug loading of 2–3 moles of drug to mAb. The drug was approved under FDA's accelerated approval program based on a response rate

Table 1

Selected examples of intracellular, ECM and vascular targets used in ADC development.

	Antigen	Reference
Tumor cells	CD30	[24]
	Can Ag	[3]
	HER2	[25]
	CD22	[26]
ECM	Fibronectin(EDA:EDB)	[27,28]
	Tenascin-C(A1)	[29]
	Periostin	[30]
	Integrins	[19]
EC	PSMA	[19]
	VEGFR-2	[19]

Table 2

Representative examples of ADC chemistries. Current ADC assembly strategies rely on bifunctional linkers. Specific release processes are responsible for active drug release. Selective scissile bonds are highlighted in yellow. The postulated active form of the released drug is depicted in red. $R_1 = \text{H, Me}$.

	Antibody	Bifunctional linkers		Release process			Ref
		mAb side	Drug side	ADC	Residual linkers	Drug release	
1							hydrolysis [33]
2							reduction [3, 25]
3							statistical proteolysis [25]
4							hydrolysis [6]
5							site-specific proteolysis [26, 38]
6							statistical proteolysis [36]

of about 30% [44]. However, it failed to confirm therapeutic benefits (i.e., higher response rates) to AML patients in clinical trials, when administered in combination with chemotherapy. Indeed a greater number of deaths occurred in the group of patients receiving Mylotarg, compared to those receiving chemotherapy. Potentially, the veno-occlusive disease observed at initial approval, worsened in the post marketing setting, causing fatalities to increase. The product was voluntarily withdrawn in June 2010 (<http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm216448.htm>).

Trastuzumab-DM1 (T-DM1) is an ADC currently in advanced stage clinical trials designed for the treatment of Her2-positive

metastatic breast cancer. T-DM1 consists of the approved anti-Her2-mAb Trastuzumab (Herceptin) directly conjugated to DM1, via a non-cleavable thioether linker present in an N-maleimido cyclohexane-1-carboxylate group (also named MCC) (Fig. 2B). Herceptin has been introduced in the market in 1998 for Her2 + breast cancer, and showed clinical benefits in combination with chemotherapy as first line or adjuvant therapy [45,46]. Despite these objective results, part of the patients treated either did not respond initially, or relapsed [47]. DM1 is a maytansinoid derivative, binds directly to microtubules and inhibits their assembly with consequent apoptosis, similar to what was observed for the Vinca alkaloids [48,49]. T-DM1

Table 3

Recent examples of linkerless ADC assembly. The cleavage process restores the unmodified antibody and the drug used for ADC development. Scissile bonds are highlighted in yellow. The postulated active form of the released drug is depicted in red.

	Antibody	Traceless linkers	Release process			Ref
			ADC	Residual linkers	Drug release	
1						hydrolysis [32]
2						reduction [22]

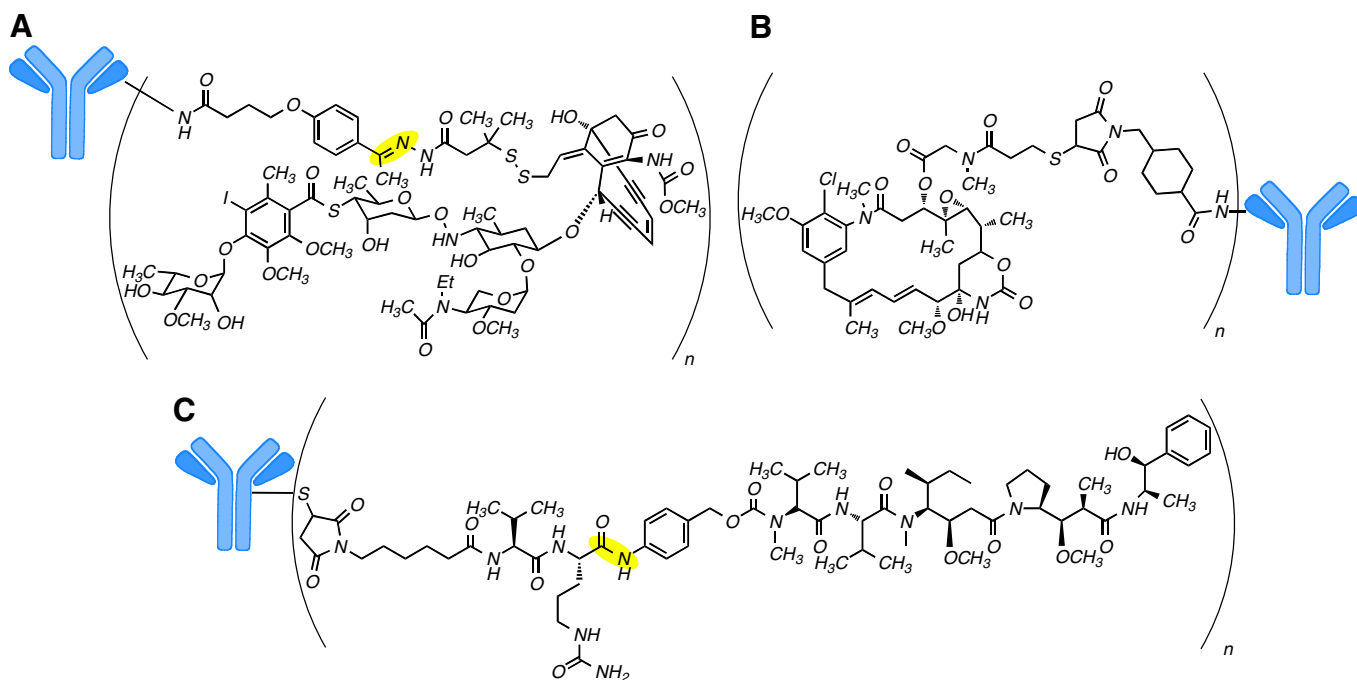


Fig. 2. Structures of selected ADCs used in cancer therapy. Mylotarg (A), T-DM1 (B), ADCETRIS™ (C). Scissile bonds are highlighted in yellow.

undergoes cell internalization and complete lysosomal proteolysis [25] and, in analogy to what has been reported for huC242-MCC-DM1 [50], (huC242 is a humanized antibody with high affinity for the carbohydrate antigen CanAg) the major active drug metabolite is believed to be the weakly membrane permeable Lys-MCC-DM1 (Table 2, entry 3).

T-DM1 carries an average of 3.5 molecules of DM1/mAb and is now used in clinical trials on heavily pre-treated patients, including Trastuzumab. A phase I dose escalation study showed partial responses in 25% of the patients when treated with doses starting from 0.3 mg/kg and going up to 4.8 mg/kg [51]. More remarkably in a phase II trial, third-line metastatic breast cancer showed a 33% objective response rate in 110 patients treated with 3.6 mg/kg T-DM1 every 3 weeks [52]. Moreover T-DM1 was well tolerated by patients with no cardiovascular toxicities. For these impressive results, approval of T-DM1 is expected in 2012, even though the FDA has refused a Biologics License Application submitted in July 2010 for accelerated approval.

In July 2011 brentuximab vedotin (SGN-35, Adcetris) was approved by the FDA for the treatment of patients with Hodgkin lymphoma after failure of autologous stem cell transplant, and for patients with systemic anaplastic large cell lymphoma after failure of at least one prior multi-agent chemotherapy regimen (<http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm268781.htm>). In SGN-35 an anti-CD30 monoclonal antibody is attached through a cathepsin-B cleavable valine-citrulline dipeptide (Val-Cit) to an average of 4 monomethyl auristatin E (MMAE) molecules (Fig. 2C). MMAE drugs are structurally related to dolastatin 10 and afford a potent antitumor activity by inhibiting tubulin polymerization [2,53]. The conjugation occurs between reduced anti-CD30 mAb and maleimido containing MMAE to form 4 thioether linkers. The ADC generated is highly homogenous and stable (half life 6 and 9.6 days in mice and monkeys) [24]. Upon binding to CD30 on the cell surface, SGN-35 is internalized, and digested inside lysosomes. Drug release begins with the selective proteolysis of the Val-Cit linker catalyzed by cathepsin-B. Following proteolytic cleavage, 1,6-elimination of the p-aminobenzyl ester group affords the unmodified drug (Table 2, entry 5) [38,54,55]. A phase I clinical trial showed tumor regressions in about 93% of treated patients, and 56% objective response rates [56,57]. A single arm phase II study for Hodgkin lymphoma on

102 patients treated with SGN-35 showed 75% objective responses and 34% complete responses [58,59]. The drug was approved under FDA's accelerated approval program, based on objective response rates (<http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm268781.htm>).

5. Concluding remarks

The area of ADC development is in rapid expansion, with large investments being made by pharmaceutical companies [60]. At present, maytansinoids and auristatins are the most frequently used drugs for ADC developments. Since ADC, at some stage, are cleared from circulation (either via the renal route or the hepatobiliary route) it is probable that future research efforts will closely evaluate therapeutic candidates not only on the basis of their tumor toxicity, but also in terms of the side-effects associated to the ADC excretion process. The complex pharmacokinetic profiles and the high cost of goods for ADC will be justified if the therapeutic performance is sufficiently good and if the disease is a severe one. In principle, advantages such as the drug release at site of disease should lead to a reduced systemic toxicity and to higher therapeutic indexes, compared to non-targeted drugs.

In the future, we envisage that novel linkers chemistry (including traceless linkers) and non-internalizing antibodies will expand the repertoire of tools for ADC development.

Competing interests statement

Giulio Casi is employed at Philochem AG.

Dario Neri is a co-founder and shareholder of Philogen SpA.

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