

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	)	MAIL STOP AMENDMENT
R. Yongxin ZHAO	)	
	)	Group Art Unit: 1643
Application No.: 14/432,073	)	
	)	Examiner: Canella, Karen A
Filed: March 27, 2015	)	
	)	Confirmation No.: 6938
For: HYDROPHILIC LINKERS AND	)	
THEIR USES FOR CONJUGATION	)	
OF DRUGS TO A CELL BINDING	)	
MOLECULES	)	

AMENDMENT UNDER 37 C.F.R. § 1.111

Commissioner for Patents
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Commissioner:

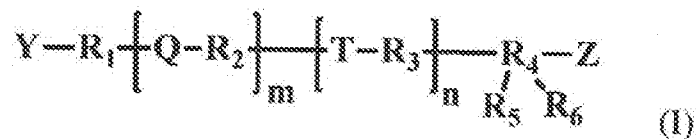
In response to the Official Action dated March 8, 2018, please amend the above identified application as follows.

AMENDMENTS TO THE CLAIMS:

This listing of claims will replace all prior versions, and listings, of claims in the application:

LISTING OF CLAIMS:

1. (Currently Amended) A hydrophilic linker of formula (I)



wherein:

Y represents a functional group that enables reaction of the hydrophilic linker with a cell-binding agent;

Q and T are either -P(=O)(OM)-, or -S(O₂)-, or -S(O)-;

m and n are integer from 0 to 5, but not 0 at the same time; provided that when m=1, n=0, Q is not -P(=O)(OM)-; ~~and when n=1, m=0, T is not -P(=O)(OM)-; and when Q or T is -S(O₂)-, m and n are not 0;~~

Z represents a functional group that enables linkage of the hydrophilic linker to a cytotoxic drug via a disulfide, thioether, thioester, hydrazone, ether, ester, carbamate, carbonate, secondary, tertiary, or quaternary amine, imine, cycloheteroalkyane, heteroaromatic, alkoxime or amide bond;

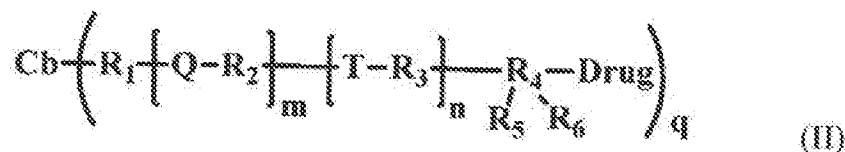
R₁, R₂, R₃, and R₄, are the same or different and are absent, linear alkyl having from 1-6 carbon atoms, branched or cyclic alkyl having from 3 to 6 carbon atoms, linear, branched or cyclic alkenyl or alkynyl, or ester, ether or amide having 2-6 carbon atoms, or polyethyleneoxy unit of formula (OCH₂CH₂)_p, wherein p is an integer from 1 to about 1000, or combination thereof,

or R₁, R₂, R₃ and R₄ are respectively a chain of two or more atoms selected from the group consisting of C, N, O, S, Si, and P that covalently connects a cell-surface binding ligand, a phosphinate or sulfonyl group, a conjugated drug and among themselves (R₁, R₂, R₃ and R₄);

R_5 and R_6 , are the same or different and are absent, H, linear alkyl having from 1-6 carbon atoms, branched or cyclic alkyl having from 3 to 6 carbon atoms, linear, branched or cyclic alkenyl or alkynyl, or ester, ether or amide having 2-6 carbon atoms, or polyethyleneoxy unit of formula $(OCH_2CH_2)_p$, wherein p is an integer from 1 to about 1000, or combination thereof;

M is H, or Na, or K, or $N^+R_1R_2R_3$ or a pharmaceutical salt.

2. (Currently Amended) A cell-binding agent-drug conjugate of formula (II):



wherein:

Cb represents a cell-binding agent;

Drug represents a drug linked to the cell-binding agent via the hydrophilic linker by a disulfide, thioether, thioester, peptide, hydrazone, ether, ester, carbamate, carbonate, cycloheteroalkane, heteroaromatic, alkoxy or amide bond;

Q and T are either $-P(=O)(OM)-$, or $-S(O_2)-$, or $-S(O)-$;

m and n are integer from 0 to 5, but not 0 at the same time; provided that when $m=1$, $n=0$, Q is not $-P(=O)(OM)-$; and when $n=1$, $m=0$, T is not $-P(=O)(OM)-$;

R_1 , R_2 , R_3 , and R_4 , are the same or different and are absent, linear alkyl having from 1-6 carbon atoms, branched or cyclic alkyl having from 3 to 6 carbon atoms, linear, branched or cyclic alkenyl or alkynyl, or ester, ether or amide having 2-6 carbon atoms, or polyethyleneoxy unit of formula $(OCH_2CH_2)_p$, wherein p is an integer from 1 to about 1000, or combination thereof,

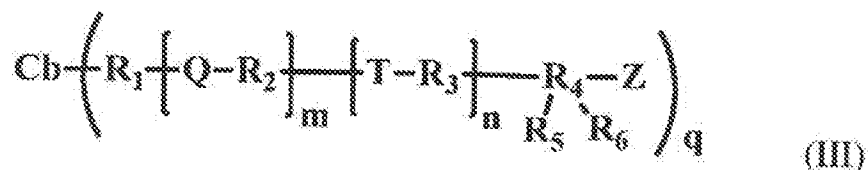
or R_1 , R_2 , R_3 and R_4 are respectively a chain of two or more atoms selected from the group consisting of C, N, O, S, Si, and P that covalently connects a cell-surface binding ligand, a phosphinate or sulfonyl group, a conjugated drug and among themselves (R_1 , R_2 , R_3 and R_4);

R_5 and R_6 , are the same or different and are absent, H, linear alkyl having from 1-6 carbon atoms, branched or cyclic alkyl having from 3 to 6 carbon atoms, linear, branched or

cyclic alkenyl or alkynyl, or ester, ether or amide having 2-6 carbon atoms, or polyethyleneoxy unit of formula $(\text{OCH}_2\text{CH}_2)_p$, wherein p is an integer from 1 to about 1000, or combination thereof;

M is H, or Na, or K, or $\text{N}^+\text{R}_1\text{R}_2\text{R}_3$ or a pharmaceutical salt.

3. (Currently Amended) A compound of formula (III):



wherein:

Cb represents a cell-binding agent;

Q and T are either $-\text{P}(=\text{O})(\text{OM})-$, or $-\text{S}(\text{O}_2)-$, or $-\text{S}(\text{O})-$;

m and n are integer from 0 to 5, but not 0 at the same time; provided that when $m=1$, $n=0$,

Q is not $-\text{P}(=\text{O})(\text{OM})-$; and when $n=1$, $m=0$, T is not $-\text{P}(=\text{O})(\text{OM})-$;

q is an integer from 1 to 20;

Z represents a functional group that enables linkage of the compound to a cytotoxic drug via a disulfide, thioether, thioester, hydrazone, ether, ester, carbamate, carbonate, secondary, tertiary, or quaternary amine, imine, cycloheteroalkane, heteroaromatic, alkoxime or amide bond;

R_1 , R_2 , R_3 , and R_4 , are the same or different and are absent, linear alkyl having from 1-6 carbon atoms, branched or cyclic alkyl having from 3 to 6 carbon atoms, linear, branched or cyclic alkenyl or alkynyl, or ester, ether or amide having 2-6 carbon atoms, or polyethyleneoxy unit of formula $(\text{OCH}_2\text{CH}_2)_p$, wherein p is an integer from 1 to about 1000, or combination thereof,

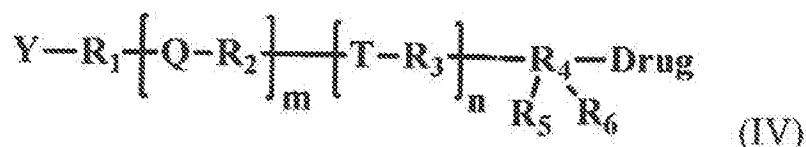
or R_1 , R_2 , R_3 and R_4 are respectively a chain of two or more atoms selected from the group consisting of C, N, O, S, Si, and P that covalently connects a cell-surface binding ligand, a phosphinate or sulfonyl group, a conjugated drug and among themselves (R_1 , R_2 , R_3 and R_4);

R_5 and R_6 , are the same or different and are absent, H, linear alkyl having from 1-6 carbon atoms, branched or cyclic alkyl having from 3 to 6 carbon atoms, linear, branched or

cyclic alkenyl or alkynyl, or ester, ether or amide having 2-6 carbon atoms, or polyethyleneoxy unit of formula $(\text{OCH}_2\text{CH}_2)_p$, wherein p is an integer from 1 to about 1000, or combination thereof;

M is H, or Na, or K, or $\text{N}^+\text{R}_1\text{R}_2\text{R}_3$ or a pharmaceutical salt.

4. (Currently Amended) A compound of formula (IV):



wherein:

Y represents a functional group that enables reaction of the compound with a cell-binding agent;

Q and T are either $-\text{P}(=\text{O})(\text{OM})-$, or $-\text{S}(\text{O}_2)-$, or $-\text{S}(\text{O})-$;

m and n are integer from 0 to 5, but not 0 at the same time; provided that when $m=1$, $n=0$, Q is not $-\text{P}(=\text{O})(\text{OM})-$; and when $n=1$, $m=0$, T is not $-\text{P}(=\text{O})(\text{OM})-$;

"Drug" represents a drug linked to the cell-binding agent via the hydrophilic linker by a disulfide, thioether, thioester, peptide, hydrazone, ether, ester, carbamate, carbonate, cycloheteroalkane, heteroaromatic, alkoxy or amide bond;

R_1 , R_2 , R_3 , and R_4 , are the same or different and are absent, linear alkyl having from 1-6 carbon atoms, branched or cyclic alkyl having from 3 to 6 carbon atoms, linear, branched or cyclic alkenyl or alkynyl, or ester, ether or amide having 2-6 carbon atoms, or polyethyleneoxy unit of formula $(\text{OCH}_2\text{CH}_2)_p$, wherein p is an integer from 1 to about 1000, or combination thereof,

or R_1 , R_2 , R_3 and R_4 are respectively a chain of two or more atoms selected from the group consisting of C, N, O, S, Si, and P that covalently connects a cell-surface binding ligand, a phosphinate or sulfonyl group, a conjugated drug and among themselves (R_1 , R_2 , R_3 and R_4);

R_5 and R_6 , are the same or different and are absent, H, linear alkyl having from 1-6 carbon atoms, branched or cyclic alkyl having from 3 to 6 carbon atoms, linear, branched or cyclic alkenyl or alkynyl, or ester, ether or amide having 2-6 carbon atoms, or polyethyleneoxy

unit of formula $(\text{OCH}_2\text{CH}_2)_p$, wherein p is an integer from 1 to about 1000, or combination thereof;

M is H, or Na, or K, or $\text{N}^+ \text{R}_1\text{R}_2\text{R}_3$ or a pharmaceutical salt.

5. (Currently Amended) The conjugate of claim 2, wherein Drug is selected from the group consisting of:

1). therapeutic agents comprising a). Alkylating agents comprising Nitrogen mustards comprising chlorambucil, chlornaphazine, cyclophosphamide, dacarbazine, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, mannomustine, mitobronitol, melphalan, mitolactol, pipobroman, novembichin, phenesterine, prednimustine, thiotepa, trofosfamide, uracil mustard; CC-1065 and adozelesin, carzelesin and bizelesin; duocarmycin, KW-2189 and CBI-TMI; benzodiazepine dimers comprising dimers of pyrrolbenzodiazepine (PBD)-or tomaymycin, indolinobenzodiazepines, imidazobenzothiadiazepines, or oxazolidinobenzodiazepines; Nitrosoureas comprising carmustine, lomustine, chlorozotocin, fotemustine, nimustine, ranimustine; Alkylsulphonates comprising busulfan, treosulfan, improsulfan and piposulfan; Triazenes; {dacarbazine}; Platinum containing compounds comprising carboplatin, cisplatin, oxaliplatin; aziridines comprising benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramidate, triethylenethiophosphoramidate and trimethylolomelamine; b). Plant Alkaloids comprising Vinca alkaloids comprising vincristine, vinblastine, vindesine, vinorelbine, navelbin; Taxoids comprising paclitaxel, and docetaxol, Maytansinoids comprising DM1, DM2, DM3, DM4, DMS, DM6, DM7, maytansine and ansamitocins, cryptophycins comprising cryptophycin 1 and cryptophycin 8; epothilones, eleutherobin, discodermolide, bryostatins, dolostatins, auristatins, tubulysins, cephalostatins; pancratistatin; a sarcodictyin; spongistatin; c). DNA Topoisomerase Inhibitors comprising Epipodophyllins comprising 9-aminocamptothecin, camptothecin, crisnatol, daunomycin, etoposide, etoposide phosphate, irinotecan, mitoxantrone, novantrone, retinoic acids, retinols, teniposide, topotecan, 9-nitrocamptothecin (RFS-2000); mitomycins; d). Anti-metabolites comprising Anti-folate comprising DHFR inhibitors comprising methotrexate,

trimetrexate, denopterin, pteropterin, and aminopterin (~~4-aminopteroic acid~~); IMP dehydrogenase Inhibitors comprising mycophenolic acid, tiazofurin, and ribavirin, EICAR; Ribonucleotide reductase Inhibitors comprising hydroxyurea and deferoxamine; Pyrimidine compounds comprising uracil compounds comprising ancitabine, azacitidine, 6-azauridine, capecitabine (~~Xeloda~~), capecitabine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, 5-Fluorouracil, floxuridine, and ratitrexed (~~Tomodex~~); Cytosine compounds comprising cytarabine, cytosine arabinoside, and fludarabine; Purine compounds comprising azathioprine, fludarabine, mercaptopurine, thiamiprine, and thioguanine; folic acid replenisher; e). Hormonal therapies comprising Receptor antagonists comprising anti-estrogen selected from the group consisting of megestrol, raloxifene, and tamoxifen; LHRH agonists comprising goserelin, and leuprolide acetate; anti-androgens comprising bicalutamide, flutamide, calusterone, dromostanolone propionate, epitio stanol, goserelin, leuprolide, mepitiostane, nilutamide, testolactone, and trilostane; Vitamin D3 compounds comprising CB 1093, EB 1089, KH 1060, cholecalciferol, and ergocalciferol; photodynamic therapies comprising verteporfin, phthalocyanine, photo sensitizer Pc4, and demethoxy-hypocrellin A; cytokines comprising Interferon-alpha, Interferon-gamma, tumor necrosis factor (~~TNFs~~), and human proteins containing a TNF domain; f). Kinase inhibitors comprising anti-EGFR/Erb2, imatinib, gefitinib, pegaptanib, sorafenib, dasatinib, sunitinib, erlotinib, nilotinib, lapatinib, axitinib, pazopanib, vandetanib, anti-VEGFR2, mubritinib, ponatinib (~~AP24534~~), bafetinib (~~INNO-406~~), bosutinib (~~SKI-606~~), cabozantinib, vismodegib, iniparib, ruxolitinib, CYT387, axitinib, tivozanib, sorafenib, bevacizumab, cetuximab, Trastuzumab, Ranibizumab, Panitumumab, and ispinesib; g). antibiotics comprising enediyne antibiotics comprising calicheamicins comprising calicheamicin γ 1, δ 1, α 1 and β 1; dynemicin selected from the group consisting of dynemicin A and deoxydynemicin; esperamicin, kedarcidin, C-1027, maduropeptin, neocarzinostatin chromophore and chromoprotein enediyne antibiotic chromomophores, aclacinomysins, actinomycins, anthramycin, azaserine, bleomycins, cactinomycin, carabacin, carminomycin, carzinophilin; chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin,

nitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; h). polyketides comprising bullatacin and bullatacinone; gemcitabine, epoxomicins, bortezomib, thalidomide, lenalidomide, pomalidomide, tosedostat, ~~zybrestat~~ ZYBRESTAT, PLX4032, STA-9090, ~~StimuVax~~ STIMUVAX, ~~allovectin-7~~ ALLOVECTIN-7, ~~Xegeva~~ XGEVA, ~~Provenge~~ PROVENGE, ~~Yervoy~~ YERVOY, Isoprenylation inhibitors, Dopaminergic neurotoxins, Cell cycle inhibitors, Actinomycin D, bleomycin A2, bleomycin B2, and peplomycin, Anthracyclines comprising daunorubicin, adriamycin, idarubicin, epirubicin, pirarubicin, zorubicin, and mitoxantrone, MDR inhibitors, Ca²⁺ ATPase inhibitors, Histone deacetylase inhibitors comprising Vorinostat, ~~Romidepsin~~ ROMIDEPSIN, Panobinostat, Valproic acid, Mocetinostat (~~MGCD0103~~), Belinostat, PCI-24781, Entinostat, SB939, Resminostat, Givinostat, AR-42, CUDC-101, sulforaphane, and Trichostatin A; Celecoxib, glitazones, epigallocatechin gallate, Disulfiram, Salinosporamide A.; Anti-adrenals comprising aminoglutethimide, mitotane, and trilostane; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; arabinoside, bestabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; eflornithine (~~DFMO~~), elfomithine; elliptinium acetate, etoglucid; gallium nitrate; gacytosine, ibandronate, lentinan; Ionidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2''-trichlorotriethylamine; trichothecenes comprising T-2 toxin, verrucarins A, roridin A and anguidine; urethane, siRNA, antisense drugs;

2). anti-autoimmune disease agents comprising cyclosporine, cyclosporine A, aminocaproic acid, azathioprine, bromocriptine, chlorambucil, chloroquine, cyclophosphamide, corticosteroids comprising amcinonide, betamethasone, budesonide, hydrocortisone, flunisolide, fluticasone propionate, fluocortolone danazol, dexamethasone, Triamcinolone acetonide, and beclomethasone dipropionate, DHEA, enanercept, hydroxychloroquine, infliximab, meloxicam, methotrexate, mofetil, mycophenylate, prednisone, sirolimus, tacrolimus;

3). anti-infectious disease agents comprising a). Aminoglycosides comprising amikacin, astromycin, gentamicin (comprising netilmicin, sisomicin, and isepamicin), hygromycin B,

kanamycin (comprising amikacin, arbekacin, bekanamycin, dibekacin, and tobramycin), neomycin (comprising framycetin, paromomycin, and ribostamycin), netilmicin, spectinomycin, streptomycin, and verdamicin; b). Amphenicols comprising azidamfenicol, chloramphenicol, florfenicol, and thiamphenicol; c). Ansamycins comprising geldanamycin, and herbimycin; d). Carbapenems comprising biapenem, doripenem, ertapenem, imipenem/cilastatin, meropenem, and panipenem; e). Cephems comprising carbacephem (~~loracarbef~~), cefacetrile, cefaclor, cefradine, cefadroxil, cefalonium, cefaloridine, cefalotin or cefalothin, cefalexin, cefaloglycin, cefamandole, cefapirin, cefatrizine, cefazaflur, cefazedone, cefazolin, cefbuperazone, cefcapene, cefdaloxime, cefepime, cefminox, cefoxitin, cefprozil, cefroxadine, ceftezole, cefuroxime, cefixime, cefdinir, cefditoren, cefepime, cefetamet, cefmenoxime, cefodizime, cefonicid, cefoperazone, ceforanide, cefotaxime, cefotiam, ceftazopran, cephalixin, cefpimizole, cefpiramide, cefpirome, cefpodoxime, cefprozil, cefquinome, cefsulodin, ceftazidime, cefteteram, ceftibuten, ceftiolene, ceftizoxime, ceftobiprole, ceftriaxone, cefuroxime, cefuzonam, cephamycin (comprising cefoxitin, cefotetan, and cefmetazole), and oxacephem (comprising flomoxef, and latamoxef); f). Glycopeptides comprising bleomycin, vancomycin (~~oritavanein, telavanein~~), teicoplanin (~~dalbavanein~~), and ramoplanin; g). Glycylcyclines; h). β -Lactamase inhibitors comprising penam (~~sulbactam, tazobactam~~), and clavam (~~clavulanic acid~~); i). Lincosamides comprising clindamycin, and lincomycin; j). Lipopeptides comprising daptomycin, A54145, and calcium-dependent antibiotics (~~CDA~~); k). Macrolides comprising azithromycin, cethromycin, clarithromycin, dirithromycin, erythromycin, flurithromycin, josamycin, ketolide (~~telithromycin, cethromycin~~), midecamycin, miocamycin, oleandomycin, rifamycins (~~rifampicin, rifampin, rifabutin, rifapentine~~), rokitamycin, roxithromycin, spiramycin, troleandomycin, and telithromycin; l). Monobactams comprising aztreonam, and tigemonam; m). Oxazolidinones; n). Penicillins comprising amoxicillin, ampicillin (~~pivampicillin, bacampicillin, bacampicillin, metampicillin, talampicillin~~), azidocillin, azlocillin, benzylpenicillin, benzathine benzylpenicillin, benzathine phenoxymethylpenicillin, clometocillin, procaine benzylpenicillin, carbenicillin (~~carindacillin~~), cloxacillin, dicloxacillin, epicillin, flucloxacillin, mecillinam (~~pivmecillinam~~), mezlocillin, meticillin, nafcillin, oxacillin, penamecillin, penicillin, pheneticillin, phenoxymethylpenicillin, piperacillin, propicillin, sulbenicillin, temocillin, and

ticarcillin; o). Polypeptides comprising bacitracin, colistin, and polymyxin B; p). Quinolones comprising alatrofloxacin, balofloxacin, ciprofloxacin, clinafloxacin, danofloxacin, difloxacin, enoxacin, enrofloxacin, floxin, garenoxacin, gatifloxacin, gemifloxacin, grepafloxacin, kano trovafloxacin, levofloxacin, lomefloxacin, marbofloxacin, moxifloxacin, nadifloxacin, norfloxacin, orbifloxacin, ofloxacin, pefloxacin, trovafloxacin, grepafloxacin, sitafloxacin, sparfloxacin, temafloxacin, tosufloxacin, and trovafloxacin; q). Streptogramins comprising pristinamycin, and quinupristin/dalfopristin; r). Sulfonamides comprising mafenide, prontosil, sulfacetamide, sulfamethizole, sulfanilimide, sulfasalazine, sulfisoxazole, trimethoprim, and trimethoprim-sulfamethoxazole (~~co-trimoxazole~~); s). Steroid antibacterials; t). Tetracyclines comprising doxycycline, chlortetracycline, clomocycline, demeclocycline, lymecycline, meclocycline, metacycline, minocycline, oxytetracycline, penimepicycline, rolitetracycline, and tetracycline; u). antibiotics comprising annonacin, arspenamine, bactoprenol inhibitors, DADAL/AR inhibitors, dictyostatin, discodermolide, eleutherobin, epothilone, ethambutol, etoposide, faropenem, fusidic acid, furazolidone, isoniazid, laulimalide, metronidazole, mupirocin, mycolactone, NAM synthesis inhibitors, nitrofurantoin, paclitaxel, platensimycin, pyrazinamide, quinupristin/dalfopristin, rifampin, tazobactam tinidazole, and uvaricin;

4). anti-viral drugs comprising a). Entry/fusion inhibitors comprising aplaviroc, maraviroc, vicriviroc, enfuvirtide, PRO 140, and ibalizumab; b). Integrase inhibitors comprising raltegravir, elvitegravir, and globoidnan A; c). Maturation inhibitors comprising bevirimat, and vivecon; d). Neuraminidase inhibitors comprising oseltamivir, zanamivir, and peramivir; e). Nucleosides & nucleotides comprising abacavir, aciclovir, adefovir, amdoxovir, apricitabine, brivudine, cidofovir, clevidine, dexelvucitabine, didanosine (~~ddI~~), elvucitabine, emtricitabine (~~FTC~~), entecavir, famciclovir, fluorouracil (~~5-FU~~), 3'-fluoro-substituted 2',3'-dideoxynucleoside compounds comprising 3'-fluoro-2',3'-dideoxythymidine (~~FLT~~) and 3'-fluoro-2',3'-dideoxyguanosine (~~FLG~~), fomivirsen, ganciclovir, idoxuridine, lamivudine (~~3TC~~), 1-nucleosides comprising β -1-thymidine and β -1-2'-deoxycytidine), penciclovir, racivir, stampidine, stavudine (~~d4T~~), taribavirin (~~viramidine~~), telbivudine, tenofovir, trifluridine valaciclovir, valganciclovir, zalcitabine (~~ddC~~), and zidovudine (~~AZT~~); f). non-nucleosides comprising amantadine, ateviridine, capravirine, diarylpyrimidines, delavirdine, docosanol, emivirine, efavirenz,

foscarnet (~~phosphonoformic acid~~), imiquimod, interferon alfa, loviride, lodenosine, methisazone, nevirapine, NOV-205, peginterferon alfa, podophyllotoxin, rimantadine, resiquimod (~~R-848~~), and tromantadine; g). protease inhibitors comprising amprenavir, atazanavir, boceprevir, darunavir, fosamprenavir, indinavir, lopinavir, nelfinavir, pleconaril, ritonavir, saquinavir, telaprevir (~~VX-950~~), and tipranavir; h). anti-virus drugs comprising ~~abzyme~~ ABZYME, ~~arbidol~~ ARBIDOL, calanolide a, ceragenin, cyanovirin-n, epigallocatechin gallate (~~EGCG~~), foscarnet, griffithsin, ~~taribavirin (viramidine)~~, hydroxyurea, KP-1461, miltefosine, pleconaril, portmanteau inhibitors, ~~ribavirin~~ RIBAVIRIN, and seliciclib;

5). ~~any of 1) to 4)~~ having a radioisotope of ^3H , ^{11}C , ^{14}C , ^{18}F , ^{32}P , ^{35}S , ^{64}Cu , ^{68}Ga , ^{86}Y , ^{99}Tc , ^{111}In , ^{123}I , ^{124}I , ^{125}I , ^{131}I , ^{133}Xe , ^{177}Lu , ^{211}At , or ^{213}Bi ; and

6). pharmaceutically acceptable salts or acids of any of 1) to 5).

6. (Previously Presented) The conjugate of claim 2, wherein Drug is selected from the group consisting of tubulysins, calicheamicins, auristatins, maytansinoids, CC-1065 compounds, morpholinos doxorubicins, taxanes, cryptophycins, epothilones, and benzodiazepine dimers, siRNA or a combination thereof, and pharmaceutically acceptable salts or acids of any of the above.

7. (Previously Presented) The conjugate of claim 2, wherein the cell binding agent is selected from the group consisting of antibodies, proteins, vitamins, peptides, polymeric micelles, liposomes, lipoprotein-based drug carriers, nano-particle drug carriers, dendrimers, and combination thereof.

8. (Currently Amended) The conjugate of claim 2, wherein the cell-binding agent is configured to bind to target cells which are selected from the group consisting of tumor cells, virus infected cells, microorganism infected cells, parasite infected cells, autoimmune cells, activated cells, myeloid cells, activated T-cells, B cells, or melanocytes; cells expressing the CD19, CD20, CD22, CD30, CD33, CD37, CD38, CD40, CD51, CD52, CD56, CD66, CD70, CD74, CD79, CD80, CD98, CD125, CD221, CD227, CD262, CD309, CD326, CEACAM3, CEACAM5

(carcinoembryonic antigen), DLL4 (~~A-like 4~~), EGFR, CTLA4, CXCR4 (~~CD184~~), Endoglin (~~CD105~~), EPCAM (~~epithelial cell adhesion molecule~~), ERBB2 (~~Epidermal Growth Factor Receptor 2~~), FCGR1, FOLR (~~folate receptor~~), GD2 ganglioside, G-28 (~~a cell surface antigen glycolipid~~), GD3 idiotype, Heat shock proteins, HER1, HER2, HLA-DR10, HLA-DRB, human chorionic gonadotropin, IGF1R (~~insulin-like growth factor 1 receptor~~), IL-2 receptor (~~interleukin 2 receptor~~), IL-6R (~~interleukin 6 receptor~~), Integrins (~~$\alpha\beta 3$, $\alpha 5\beta 1$, $\alpha 6\beta 4$, $\alpha 11\beta 3$, $\alpha 5\beta 5$, $\alpha\beta 5$~~), MAGE-1, MAGE-2, MAGE-3, MAGE 4, anti-transferrin receptor, p97, MS4A1 (~~membrane-spanning 4 domains subfamily A member 1~~), MUC1 or MUC1-KLH, MUC16 (~~CA125~~), CEA, gp100, MART1, MPG, MS4A1 (~~membrane-spanning 4 domains subfamily A~~), Nucleolin, Neu oncogene product, P21, Paratope antibody having a paratope of anti-(N-glycolyneuraminic acid), PLAP-like testicular alkaline phosphatase, PSMA, PSA, ROBO4, TAG 72 (~~tumor associated glycoprotein 72~~), T cell transmembrane protein, Tie (~~CD202b~~), TNFRSF10B (~~tumor necrosis factor receptor superfamily member 10B~~), TNFRSF13B (~~tumor necrosis factor receptor superfamily member 13B~~), TPBG (~~trophoblast glycoprotein~~), TRAIL-R1 (~~Tumor necrosis apoptosis-inducing ligand Receptor 1~~), VCAM-1 (~~CD106~~), VEGF, VEGF-A, VEGF-2 (~~CD309~~), CanAg, CALLA, and cells expressing insulin growth factor receptor, or epidermal growth factor receptor.

9. (Previously Presented) The conjugate of claim 2, wherein the cell-binding agent is an antibody, a single chain antibody, an antibody fragment that binds to a target cell, a monoclonal antibody, a single chain monoclonal antibody, or a monoclonal antibody fragment that binds the target cell, a chimeric antibody, a chimeric antibody fragment that binds to the target cell, a domain antibody, a domain antibody fragment that binds to the target cell, a resurfaced antibody, a resurfaced single chain antibody, or a resurfaced antibody fragment that binds to the target cell, a humanized antibody or a resurfaced antibody, a humanized single chain antibody, or a humanized antibody fragment that binds to the target cell, a lymphokine, a hormone, a vitamin, a growth factor, a colony stimulating factor, or a nutrient-transport molecule.

10. (Previously Presented) The conjugate of claim 8, wherein the tumor cells are selected from

the group consisting of lymphoma cells, myeloma cells, renal cells, breast cancer cells, prostate cancer cells, ovarian cancer cells, colorectal cancer cells, gastric cancer cells, squamous cancer cells, small-cell lung cancer cells, testicular cancer cells, and cells that grow and divide at an unregulated, quickened pace to cause cancers.

11. (Previously Presented) A pharmaceutical composition comprising a therapeutically effective amount of the conjugate of claim 2, and a pharmaceutically acceptable salt, carrier, diluent, or excipient therefor.

12. (Previously Presented) The conjugate of claim 2, wherein the drug is selected from the group consisting of a toxin, a chemotherapeutic agent, a drug moiety, an antibiotic, a radioactive isotope, and a nucleolytic enzyme.

13. (Canceled)

14. (Currently Amended) The conjugate of claim 2, wherein the conjugate comprises one or more linker components of 6-maleimidocaproyl-(MC), maleimidopropanoyl-(MP), valine-citrulline-(~~val-cit~~), alanine-phenylalanine-(~~ala-phe~~), p-aminobenzyloxycarbonyl-(PAB), 4-thio-pentanoate-(SPP), 4-(N-maleimidomethyl)cyclohexane-1-carboxylate-(MCC), 4-thio-butyrates-(SPDB), maleimidoethyl ~~containing~~-(ME), 4-thio-2-hydroxysulfonyl-butyrates-(~~2-Sulfo-SPDB~~) and (4-acetyl)aminobenzoate-(SIAB).

15. (Currently Amended) The conjugate of claim 2, wherein Drug is selected from the group consisting of tubulysins, maytansinoids, taxanoids-(~~taxanes~~), CC-1065 compounds, daunorubicin and doxorubicin compounds, benzodiazepine dimers, calicheamicins and the enediyne antibiotics, actinomycin, azaserines, bleomycins, epirubicin, tamoxifen, idarubicin, dolastatins/auristatins, duocarmycins, thiotepa, vincristine, hemiasterlins, and esperamicins.

16. (Previously Presented) The conjugate of claim 2, having in vitro, in vivo or ex vivo cell

killing activity.

17. (Previously Presented) The conjugate of claim 2, wherein the conjugate comprises either a peptide of 1-20 units of natural or unnatural amino acids, or a p-aminobenzyl unit, or a 6-maleimidocaproyl unit, or a disulfide unit, or a thioether unit, or a hydrozone unit, or an alkoxime unit.
18. (Previously Presented) The conjugate of claim 2, wherein the conjugate is cleavable by a protease.
19. (Previously Presented) A pharmaceutical composition comprising the conjugate of claim 2, and a pharmaceutically acceptable carrier.
20. (Previously Presented) A pharmaceutical composition comprising a therapeutically effective amount of the conjugate of claim 2, and a chemotherapeutic agent, radiation therapy agent, immunotherapy agent, autoimmune disorder agent, or anti-infectious agent.
21. (Previously Presented) The hydrophilic linker of claim 1, wherein Z comprises a thiol, disulfide, amino, carboxy, aldehydes, maleimido, haloacetyl, hydrazine or hydroxyl group.
22. (New) The conjugate of claim 2, wherein the drug is derived from a drug containing a -SH, -CONHNH₂, -≡, -COR'' or -NHR'', wherein R'' is H, CH₃, Et, Pr, Bu, or Ph; and/or the cell-binding agent is an antibody or protein.
23. (New) The conjugate of claim 3, wherein the cytotoxic drug is a drug containing a -SH, -CONHNH₂, -≡, -COR'' or -NHR'', wherein R'' is H, CH₃, Et, Pr, Bu, or Ph; and/or the cell-binding agent is an antibody or protein.

24. (New) The conjugate of claim 4, wherein the drug is derived from a drug containing a -SH, -CONHNH₂, -≡, -COR'' or -NHR'', wherein R'' is H, CH₃, Et, Pr, Bu, or Ph; and/or the cell-binding agent is an antibody or protein.

REMARKS

Entry of the foregoing, reconsideration of the subject matter identified in caption, as amended, pursuant to and consistent with 37 C.F.R. §1.111, and in light of the remarks which follow, are respectfully requested.

In the present Amendment, claim 1 has been amended to further define R₁-R₆, Q, T, m and n. Claims 2-4 also have been amended to further define R₁-R₆. Support for these amendments can be found throughout the Specification, particularly, the Examples (see, e.g., Examples 69, 75, 86, 116, 122, 128, 135, 138, 142 and 147). Claims 5, 8, 14 and 15 have been amended to further improve their form. New claims 22-24 have been added. Support for claims 22-24 can be found throughout the Specification, particularly, Figs. 6, 7, 11, 13, 15, 16 and 18. Claim 13 was previously cancelled. No new matter has been added. Upon entry of the Amendment, claims 1-12 and 14-24 will be all the claims pending in the application.

I. Response to Claim Objection

Claim 5 was objected to for informalities. Applicant respectfully submits that claim 5 as amended does not contain informalities.

In the Amendment, claim 5 has been amended to correct a typographical error, by replacing "Xegeva" with --XGEVA--, as suggested by the Examiner. Accordingly, reconsideration and withdrawal of the objection are respectfully requested.

II. Response to Rejections under 35 U.S.C. § 112

a. Claims 5, 8, 14, and 15 were rejected under 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite. Applicant respectfully submits that the claims as amended are not indefinite.

In the Amendment, claims 5, 8, 14, and 15 have been amended to delete certain parentheses therein. Claim 14 has been amended to delete the word "containing." Claim 8 has been amended to specify "antibody having a paratope." Further, claim 5 has been amended to capitalize trademarks therein. Accordingly, reconsideration and withdrawal of the rejection are respectfully requested.

b. Claim 5 was rejected under 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. Applicant respectfully submits that claim 5 as amended complies with the written description requirement.

In the Amendment, claim 5 has been amended to recite "a radioisotope." Accordingly, reconsideration and withdrawal of the rejection are respectfully requested.

c. Claims 2-12 and 14-21 were rejected under 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the enablement requirement. Applicant respectfully traverses the rejection for the following reasons.

The Office Action asserts that:

The specification has described conjugation the synthesis of bi-functional mono, bi, tri and tetraphosphinate linkers enabling conjugation to a drug via reaction of a thiol group on a drug moiety and conjugation of a protein or antibody via a maleimido [sic, maleimido] group on the opposite end of the linker. The use of the maleimido group in protein and antibody conjugation is well known in the art. However, claim 5 is not limited to drugs or biologically active proteins having a free thiol group ... The specification provides no teaching or guidance on the modification of the multitude of drugs ... to provide a free thiol group for conjugation to the linker such that the modification and/or the conjugation of the drug, products or cells preserves the required function of the drug, multicomponent products or cells. Thus, one of skill in the art would be required to determine how to modify all the drugs and multicomponent products required in claims [sic, claim] 5 such that the expected biological functions of the drugs and products are maintained and if such modification was possible. Based on this requirement, one of skill in the art would be subject to undue experimentation without reasonable expectation of success in order to make and use the broadly claimed compounds of claim 5. (Office Action at pages 4-5)

Applicant respectfully disagrees. As set forth in the previous Amendment, one skilled in the art would be able to synthesize conjugates of the drugs recited in claim 5, through an appropriate chemical reaction with the linker to form a disulfide, thioether, thioester, peptide, hydrazone, ether, ester, carbamate, carbonate, secondary, tertiary, or quaternary amine, imine, cycloheteroalkane, heteroaromatic, alkoxime or amide bond.

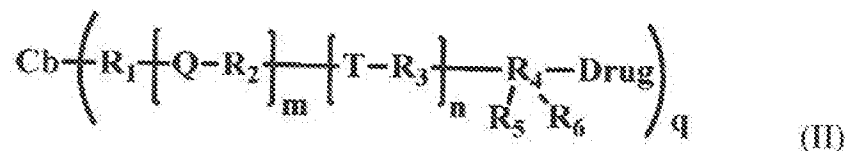
The present specification also provides specific examples of reacting the linker with various drugs, besides the drugs with a -SH group. For example, in Fig. 11, Drug-CO-NHNH₂

is used to convert Compounds 150 and 152 into Compounds 154 and 153, respectively; in Fig. 13, Drug≡ is used to convert Compound 166 into Compound 167; in Fig. 15, Drug-CO-R" is used to convert Compounds 193 and 196 into Compounds 194 and 195, respectively; in Fig. 16, Drug-CO-R" is used to convert Compounds 201 and 204 into Compounds 202 and 203, respectively; and in Fig. 18, Drug-NH-R" is used to convert Compound 228 into Compound 229.

The Office Action also asserts that:

Claim 5 ... the "drug" includes an anti-infectious disease agent or an anti-viral agent ... The conjugate of the instant invention requires a cell binding agent, but for recognition of non-retroviral or non-lentiviral viral infections, the virus itself will be bound, rather than the cell harboring the virus. As a free virus is not a cell per se, the targeting of a free virus cannot be carried out by a "cell-binding agent" required by the conjugate of instant claim 2.... (Office Action at page 5)

The Examiner appears to misread the claim language. Specifically, claim 2, from which claim 5 depends, recites a cell-binding agent-drug conjugate of formula (II):



Note that Drug and the cell-binding agent Cb are both present in formula (II). Claim 5 further specifies that Drug includes, among others, an anti-infectious disease agent or an anti-viral drug. However, the claim does not require that when Drug is an anti-infectious disease agent or an anti-viral drug, the cell-binding agent bind to the target virus.

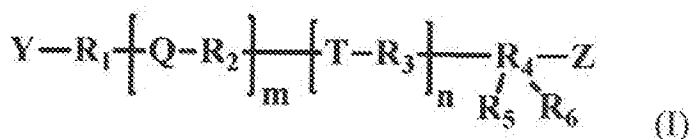
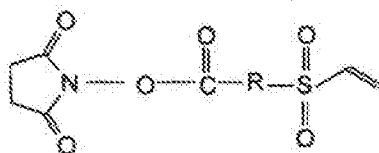
Applicant advises that both bacterial and viral cells express certain antigens on their cell surfaces, the antibody-drug conjugates therefore can bind, be endocytosed and then kill the abnormal cells, the same as for tumor cells. See, e.g., Pincus, et al., *Design and In Vivo Characterization of Immunoconjugates Targeting HIV gp160*, Journal of Virology, online publication, October 19, 2016 (51 pages); and Lehar, et al., Novel antibody-antibiotic conjugate eliminates intracellular *S. aureus*, Nature, 2015, 527, 323-328, copies of which are attached hereto.

In view of the foregoing, reconsideration and withdrawal of the rejection are respectfully requested.

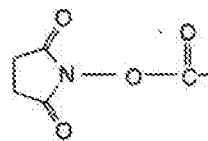
III. Response to Rejection under 35 U.S.C. § 102

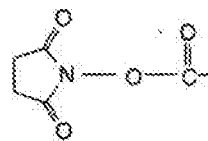
Claim 1 was rejected under pre-AIA 35 U.S.C. 102(b) as being anticipated by WO 97/41897 to Lees. Applicant respectfully submits that claim 1 as amended is novel and patentable over Lees for at least the following reasons.

Lees discloses protein/polysaccharide conjugates prepared via a heterobifunctional vinylsulfone, such as an N-hydroxysuccinimide vinylsulfone having the following structure:



This compound corresponds to



recited in claim 1 of the present application, where Y is , Q (or T) is $-S(O_2)-$, m (or n) is 1, n (or m) is 0, $R_2(R_3)$ and R_4-R_6 are absent, and Z is .

On the other hand, claim 1 of the present application recites that when Q or T is $-S(O_2)-$, m and n are not 0, thereby excluding the above noted compound described in Lees.

In view of the above, reconsideration and withdrawal of the rejection are respectfully requested.

IV. New Claims

Newly added claims 22-24 depend from claim 2, 3 or 4, and thus are patentable at least for the reasons set forth above.

V. Conclusion

From the foregoing, further and favorable action in the form of a Notice of Allowance is believed to be next in order and such action is earnestly solicited. If there are any questions

concerning this paper or the application in general, the Examiner is invited to telephone the undersigned at his earliest convenience.

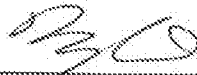
The Director is hereby authorized to charge any appropriate fees under 37 C.F.R. §§ 1.16, 1.17 and 1.20(d) and 1.21 that may be required by this paper, and to credit any overpayment, to Deposit Account No. 02-4800.

Respectfully submitted,

BUCHANAN INGERSOLL & ROONEY PC

Date: March 20, 2018

By: _____



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Design and In Vivo Characterization of Immunoconjugates Targeting HIV gp160

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Running Head: Anti-HIV Immunoconjugates

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Word Count: Abstract, 212 words. Main Text: 6263 words

ABSTRACT

The envelope (Env) glycoprotein of HIV is expressed on the surface of productively-infected cells, and can be used as a target for cytotoxic immunoconjugates (ICs), in which cell-killing moieties including toxins, drugs, or radionuclides are chemically or genetically linked to monoclonal antibodies (MAbs) or other targeting ligands. Such ICs could be used to eliminate persistent reservoirs of HIV infection. We have found that MAbs which bind to the external loop of gp41, e.g. MAb 7B2, make highly effective ICs, particularly when used in combination with soluble CD4. We have evaluated toxicity, immunogenicity, and efficacy of the ICs targeted with 7B2 in mice and in SHIV-infected macaques. In the macaques, we tested immunotoxins (ITs), consisting of protein toxins bound to the targeting agent. ITs were well tolerated, initially efficacious, but ultimately limited by immunogenicity. In an effort to decrease immunogenicity, we tested different toxic moieties, including recombinant toxins, cytotoxic drugs, and tubulin inhibitors. ICs containing deglycosylated ricin A chain prepared from ricin toxin extracted from castor beans were the most effective in killing HIV-infected cells. Having identified immunogenicity as a major concern, we show that conjugation of IT to polyethylene glycol limits immunogenicity. These studies demonstrate that cytotoxic ICs can target virus-infected cells *in vivo*, but also highlight potential problems to be addressed.

IMPORTANCE

It is not yet possible to cure HIV infection. Even after years of fully effective antiviral therapy, a persistent reservoir of virus-infected cells remains. Here we propose that a targeted conjugate, consisting of an anti-HIV antibody bound to a toxic moiety, could function to kill the HIV-infected cells that constitute this reservoir. We tested this approach in HIV-infected cells grown in the lab, and in animal infections. Our studies demonstrated that these immunoconjugates are effective both in vitro and in test animals. In particular, ITs constructed with the deglycosylated A chain prepared from native ricin were the most effective in killing cells. But their utility was blunted because they provoked immune reactions that interfered with the therapeutic effects. We then demonstrated that coating ITs with polyethylene glycol minimizes immunogenicity, as has been demonstrated with other protein therapies.

INTRODUCTION

The HIV envelope glycoprotein (Env, consisting of precursor gp160, transmembrane gp41 and surface gp120) is expressed on virions and infected cells, and is the target of HIV-neutralizing antibodies (Abs). HIV-infected cells present in virus reservoirs may be targeted with anti-Env monoclonal Abs (MAbs), either as unmodified MAbs exerting cytotoxic effects through Fc-mediated mechanisms, or as cytotoxic conjugates. If the toxic moiety is a protein derived from a plant, bacterial or animal source, the IC is termed an immunotoxin (IT). If it is a small drug, then the term Ab-drug conjugate (ADC) is commonly applied. ICs containing high-energy isotopes are known as radio-ICs and unlike ADCs and ITs do not require internalization to kill cells. Anti-Env ITs and radio-ICs have been shown to have potent *in vitro* anti-HIV activity at therapeutically obtainable concentrations, when tested in a variety of different cell types, including primary T cells and macrophages, and tested against clinical virus isolates. Efficacy has also been demonstrated in murine models of active and persistent HIV infection (1-13). Expression of Env on the cells that constitute persistent reservoirs in ART-suppressed patients may be through ongoing low-level, anatomically-localized transcription of viral RNA, or may require pharmacologic activation of latent provirus utilizing so-called "activate and purge" protocols (14-17). We propose the use of cytotoxic Abs and ICs to actively purge cells expressing Env.

We have previously reported that the most effective ITs were obtained using MAbs that bind to epitopes located in the extracellular loop domain of gp41, such as MAb 7B2. These ITs are especially effective when used in association with soluble CD4 (sCD4), which destabilizes the interaction between gp120 and gp41, causing increased exposure of this partially obscured epitope (4, 8, 18). Others propose targeting the CD4 binding-site (CD4bs) with either MAbs, or sCD4 itself (1, 5-7, 9-11, 13). In a companion publication, we again confirmed the efficacy of MAb 7B2 + sCD4 in killing infected cells and cells stably expressing transfected Env as compared to highly potent broadly-neutralizing MAbs that only killed cells stably expressing Env,

93 but not infected cells (Pincus, S.H., *et al*, Identification of human anti-HIV gp160 MAbs that
 94 make effective ITs, submitted for publication). We also demonstrated that size limited the ability
 95 of ITs targeted against the CD4bs to kill HIV-infected cells. Smaller ITs using Fv and Fab
 96 fragments of MAbs were effective, but larger ITs using the same V regions in intact IgG were
 97 not. Size did not affect the function of ITs targeted elsewhere on Env.

98 In this publication, we describe in vivo efficacy studies of anti-Env ITs, using both a SCID
 99 mouse model and SHIV-infected macaques. We compared MAb 7B2, which binds to the
 100 external disulfide loop of gp41, conjugated to deglycosylated ricin A chain (RAC) to ITs that
 101 target the CD4bs. We provide evidence that ITs were well-tolerated and initially efficacious in
 102 SHIV-infected macaques, but that immunogenicity blunted this efficacy within 2-3 weeks.
 103 Effective doses of IT were <1% the dose of "naked" Abs shown to have potent antiviral effects in
 104 similar SHIV infections (19, 20), or in HIV infected humans (21). To minimize immunogenicity,
 105 we made 7B2-based ITs with recombinant toxins and produced ADCs with 7B2, which were
 106 effective, but required higher concentrations. However, coupling the ITs with polyethylene glycol
 107 decreased immunogenicity without compromising specific cytotoxicity. The data support the
 108 continued development of ICs capable of killing HIV infected cells expressing Env.

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MATERIALS AND METHODS

Human Samples

Human blood was obtained from adult volunteers providing written consent. Blood was drawn by study nurses who provided blinded samples to investigators. The IRBs of LSU Health Sciences Center (IRB# 5030) and Children's Hospital, New Orleans (ARC# 01-140) approved the drawing of blood from healthy donors for research purposes. Leukapheresis packs were obtained from Research Blood Components, LLC (Brighton, MA). Cells were from de-identified individuals tested to be negative for known infections prior to leukapheresis. Commercially obtained, de-identified cells are exempt as human subjects research.

Animal Studies

Macaca nemestrina (pigtailed) and *M. mulatta* (rhesus) were studied. Animals were infected with SHIV 162.P3, KU-1, or 89.6p. Animals were housed at the Washington National Primate Research Center (WaNPRC) and at Advanced BioScience Laboratories. All studies were performed in compliance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals", were approved by the appropriate Institutional Animal Care and Use Committees, and all procedures were performed according to protocols pre-approved by the IACUC. Animals were under the care of a licensed veterinarian and all efforts were made to minimize animal pain and suffering, in accordance with the recommendations of the Weatherall report. Analgesics, anesthetics and/or tranquilizers were used to limit discomfort, distress, or pain associated with experimental procedures. Facilities were accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International and registered as a USDA Class R research facilities. WaNPRC is certified by the NIH Office of Laboratory Animal Welfare (OLAW A3464-01), and experiments were performed under protocols 2370-12, 2370-20, 2370-18 and 2984-04, all approved by the University of Washington IACUC. Animal studies

136 at Advanced BioScience Laboratories (OLAW A3467-01) were performed under IACUC-
137 approved protocol AUP 315. Details of animal husbandry at each site have been published (22).

138 Macaques used in these studies were previously enrolled in vaccine studies. All had
139 been infected with SHIV for several years, and many were developing symptomatology and/or
140 laboratory findings consistent with early AIDS. Rather than euthanize these animals, those that
141 were not acutely ill, suffering, or in pain, were chosen for these studies. ITs were administered
142 to sedated animals by IV injection over a 10-20 min period. Vital signs were measured prior to
143 the administration of ITs, and in the latter stages of sedation. Animals were observed clinically
144 throughout the following day, and daily thereafter. Blood chemistry and CBC, were monitored 1-
145 2X per week, CD4 counts and viral load were tested weekly. Animals were weighed weekly.

146 Mice used for testing ITs and ADCs for anti-HIV efficacy and for studying
147 immunogenicity were housed at the Research Institute for Children, New Orleans (OLAW
148 assurance A-4336-01). Studies were performed under IACUC approved protocols 108 (HIV
149 studies) and 109 (immunization). NSG mice (NOD/SCID/gamma-chain KO) were obtained from
150 Jackson Labs, and bred in our facilities. Using our published protocols (8, 23), NSG mice were
151 treated with cyclophosphamide (100µg/g ip), and 24hr later two separate tumors in different
152 body compartments were implanted in Matrigel. The first contained infected H9/NL4-3, the other
153 (if implanted) uninfected H9. For the injection, mice were anesthetized with
154 telazol/acepromazine. Conjugates were administered by intraperitoneal (ip) injection in
155 PBS/1%BSA on the days indicated in figure captions. Following the injection of human tumor
156 cells, mice were observed daily for any clinical signs of distress (hunching, ruffled hair, difficulty
157 moving, unwillingness to move, or weight loss). If the animal technician, who was unaware of
158 the experimental protocol, determined that the animals were in pain or distress, they were
159 euthanized. We have carefully studied the kinetics of tumor growth and HIV spread, and only
160 rarely did mice show evidence of pain or distress. At sacrifice, mice were bled by intracardiac
161 puncture while under isoflurane anesthesia, then euthanized by cervical dislocation while still
162 under anesthesia. Spleens were removed, single cell suspensions made and cryopreserved. In

163 experiments measuring immunogenicity of ITs, outbred SW mice (NCI) were injected ip with IT
 164 in saline, using a regimen of 10µg/mouse 3 times at weekly intervals. Mice were bled by
 165 saphenous venipuncture prior to, and 3, 5, and 7 weeks post injection.

167 **Cells and Reagents**

168 H9/NL4-3 cells are H9 CD4+T-cell lymphoma culture lines that are persistently infected
 169 with the NL4-3 molecular clone of HIV (24). H9/NL4-3 cells maintain a productive infection in all
 170 cells, even during prolonged passage in tissue culture (8, 25). 293T cells stably express native
 171 gp120/gp41 Env trimers derived from clinical isolates 92UG and C97, and were the generous
 172 gift of Bing Chen and Hanqin Peng (26). C8166.R5 cells are CD4+ CXCR4+ lymphoma cell line
 173 engineered to express CCR5 (27). Activated T-cell cultures were obtained by culturing
 174 peripheral blood mononuclear cells with phytohemagglutinin (PHA, 10 µg/ml), and maintained
 175 with IL-2 200U/ml (27). All cells were cultured at 37° in 5% CO₂ in RPMI 1640 medium with 10%
 176 fetal bovine serum (FBS) (Life Technologies, Inc).

177 Table 1 describes the constituent reagents that were used to construct the ICs used in
 178 these studies. MAbs 7B2 (human anti-gp41) and 924 (murine anti-gp120, V3-loop) were
 179 secreted by hybridoma cells in tissue culture using low IgG FBS, and purified on protein G-
 180 sepharose, as described (8). Alexa₄₈₈-conjugated RAC18 MAb to ricin A chain was prepared as
 181 previously described (28). Soluble CD4-IgG2 (Pro542) was from Progenics Pharmaceuticals,
 182 Tarrytown NY (29, 30). Goat anti-human or anti-mouse IgG (heavy + light chains) was
 183 conjugated to either alkaline phosphatase (AP) or fluorescein isothiocyanate (FITC, Life
 184 Technologies, Inc). Mouse anti-doxorubicin MAb was obtained from U.S. Biological (Salem,
 185 MA).

186 Chimeric CD4-pseudomonas exotoxin₄₀ (CD4-PE40) was obtained from Upjohn
 187 Laboratories (Kalamazoo, MI). Single chain variable domain (scFv) PE-based IT HY-PE38, HY-
 188 (PE38)-KDEL, and HY-(PE38)mut were produced in *E. coli* and chemically refolded as
 189 described (6, 10). HY is derived from the CD4bs MAb b12, through two rounds of affinity

190 maturation (b12 to 3B3 to HY). HY-KDEL contains a carboxy-terminus endoplasmic reticulum
 191 targeting sequence (KDEL)

192 Ricin A chain was prepared in two ways. Deglycosylated RAC was prepared from castor
 193 bean extracts, as described previously (31, 32). Recombinant ricin toxin A chain (RTA) was
 194 produced in BL21(DE3)RIL cells (Novagen) from plasmid pETtrx, which encodes *E. coli*
 195 thioredoxin and a tobacco etch virus (TEV) protease-cleavable 6xHis affinity tag in frame with
 196 and N-terminal to the RTA coding sequence. Cells harboring the pETtrx plasmid were grown in
 197 LB at 37°C in the presence of 50µg/mL kanamycin sulfate and 30µg/mL chloramphenicol. When
 198 the culture reached an optical density (600nm) of 0.7, the temperature was reduced to 27°C and
 199 0.5 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce protein
 200 expression. After 5 hr., the cells were harvested by centrifugation and resuspended in 300 mM
 201 NaCl, 20 mM Tris-HCL pH 8.0, 15mM imidazole, and 5% glycerol (N1 buffer). The cells were
 202 lysed at 4°C using an Emulsiflex-C5 high pressure homogenizer (Avestin) and the lysate was
 203 then centrifuged for 45min at 260,000g at 4°C. The supernatant was loaded onto a 5ml HisTrap
 204 HP column (GE Healthcare) equilibrated to N1 buffer, and after extensive washing with N1
 205 buffer, the protein was eluted with N1 buffer containing an additional 300 mM imidazole. TEV
 206 protease was added to the eluate and the proteins were dialyzed overnight at 4°C against N1
 207 buffer. The following morning, dialyzed proteins were again loaded onto a 5ml HisTrap HP
 208 column equilibrated to N1 and the untagged RTA was collected in the flow-through,
 209 concentrated, and loaded onto a 26/60 Sephacryl-100 gel filtration column (GE Healthcare)
 210 equilibrated to 100mM NaCl, 10mM KCL, 10mM Na/K-phosphate (pH 7.0), 2mM β-
 211 mercaptoethanol. Sephacryl-100 fractions containing RTA were identified using SDS-PAGE
 212 followed by Coomassie brilliant blue staining then pooled and concentrated to 14 mg/mL. The
 213 purified RTA migrated as a single band as determined by SDS-PAGE, and crystallized in 1M
 214 Li₂SO₄ and 2.5% polyethylene glycol MW 8,000; forming apparently tetragonal crystals that
 215 measure up to 0.5 x 0.5 x 0.8 mm. It should be noted that many of the ricin A chain X-ray
 216 structures deposited in the Protein Data Bank arose from crystals belonging to space group

217 P4₁2₁2 (Tetragonal) with unit cell parameters $a \approx 67\text{\AA}$, $b \approx 67\text{\AA}$ and $c \approx 137\text{\AA}$ (e.g. 2R2X, 2PJO,
218 3SRP, etc.). Both RAC and RTA were conjugated to intact Ab at a 2.5:1 molar ratio using the
219 heterobifunctional cross-linking reagent succinimidyl 6-[3(2-pyridyldithio) propionamido]
220 hexanoate (SPDP, Pierce), as described elsewhere (3, 18, 33). Polyethylene glycol (PEG)₅₀₀₀-
221 NHS was purchased from Shearwater Pharmaceuticals.

222 The chemical conjugation and purification of recombinant gelonin (Gel) conjugated to
223 7B2 and 924 were performed essentially as described previously (34). Briefly, a 3 fold molar
224 excess of the SPDP cross-linker was added to 2 ml of PBS containing 10 mg of purified MAb
225 and incubated at room temperature for 30 min. Excess SPDP was removed by gel filtration and
226 Ab was slowly added to purified Gel which had been reduced by addition of DTT. The
227 conjugation was allowed to proceed for 6 hr at 4⁰ under nitrogen. The 7B2-Gel conjugate was
228 then purified by sequential chromatography on Superose S-75 and blue sepharose columns.
229 The final conjugate preparations were aliquoted and stored at -20C.

230 ADCs of MAb 7B2 and 924 conjugated to monomethyl aueromycin E (MMAE),
231 duocarmycin, or maytansine, were prepared using non-cleavable or cathepsin-B cleavable
232 linkers. The conjugates were purified on PD-10 columns to remove free drug and the degree of
233 conjugation quantified using hydrophobic interaction HPLC, with a TSKgel Butyl-NPR column,
234 buffer A 25 mM sodium phosphate, 1.5 mM ammonium sulfate (pH 7.0); buffer B 75% v/v 25
235 mM sodium phosphate (pH 7.0), 25% v/v isopropanol, and a linear gradient (10-70% buffer B in
236 10 min, to 100% buffer B in 15 minutes). The ADCs were desalted on a PD-10 column, and
237 characterized by size exclusion and hydrophobic interaction HPLC and CE-IEF. Drug:Ab ratios
238 of 2-3 were obtained.

239 Epirubicin-(C₃-amide)-7B2 was produced employing a 2-phase synthesis regimen with
240 succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate using established protocols (35-
241 37). Residual un-reacted reagents were removed by micro-scale column chromatography, and
242 the percent of non-immunoglobulin bound epirubicin was measured in a chloroform/propanol
243 supernatant extract following centrifugation of the resulting protein precipitate. Percent

covalently bound epirubicin was above 97%, the molar-incorporation-index was 57%.
 Gemcitabine-(C₂-amide)-SS-7B2 was produced employing a 2-phase synthesis regimen with
 succinimidyl 2-[(4,4'-azipentanamido)ethyl]-1,3'-dithiopropionate using established protocols
 (35-37). Residual un-reacted reagents were removed by micro-scale column chromatography.
 Percent bound Gemcitabine ranged upward from 81%.

Enzyme-linked immunosorbent assay (ELISA)

Anti-IT antibody responses were measured using an indirect ELISA performed on plates
 coated with IT, MAb, or RAC using established protocols (33). Immulon 2HB plates (Thermo,
 Waltham, MA) were coated for 18-24 hr with 1 µg/ml of antigen in PBS, then blocked with
 PBS/1% BSA/0.01% sodium azide (PBA) 200µl for 8-16 hours at 4°. After blocking, 100µl of
 each test MAb was added and incubated at 4° overnight. The plates were washed and AP-
 conjugated goat anti-human or mouse IgG secondary antibody was added at 1µg/ml in PBA and
 incubated for 6 hr. Substrate (4-nitrophenyl phosphate, Sigma Chemical) was dissolved in 9.8%
 diethanoleamine buffer (pH=9.8) to a concentration of 0.5mg/ml. After a final wash, 100µl of
 substrate was added to each well. ELISA plates were read at 405nm at room temperature in a
 BioTek EL320 microplate reader (BioTek, Winooski, VT) at 5-15 minute intervals. Plasma and
 tissue culture p24 levels were measured with an antigen capture ELISA sensitive to 7.5 pg/ml,
 (8, 23, 38).

Immunofluorescence and flow cytometry

H9/NL4-3 cells (1x10⁵) were stained for flow cytometry in 100µl in round-bottom 96 well
 plates (Costar, Lowell, MA) (4). To perform indirect immunofluorescence, the IT or ADC was
 added to the cells in PBA. Cells were incubated 1-2 hr. at 4°, washed, and stained with FITC-
 conjugated goat anti-human IgG secondary antibody for 1-4 hr., or with murine MAbs to
 doxorubicin or ricin A chain. Those cells incubated with murine MAbs were washed again, then
 stained with FITC goat anti-mouse Ig. All cells were washed a final time and fixed in 100µl of 2%

271 paraformaldehyde. Cells were analyzed on a Becton-Dickson LSR II (BD, Franklin Lakes, NJ)
 272 with HTS plate reader, and gated on forward scatter and side scatter. 10000 gated events were
 273 collected and data analyzed by Flow-Jo software (Treestar, Ashland, OR). None of the MABs,
 274 ITs or ADCs bound to uninfected H9 cells, nor did the FITC-conjugated anti-mouse bind to cells
 275 bound by human MAb 7B2.

276

277 **Cytotoxicity of ITs, ADCs, and drugs**

278 Uninfected H9 (1.5×10^4) and H9/NL4-3 cells (8×10^3) were plated in triplicate in RPMI
 279 1640 medium + 10% FBS in 96 well flat-bottom tissue culture plates (Costar). Alternatively
 280 nontransfected 293T, 293T/92UG, or 293T/C97 (all at 3×10^4) were plated. Serial dilutions of
 281 conjugates were incubated with cells for 72 hr. 37° in the presence or absence of 300ng/ml of
 282 sCD4₁₈₃. Controls included: no cells (background) and cells in the absence of IC (uninhibited).
 283 When cytotoxicity of small drugs was tested, the drug was added to the indicated concentration
 284 at the start of the assay. For the final 6 hr. of incubation, MTS/PMS substrate (CellTiter 96®
 285 AQueous Non-Radioactive Cell Proliferation Assay Promega, Madison, WI) was added to each
 286 well and absorbance at 490nm determined hourly. Percent inhibition was calculated as: $[1 -$
 287 $(A_{\text{MAB}} - A_{\text{no cells}}) / (A_{\text{No MAB}} - A_{\text{no cells}})] * 100$.

288

289 **Inhibition of virus propagation in tissue culture**

290 To test the efficacy of ITs against primary HIV isolates, we studied the spread of
 291 infection in cell culture by measuring p24 production. Virus was cultured with PHA blasts or a
 292 cell line (C8166.R5) that provides comparable results to PHA blasts, but with greater
 293 consistency (27). Primary clade B isolates, R5-tropic 208K8 (39) and dual-tropic HT/92/594
 294 (ARP, from Dr. Neal Halsey and DAIDS), were amplified in PHA blast culture. Supernatants
 295 were harvested, filtered, aliquoted, titered, and frozen. To assay the efficacy of ITs, virus was
 296 mixed with cells in a 2 ml culture, incubated for 24hr, cells washed, and IT added. Supernatants

297 were removed at different time points, and levels of p24 measured by ELISA. Data are from the
298 early log phase of p24 accumulation, and exact times in the figure legends.

299

300 **Determination of plasma viremia in macaques**

301 Conventional nucleic acid sequence based amplification (NASBA) and quantitative real-
302 time reverse transcriptase PCR assays were used to measure plasma viral load at ABL and
303 WaNPRC, respectively (40-42). The limit of quantification for studies performed at WaNPRC
304 was 100 copy/ml, at ABL 50 copy/ml.

RESULTS

Murine analyses confirm in vitro studies

We have previously described a murine model for testing the efficacy of anti-HIV therapies, including ITs, and have used the model to show that 7B2-RAC plus CD4-IgG2 was at least 10-fold more effective in vivo than CD4-PE40 (8, 23). In Fig. 1, we demonstrate that *in vitro* efficacy was predictive of results in mice, measuring plasma p24 levels as an indicator of infection. Experiment 1 shows that the *in vivo* activity was due to the combination of 7B2-RAC and CD4-IgG2, but not to either component alone, nor to MAb-mediated neutralization. In experiment 2, we observed that although the scFv HY-KDEL had activity equivalent to that of 7B2-RAC/CD4-IgG2 in vitro, HY-KDEL had no *in vivo* activity, while the anti-gp41 IT was effective. We also measured the number of cells secreting infectious HIV in the spleens of these animals. Similar results were observed when measuring infectious centers rather than plasma p24 results, but due to large variance, differences among groups were not significant, except combined treatment with CD4-IgG2+7B2-RAC which completely eliminated infected cells (compared to a mean of 235,000/spleen in saline-treated animals).

The lack of activity of scFv HY-KDEL may be due to its structure; since an scFv has a shorter plasma residence time (2-3hr) than an intact Ig (2-3d), and thus a shorter window of therapeutic opportunity (8). Targeting the CD4bs using ITs constructed from intact IgGs (HY-RAC and CD4-IgG2-RAC) was ineffective both in vitro and in vivo, confirming our observation that killing by ITs directed against the CD4bs is limited by size, i.e. scFv ITs function in vitro, whereas those based on intact IgGs do not (Pincus, S.H., *et al*, Identification of human anti-HIV gp160 MAbs that make effective ITs, submitted for publication). The lack of efficacy of IgG-based ITs to the CD4bs was not an artifact of the conjugation process, because the binding of the ITs to HIV-infected cells measured by flow cytometry was not affected by conjugation to ricin, and RAC-based ITs directed against other gp160 epitopes conjugated side-by-side, using the same linker and batch of ricin, were highly effective.

332

333 **Studies in macaques**

334 Although studies in mice are useful in evaluating in vivo anti-HIV effects, mice are not
335 ideal. Studies in macaques are believed to be most predictive of efficacy in humans. Because
336 there are considerable antigenic differences between HIV and SIV Envs, and the ITs target HIV
337 Env, we have tested the ITs in macaques infected with chimeric SHIV, expressing X4 and R5
338 tropic HIV Envs (Supplemental Table). The macaques we studied have AIDS and are not meant
339 to be a model for the use of ITs to eliminate latent and persistent reservoirs. Although we have
340 chosen to test the ITs in SHIV-infected macaques, it must be emphasized that these studies
341 were not designed as efficacy studies (insufficient power), but rather to test toxicity,
342 pharmacokinetics, and immunogenicity, in a phase I preclinical trial. However, we did measure
343 viral load and CD4 counts, in order to determine if there were signs of efficacy (Fig. 2,
344 Supplemental Table).

345 Macaques were treated with escalating doses of different ITs. Animals were monitored
346 clinically and in the laboratory by CBC, chemistry, CD4/CD8 counts, plasma viral load, and
347 PBMC provirus. Antibody responses against the administered ITs were measured before and at
348 different times following administration of the ITs. In experiment 1 (Supplemental Table), we
349 compared 7B2-RAC/CD4-IgG2 to CD4-PE40. The latter has been used in human clinical trials,
350 and was found to be hepatotoxic at very low doses, with a $t_{1/2}$ of ~90 min, and no evidence of
351 clinical efficacy (43, 44). Macaques receiving CD4-PE40 developed dose-related elevations of
352 serum hepatic transaminases 3 days after administration, which returned to normal by 7 days,
353 which is similar to observations in patients (43, 44). Escalating doses of CD4-PE40 were halted
354 at 16 $\mu\text{g}/\text{kg}$. In contrast, 7B2-RAC/CD4-IgG2 was tolerated at doses as high as 65 $\mu\text{g}/\text{kg}$ 3X per
355 week (Experiments 2 and 3, Supplemental Table) with no clinical or laboratory evidence of
356 toxicity beyond moderate decreases in serum albumin at higher IT doses. PE was the toxic
357 moiety used in all the ITs based on scFv HY, which were tolerated by most animals in doses as
358 high as 500 $\mu\text{g}/\text{kg}$ (Experiment 4, Supplemental Table), thus indicating that the toxicity of CD4-

359 PE40 was probably unique to that molecule, and not a function of PE. One animal receiving HY-
360 KDEL did develop capillary leak syndrome at the highest dose, which was only diagnosed upon
361 necropsy following euthanasia. This diagnosis was based on decreased plasma albumin,
362 ascites, and pulmonary edema. Many of the animals receiving the ITs had early AIDS, with
363 increasing weight loss, diarrhea, and opportunistic infections. Yet most tolerated the ITs well,
364 even at very high doses. These results are consistent with observations in healthy, uninfected
365 macaques dosed with 3B3-PE38, a precursor of HY-KDEL, and with CD4-PE40 (45).

366 We studied the immunogenicity of the ITs in the macaques, using ELISA to measure
367 Abs that bind to the IT (Fig. 2). In panel A, we show the relationship of plasma viral load (left
368 axis, bars) and antibody response (right axis, lines) in three macaques from experiment 3. By 3
369 weeks, 2 of the macaques had developed a high level of anti-IT Ab (J97183 and J98124), one
370 had a lower, but measurable response (K98099). All macaques appeared to have an initial
371 decline in viral load with initiation of IT therapy, but in only K98099 was that suppression of
372 viremia maintained beyond the third week. These data suggest an inverse relationship between
373 IT efficacy and immunogenicity. In Fig. 2B and C, we show the relationship between CD4 cell
374 count and anti-IT antibody responses. All but one of the animals with normal CD4 counts
375 (>300), developed anti-IT antibodies, and the correlation between CD4 counts and antibody
376 levels was highly significant.

377 Serial plasma viral loads are shown in the Supplemental Table. In 6 of 11 animals
378 receiving adequate doses of IT (experiments 3 and 4), there was a >50% decline in viral load 7
379 days post treatment, when compared to the average of three pretreatment values. There was a
380 mean 75% reduction in viral load at day 7 in animals receiving 7B2-RAC+CD4-IgG (32-64µg/kg)
381 in experiment 3, compared to a 10% mean reduction in those animals receiving HY-PE or HY-
382 KDEL (125-500 µg/kg, experiment 4). These differences were not statistically significant. Nor
383 was there a significant difference in pre vs post-treatment VLs by paired t tests. However, these
384 studies were insufficiently powered to test the efficacy of the ITs. Only a total of 11 animals
385 (experiments 2, 3, and 4) received a sufficient amount of IT to expect an effect. Interestingly, the

386 degree of response to IT therapy at day 7 was significantly negatively correlated with initial viral
387 load ($R^2=.49$, $P=.016$), suggesting that free virus in the plasma may act as an immunological
388 “sink”, binding to the IT before it can reach the target cell. The effect of immunogenicity on the
389 efficacy of the IT was also demonstrated: there was a marginally significant negative correlation
390 between levels of anti-IT antibody and the therapeutic responses to IT at day 21 ($R^2=.37$,
391 $P=.04$), but not at earlier time points.

392 These data show that ITs using RAC or PE were well tolerated, even in sick animals.
393 The ITs were immunogenic if animals had a functional immune system, and this immunogenicity
394 likely affected efficacy. The best responses were observed in animals treated with 7B2-RAC +
395 CD4-IgG2, even though the maximal dose was 8-fold less than that used for HY-PE or HY-
396 KDEL. The HY ITs are single chain constructs, whereas 7B2-RAC uses intact IgG. The longer
397 $T_{1/2}$ of Fc containing molecules could explain the increased efficacy of 7B2-RAC. The dose of
398 7B2-RAC used was 2-3 orders of magnitude lower than the amount of MAbs used in recent
399 macaque studies that demonstrated the therapeutic efficacy of neutralizing MAbs in treating
400 active SHIV infection (19, 20).

401

402 **Recombinant Toxins**

403 The major limitation to the use of repeated doses of ITs is immunogenicity, not toxicity.
404 Less immunogenic alternatives to the use of toxins have been developed for cancer therapy,
405 and we have sought to apply these to the development of anti-HIV ITs. One approach is the use
406 of recombinant toxins. Gel, a single chain protein toxin has the same N-glycosidase activity as
407 ricin A chain, but lacks a cell-binding B chain. ITs targeting CD33 conjugated to Gel have
408 recently completed phase I clinical trials demonstrating excellent in vitro cytotoxicity against
409 target cells and low immunogenicity in patients, despite multiple injections (46). Figs. 3 and 4
410 show results comparing 7B2-RAC to 7B2-Gel in vitro and in vivo. Direct toxicity of the ITs on
411 H9/NL4-3 and uninfected parental H9 cells (Fig. 3A) showed that the RAC-based IT was 5-10X
412 more effective than its Gel counterpart. Yet Gel should have equimolar toxic activity to RAC,

413 since both have the same enzymatic activity. Because the Gel was recombinant, we asked
 414 whether recombinant toxins function less well than native when used in an IT, Fig. 3B. Two
 415 forms of 7B2-ricin A chain were prepared, one using deglycosylated ricin A chain obtained from
 416 castor beans (RAC), the other with recombinant ricin toxin A (rRTA), made in *E. coli*. Two
 417 different highly-purified preparations of rRTA were tested. One was of sufficient purity to yield
 418 high-grade crystals (Fig. 3B, left). RAC outperformed the rRTA by threefold (Fig. 3B, right).

419 We tested the comparative efficacy of RAC vs Gel ITs in suppressing infection with
 420 primary isolates in tissue culture cell lines (Fig. 3C) and PHA blasts (3D), showing similar
 421 results, with greater activity demonstrated by the IT using RAC. Studies in mice demonstrated
 422 that the RAC IT had greater anti-HIV activity than Gel, measured both as plasma p24 (Fig. 4A)
 423 and infectious centers (mean values: untreated 15845 infectious cells/spleen, 7B2-Gel 3039,
 424 7B2-RAC 184, differences are not significant). Both RAC and Gel had similar immunogenicity
 425 (Fig. 4B). These results indicate that the use of Gel has little effect on immunogenicity, but
 426 results in a less effective IT compared to deglycosylated RAC.

428 **ADCs**

429 Because ADCs are even less likely to be immunogenic, we have also studied the
 430 efficacy of MAb 7B2 conjugated to small drug molecules. A concern about the use of ADCs for
 431 targeting HIV-reservoirs, is that the current generation of ADCs utilize agents that target dividing
 432 cells (e.g. tubulin inhibitors) and it is likely that persistent reservoirs of HIV include resting cells.
 433 Fig. 5A shows the molar toxicity on H9 cells of ricin and potent cytotoxic drugs used in the
 434 therapy of lymphomas and leukemia's. Ricin is 3 logs more cytotoxic than gemcitabine, the most
 435 effective conventional chemotherapeutic agent. Four different ADCs were prepared (Fig. 5B),
 436 conjugating epirubicin or gemcitabine to anti-Env MAbs, using either non-cleavable or peptide
 437 cleavable linkers. The first ADC that we tested was 7B2 conjugated to epirubicin. The ADC
 438 bound to infected cells, because both Ig and epirubicin were detected on cells by flow cytometry
 439 (Fig 5C). But no cytotoxicity was observed (Fig 5D and E). We reasoned that a more effective

440 drug would be needed. But conjugation to gemcitabine also failed to produce an effective
441 conjugate (Fig 5F). The non-specific toxicity observed at very high doses of ADC, suggests that
442 the small drugs attached to the MAbs retained their cytotoxic activity following conjugation.

443 Having failed with conventional chemotherapeutic agents, we next made conjugates with
444 the newer agents used in recently developed ADCs (47-49), including monomethyl aueromycin
445 E (MMAE), duocarmycin, or maytansine, with non-cleavable or cathepsin-B cleavable linkers
446 (Fig. 6). Titration of cytotoxicity by free drug (Fig. 6A) shows that these agents are 2-3 orders of
447 magnitude more toxic to H9 cells than gemcitabine, but still >1 log less toxic than ricin. Ab to
448 drug ratios of 2-3:1 were obtained with no loss of Ab-binding activity (Fig. 6B), but there was no
449 evidence of cytotoxicity in persistently infected H9/NL4-3 cells or of in vivo efficacy (Fig. 6C and
450 D). Similar conjugates were made with anti-gp120 MAb 924, and they also failed to demonstrate
451 cytotoxicity (not shown). It should be noted that H9/NL4-3 cells are rapidly dividing (doubling
452 time ~18 hr), and so would be expected to be susceptible to the effects of these drugs.
453 Because cells transfected to stably express native HIV Env on the cell surface are more
454 sensitive to the cytotoxic effects of ITs (Pincus, S.H., *et al*, Identification of human anti-HIV
455 gp160 MAbs that make effective ITs, submitted for publication), we tested the ADCs in 293T
456 cells stably expressing gp160 of the clinical isolates 92UG and C97 (26). As shown in Fig. 6E,
457 the ADCs exhibited specific cytotoxicity against the Env-expressing cells at high concentrations
458 of IC (>1µg/ml). The ADCs killed neither non-transfected 293T, nor H9/NL4-3 cells. At these
459 very high concentrations, 7B2-RAC was toxic to non-transfected 293T, but the IT retained
460 specific toxicity even in nanogram concentrations on cells stably expressing gp160-expressing
461 (Pincus, S.H., *et al*, Identification of human anti-HIV gp160 MAbs that make effective ITs,
462 submitted for publication).

463

464 **Decreasing immunogenicity of ITs by conjugation to PEG**

465 Although the use of RAC in ITs is confounded by its immunogenicity, it does offer distinct
466 advantages over small drugs for use against HIV. On a molar basis ricin is more toxic to cells

467 (Figs. 5A and 6A) and can kill non-proliferating cells, which could be critical for targeting
468 persistent reservoirs. To improve the utility of RAC for use in anti-HIV ITs, we have sought to
469 lessen immunogenicity by conjugating the IT to PEG (Fig. 7). The results showed that
470 conjugation ratios of 10:1 did not decrease the specific cytotoxic activity of the IT, and that the
471 development of an anti-IT Ab response in mice was partly suppressed by PEG conjugation,
472 despite incomplete PEGylation. These results are consistent with the effects of PEGylation on
473 other therapeutic proteins (50-52). Other approaches to limit immunogenicity have also been
474 proposed (53-58).

475

DISCUSSION

The long-term objective of these studies is to develop cytotoxic ICs for use in purging persistent reservoirs of HIV infection. The activation of latent provirus may be necessary to induce expression of sufficient Env to result in cell killing. Using histone deacetylase inhibitors, investigators have demonstrated activation of latent reservoirs in patients whose viremia was fully suppressed by anti-retroviral therapy (14-17). However, relying on viral cytopathic effect and/or host defenses to eliminate activated cells, there was no evidence that such treatment reduced the reservoirs in these patients. We propose that a more active approach to eradicating persistently infected cells will be necessary. Our efforts have primarily focused on comparing different anti-Env MAbs for their ability to target ITs to infected cells. We have consistently found that those targeted to the gp41 external helix-loop region (for example, MAb 7B2) when used with sCD4, were the most efficacious MAbs for delivering anti-HIV ITs to HIV-infected cells (4, 8, 18, 33 and Pincus, S.H., et al, Identification of human anti-HIV gp160 MAbs that make effective ITs, submitted for publication). To extend this observation, we tested ITs and ADCs based upon MAb 7B2 for safety and efficacy, in both mice and SHIV-infected macaques. The animal models reflected active, rather than persistent/latent infection, but they allowed us to compare different ICs and to identify potential problems and solutions for the use of ITs in HIV infection.

The major findings to emerge from these studies are: A) Anti-HIVs ITs were well-tolerated in SHIV-infected macaques, even with relatively advanced AIDS. The demonstration of safety is reassuring given the toxicity shown by CD4-PE40 in the only human trials of an anti-HIV IT (43, 44). Dose limiting toxicity with CD4-PE40 in macaques was similar to that observed in humans (15µg/kg) whereas other anti-HIV ITs were tolerated at much higher doses. B) In vivo efficacy of 7B2-RAC+CD4-IgG2 in immunodeficient mice was clearly demonstrated. The data suggest that the ITs showed therapeutic efficacy in macaques, but that any benefit appeared to be short-lived and blunted by immunogenicity elicited by the toxins. C) ADCs were marginally effective in cytotoxicity assays relative to ITs. No specific cytotoxicity of ADCs was observed on

503 infected cells, but was clearly demonstrated at high ADC concentrations on that stably express
504 Env, which are more sensitive to ICs. D) The immunogenicity of ITs can be suppressed by
505 PEGylation, suggesting a potential solution to this problem.

506 ITs to the CD4bs, all single-chain constructs, have been shown effective in vitro and in
507 murine models (1, 5-7, 9-11, 13). We found that scFv and other monomeric constructs targeted
508 to the CD4bs functioned well in vitro as ITs, whereas the same V-regions in the context of intact
509 Ig were 10-100X less cytotoxic to infected cells (Pincus, S.H., et al, Identification of human anti-
510 HIV gp160 MAbs that make effective ITs, submitted for publication). In contrast, intact IgG MAbs
511 against the V3-loop of gp120 (MAb 924) and to the gp41 helix-loop region (MAb 7B2) were
512 highly lethal to H9/NL4-3 cells. On the basis of our in vivo studies, we believe that ITs prepared
513 with intact IgG are more effective than those using scFv or other single chain, Fc-lacking
514 constructs. This was observed in mice (8) and by the relative lack of efficacy of HY constructs in
515 macaques, despite use at much higher doses. The greater in vivo effect of ITs using intact Ig is
516 most-likely due to increased plasma residence time (days for Ig vs hrs. for scFv), as we have
517 previously demonstrated (8). Thus the requirement for using single chain constructs to target
518 the CD4bs may limit the utility of such ITs.

519 Our goal is to use Abs to eliminate persistently infected cells, rather than to protect
520 uninfected cells by neutralizing virus infectivity. Yet results from the treatment of active infection
521 with highly effective neutralizing MAbs (19-21) have been extrapolated to HIV eradication
522 efforts, placing great emphasis on the role of virus neutralization (59). However in the case of
523 ICs, this focus on neutralization may be misleading. Clearing persistent reservoirs will require
524 the elimination of cells containing functional provirus. To obtain this cytotoxic effect using MAbs
525 will require either the stimulation of Fc-mediated Ab effector functions (complement-mediated
526 killing, ADCC) or the delivery of toxic moieties to cells. Studies in SCID mice indicate that Fc-
527 mediated effector function can play a role in eliminating HIV reservoirs (60). The direct delivery
528 of toxic agents should enhance the killing of persistently infected cells by MAbs. The different
529 forms of cytotoxic ICs each have merits and disadvantages for use in eradicating HIV infection.

530 Anti-HIV ITs have been extensively tested in vitro, showing activity against different cell types
531 and clinical isolates at therapeutically achievable concentrations; efficacy in murine models has
532 also been shown. But the immunogenicity of ITs could limit their therapeutic utility (61). Anti-IT
533 antibodies rarely result in anaphylactic or other reactions, rather they lead to enhanced
534 clearance and neutralization of IT function. However ITs hold some advantages over the less
535 immunogenic ADCs, including greater cytotoxicity on a molar basis than the best small drugs
536 and ability to kill non-proliferating cells. Radio-ICs do not require internalization for cell killing,
537 but can cause bystander killing and must be used within hours of production due to short
538 isotope half-life. Preclinical studies in a relevant macaque model of latency and activation will be
539 required to determine the relative merits of these approaches.

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FIG. LEGENDS

Fig. 1. Efficacy of anti-HIV ITs in vitro and in mice.

Experiment 1. Demonstration that IT efficacy required conjugation to RAC, and was not due to 7B2 or CD4-IgG2 alone. Top: In vitro measurement of cytotoxicity. HIV-infected H9/NL4-3 cells were incubated with indicated concentrations of MAbs or ITs and cell viability assessed by MTS dye-reduction on day 3. Data are presented as the mean and SEM of triplicate samples. If no error bars are visible, then the error is smaller than the symbol (as with all figures in this manuscript). Representative of >5 experiments. Bottom: Groups of 6-9 animals were injected with 2.5×10^5 H9/NL4-3 cells ip in 40% Matrigel one day after being treated with cyclophosphamide (100 μ g/g). Animals received ip injections of IT (10 μ g), MAb (10 μ g) and/or CD4-IgG2 (6 μ g) on day 1, 3, and 5. Mice were sacrificed on day 7. Plasma p24 was determined by antigen capture ELISA. Group means and standard errors are shown. Statistical comparisons used the Mann-Whitney non-parametric t test. * $P < .05$, ** $P < .01$, if bars not marked, then NS. Representative of 2-3 experiments (depending upon IT).

Experiment 2. ITs targeted against gp41 were effective in vivo, whereas those to the CD4bs were not. Top: In vitro assays show ITs targeted to the CD4bs are effectively delivered by scFv, but not intact IgG. Representative of >5 experiments, depending upon the IT. Bottom: Groups of 8 cyclophosphamide-treated animals were injected with 10^7 H9 (ip) and 3×10^5 H9/NL4-3 (sc) cells in 40% Matrigel. ITs (5 μ g/dose) were administered ip on days 1, 3, 5, 7, 9, and 11. Animals were sacrificed on day 12. Plasma p24, mean and SEM are shown. Although scFv HY-KDEL was highly efficacious in vitro, it showed no effect in vivo. Representative of 2-3 experiments (depending upon the IT).

Fig. 2. Immunogenicity of anti-HIV ITs in macaques. (A) Relationship of antiviral efficacy of IT and anti-IT Ab response in experiment 3. Vertical arrows beneath the time scale indicate

799 times of injection of 7B2-RAC + CD4-IgG, 35µg/kg for the first three doses, 64µg/kg thereafter.
800 Day1 is the first administration of the IT. Plasma viral loads are shown on the left axis and as
801 vertical bars. The time zero viral load represents the mean and SEM of three pre-treatment
802 determinations. Anti-IT Ab is shown on the right axis, using the line graph. Ab is represented as
803 the absorbance obtained in ELISA, with RAC-coated wells and a 1:500 plasma dilution. (B and
804 C) Relationship of CD4 count and anti-IT Ab response. B shows results from individual
805 macaques, C shows the overall correlation. Ab is shown as the change in absorbance between
806 the pre-bleed and the peak antibody level (Delta A₄₀₅). The specific IT the animal received was
807 used as antigen. The F test was used to determine the statistical significance of the correlation.
808

809 **Fig. 3. In vitro efficacy of ITs prepared with deglycosylated RAC and recombinant protein**
810 **toxins.** (A) The cytotoxic effect of 7B2 conjugated to deglycosylated RAC or Gel. Cytotoxicity of
811 the ITs (in the presence of CD4-IgG2) was compared in uninfected H9 cells (left) or persistently-
812 infected H9/NL4-3 (right). An MTS dye-reduction assay was used to measure cytotoxicity on
813 triplicate samples. Mean and SEM are shown. Representative of >5 experiments. (B) The
814 cytotoxic effect of 7B2 conjugated to deglycosylated RAC and rRTA (two different preparations).
815 The purity of the rRTA is demonstrated by its ability to form crystals in 1M Li₂SO₄ and 2.5%
816 polyethylene glycol MW 8,000. Tetragonal crystals measuring up to 0.5 x 0.5 x 0.8 mm were
817 formed. Representative of 3-4 experiments. (C and D) Ability of IT's to inhibit spread of HIV
818 infection. HIV spread in tissue culture was tested in C8166.R5 cells (C) or PHA blasts (D). Virus
819 stocks were grown in PHA blasts. C8166.R5 cells were incubated with virus stock and grown for
820 3 days. Cells were washed, and 10⁴ "infected" cells were mixed with 10⁵ uninfected cells, in the
821 presence of the indicated IT (200 ng/ml) and CD4-IgG2 (200 ng/ml). Cells were incubated for 5
822 days, with aliquots removed daily for p24 antigen testing. Samples were run in triplicate, day 3
823 results are shown, mean and SEM. Representative of 2 experiments, 3 time points per
824 experiment. PHA blast cultures were infected for 24 hr. with cell free supernatant, the cells
825 washed, and triplicate cultures established with indicated ITs. Culture supernatants were

826 sampled on day 5 and 7, day 7 results are shown. Statistically significant (student t) results are
827 indicated: * $P < .05$, ** $P < .01$, *** $P < .001$. Unmarked comparisons are not statistically different.

828

829 **Fig. 4. In vivo effects of RAC and Gel ITs.** (A) Efficacy. Groups of ten mice were injected with
830 10^7 H9 ip and 3×10^5 H9/NL4-3 sc in 0.2ml 40% Matrigel and were treated with the indicated IT
831 (10 μ g/mouse) and CD4-IgG2 (5 μ g) on days 1, 3, 5, 8, and 10. Animals were sacrificed on day
832 12, and plasma p24 determined. Statistical comparisons used the Mann-Whitney test.
833 Representative of 3 experiments. (B) Immunogenicity. Groups of 3 mice received injections of
834 ITs using the murine anti-gp120 MAb 924 conjugated to either RAC or Gel. Mice received 3
835 weekly ip injections of IT (10 μ g) in saline. The development of antibody responses against both
836 components of the IT (MAb 924 and toxin) were measured by ELISA. Results are mean, SEM of
837 triplicate determinations on 1:1000 plasma dilutions. Each line represents an individual mouse.
838 The open circles indicate plasma from a control mouse treated with the inappropriate IT.

839

840 **Fig. 5. Testing of ADCs utilizing the chemotherapeutic agents epirubicin and**
841 **gemcitabine.** (A) Cytotoxicity of unconjugated small drugs used in ADCs, compared to ricin. H9
842 cells were incubated with serial dilutions of conventional chemotherapeutic agents used to treat
843 lymphoma or with ricin. Toxicity was tested in a 3 day MTS dye-reduction assay, and the results
844 converted to % viability. Representative of 2-3 experiments. (B) Characterization of ADCs. (C)
845 Binding of 7B2-RAC and 7B2-epirubicin to H9/NL4-3 cells. Cells were first incubated with
846 unconjugated 7B2 or ADC, washed, and detected using the indicated secondary Abs. MAb and
847 ADC and IT were detected equally with anti-Hu IgG, whereas only the appropriate conjugate
848 gave a signal with RAC or epirubicin specific MAbs. (D) Cytotoxicity of ICs on H9/NL4-3 cells. A
849 3 day MTS assay was performed in the presence of CD4-IgG2. Results are $A_{490} - \text{Bkgd}$, mean
850 and SEM of triplicate samples. Representative of 5 experiments. (E and F). Cytotoxicity of anti-
851 gp120 MAb 924 to epirubicin (E), gemcitabine (F), compared to RAC. The indicated ICs were
852 incubated with uninfected H9 cells, or persistently-infected H9/NL4-3 for 3 days, and cell viability

853 measured by MTS dye reduction. Mean and SEM of triplicate values. Representative of at least
854 two experiments.

855

856 **Fig. 6. Preparation of ADCs using newer cytotoxic agents.** (A) Cytotoxicity of unconjugated
857 small drugs used in ADCs, compared to ricin, as performed in Fig. 5A. Representative of 2
858 experiments. (B) At the top, a tracing from hydrophobic interaction chromatography
859 demonstrates an ADC consisting of a mixture of 0, 2, 4, or 6 small drugs per Ab. The table
860 shows the average drug:MAb ratio calculated from such tracings, as well as the linker type. (C)
861 Cytotoxicity of ADCs and a RAC-conjugate on uninfected H9 and H9/NL4-3, tested in the
862 presence of CD4-IgG2, using MTS dye reduction assay, mean and SEM of triplicate samples.
863 Representative of 2-4 experiments. (D) In vivo efficacy of anti-HIV ICs. Mice received the
864 indicated conjugate in the presence of CD4-IgG2. The first 3 columns on the left are replicated
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868 sCD4 (300ng/ml). Representative of 3 experiments.

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872 **Fig. 7. Effects of PEGylating 7B2-RAC.** PEG₅₀₀₀ was conjugated to 7B2-RAC at 30, 10, or 3X
873 molar ratios. (A) Microcapillary electrophoresis of reduced protein preparations. Results show
874 incomplete PEGylation (arrow heads). Antibody L chain and RAC co-migrate at MW ~28,000.
875 (B) Cytotoxicity of PEGylated ITs. Killing by the 30:1 conjugate was inhibited in MTS assay on
876 H9/NL4-3 cells. (C) Immunogenicity of PEGylated ITs. IT alone or the 10:1 PEG-conjugate was
877 administered to mice (4 mice/group, 2 weekly injections, and bled 1 week later). Murine
878 antibodies against human Ig and to RAC were measured by ELISA.

879

880 **Table 1. Immunoconjugates and their constituents.**
881

Cytotoxic immunoconjugate (IC)	An IC consists of a MAb or other targeting agent linked to a moiety that is toxic to cells. These may include protein toxins, small drugs, or high-energy radionuclides.
Immunotoxin (IT)	An IC wherein the toxic moiety is a protein toxin usually derived from bacteria or plants. Toxins used here include: • Ricin A chain: deglycosylated native ricin A chain (RAC), recombinant ricin toxin A (rRTA) • Pseudomonas exotoxin A: PE40 and PE38 are domain I deletion mutants, lacking receptor binding activity. PE38-KDEL has a C-terminal endoplasmic reticulum-targeting domain • Gelonin (Gel) is a single chain toxin with N-glycosidase activity similar to ricin A chain
Antibody drug conjugate (ADC)	An IC wherein the toxic moiety is a small drug. ADCs used in this study include: • Conventional chemotherapeutics agents: Epirubicin (Epi) and Gemcitabine (Gem) • Potent inhibitors of mitosis: Duocarmycin, Maytansine, and Monomethyl aueromycin E (MMAE)
Radioimmunoconjugate	An IC wherein the toxic moiety is a high energy radionuclide
Targeting agents	Deliver the toxic moiety of an IC to the appropriate target cell. For HIV these include: • Anti-gp160 MAbs used here include: 7B2 (gp41 external domain), HY (gp120 CD4bs), and 924 (gp120 V3 loop) • CD4

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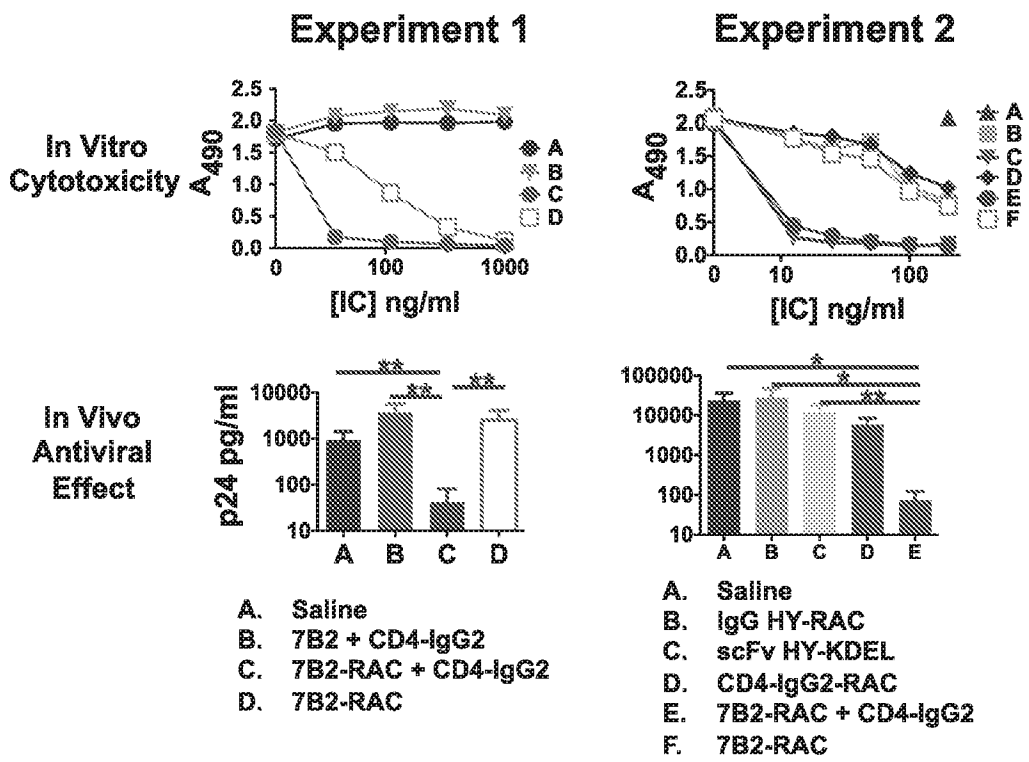


Fig. 1. Efficacy of anti-HIV ITs in vitro and in mice.

Experiment 1. Demonstration that IT efficacy required conjugation to RAC, and was not due to 7B2 or CD4-IgG2 alone. Top: In vitro measurement of cytotoxicity. HIV-infected H9/NL4-3 cells were incubated with indicated concentrations of MAbs or ITs and cell viability assessed by MTS dye-reduction on day 3. Data are presented as the mean and SEM of triplicate samples. If no error bars are visible, then the error is smaller than the symbol (as with all figures in this manuscript). Representative of >5 experiments. Bottom: Groups of 6-9 animals were injected with 2.5×10^5 H9/NL4-3 cells ip in 40% Matrigel one day after being treated with cyclophosphamide (100 μ g/g). Animals received ip injections of IT (10 μ g), MAb (10 μ g) and/or CD4-IgG2 (6 μ g) on day 1, 3, and 5. Mice were sacrificed on day 7. Plasma p24 was determined by antigen capture ELISA. Group means and standard errors are shown. Statistical comparisons used the Mann-Whitney non-parametric t test. * $P < .05$, ** $P < .01$, if bars not marked, then NS. Representative of 2-3 experiments (depending upon IT).

Experiment 2. ITs targeted against gp41 were effective in vivo, whereas those to the CD4bs were not. Top: In vitro assays show ITs targeted to the CD4bs are effectively delivered by scFv, but not intact IgG. Representative of >5 experiments, depending upon the IT. Bottom: Groups of 8 cyclophosphamide-treated animals were injected with 10^7 H9 (ip) and 3×10^5 H9/NL4-3 (sc) cells in 40% Matrigel. ITs (5 μ g/dose) were administered ip on days 1, 3, 5, 7, 9, and 11. Animals were sacrificed on day 12. Plasma p24, mean and SEM are shown. Although scFv HY-KDEL was highly efficacious in vitro, it showed no effect in vivo. Representative of 2-3 experiments (depending upon the IT).

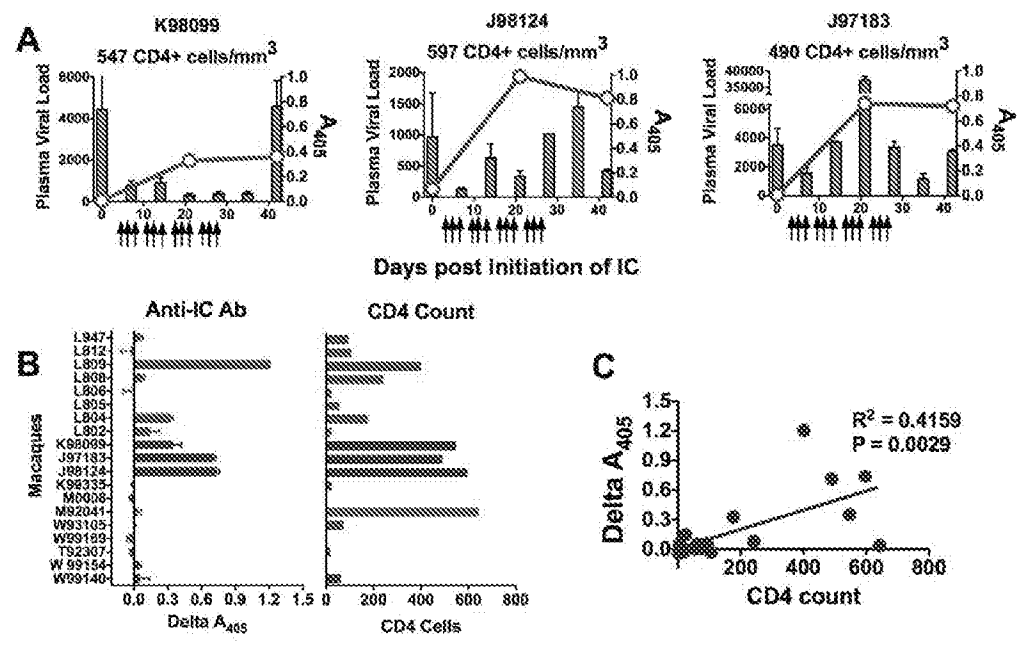


Fig. 2. Immunogenicity of anti-HIV ITs in macaques. (A) Relationship of antiviral efficacy of IT and anti-IT antibody response in experiment 3. Vertical arrows beneath the time scale indicate times of injection of 7B2-RAC + CD4-IgG, 35µg/kg for the first three doses, 64µg/kg thereafter. Day1 is the first administration of the IT. Plasma viral loads are shown on the left axis and as vertical bars. The time zero viral load represents the mean and SEM of three pre-treatment determinations. Anti-IT Ab is shown on the right axis, using the line graph. Ab is represented as the absorbance obtained in ELISA, with RAC-coated wells and a 1:500 plasma dilution. (B and C) Relationship of CD4 count and anti-IT Ab response. B shows results from individual macaques, C shows the overall correlation. Ab is shown as the change in absorbance between the pre-bleed and the peak antibody level (Delta A₄₀₅). The specific IT the animal received was used as antigen. The F test was used to determine the statistical significance of the correlation.

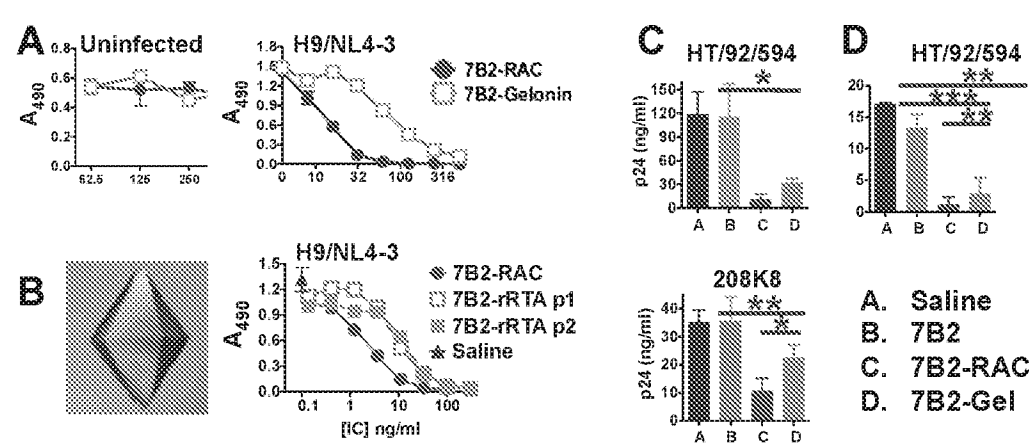


Fig. 3. In vitro efficacy of ITs prepared with deglycosylated RAC and recombinant protein toxins. (A) The cytotoxic effect of 7B2 conjugated to deglycosylated RAC or Gel. Cytotoxicity of the ITs (in the presence of CD4-IgG2) was compared in uninfected H9 cells (left) or persistently-infected H9/NL4-3 (right). An MTS dye-reduction assay was used to measure cytotoxicity on triplicate samples. Mean and SEM are shown. Representative of >5 experiments. (B) The cytotoxic effect of 7B2 conjugated to deglycosylated RAC and rRTA (two different preparations). The purity of the rRTA is demonstrated by its ability to form crystals in 1M Li_2SO_4 and 2.5% polyethylene glycol MW 8,000. Tetragonal crystals measuring up to 0.5 x 0.5 x 0.8 mm were formed. Representative of 3-4 experiments. (C and D) Ability of IT's to inhibit spread of HIV infection. HIV spread in tissue culture was tested in C8166.R5 cells (C) or PHA blasts (D). Virus stocks were grown in PHA blasts. C8166.R5 cells were incubated with virus stock and grown for 3 days. Cells were washed, and 10^4 "infected" cells were mixed with 10^5 uninfected cells, in the presence of the indicated IT (200 ng/ml) and CD4-IgG2 (200 ng/ml). Cells were incubated for 5 days, with aliquots removed daily for p24 antigen testing. Samples were run in triplicate, day 3 results are shown, mean and SEM. Representative of 2 experiments, 3 time points per experiment. PHA blast cultures were infected for 24 hr. with cell free supernatant, the cells washed, and triplicate cultures established with indicated ITs. Culture supernatants were sampled on day 5 and 7, day 7 results are shown. Statistically significant (student t) results are indicated: * $P < .05$, ** $P < .01$, *** $P < .001$. Unmarked comparisons are not statistically different.

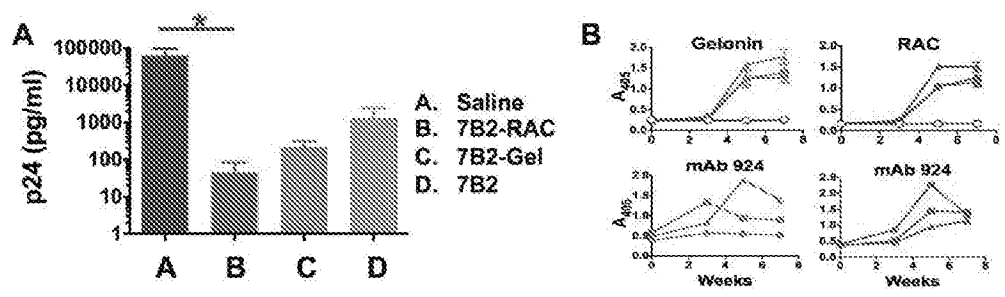


Fig. 4. In vivo effects of RAC and Gel ITs. (A) Efficacy. Groups of ten mice were injected with 10^7 H9 ip and 3×10^5 H9/NL4-3 sc in 0.2ml 40% Matrigel and were treated with the indicated IT (10 μ g/mouse) and CD4-IgG2 (5 μ g) on days 1, 3, 5, 8, and 10. Animals were sacrificed on day 12, and plasma p24 determined. Statistical comparisons used the Mann-Whitney test. Representative of 3 experiments. (B) Immunogenicity. Groups of 3 mice received injections of ITs using the murine anti-gp120 MAb 924 conjugated to either RAC or Gel. Mice received 3 weekly ip injections of IT (10 μ g) in saline. The development of antibody responses against both components of the IT (MAb 924 and toxin) were measured by ELISA. Results are mean, SEM of triplicate determinations on 1:1000 plasma dilutions. Each line represents an individual IT. The open circles indicate plasma from a control mouse treated with the inappropriate IT.

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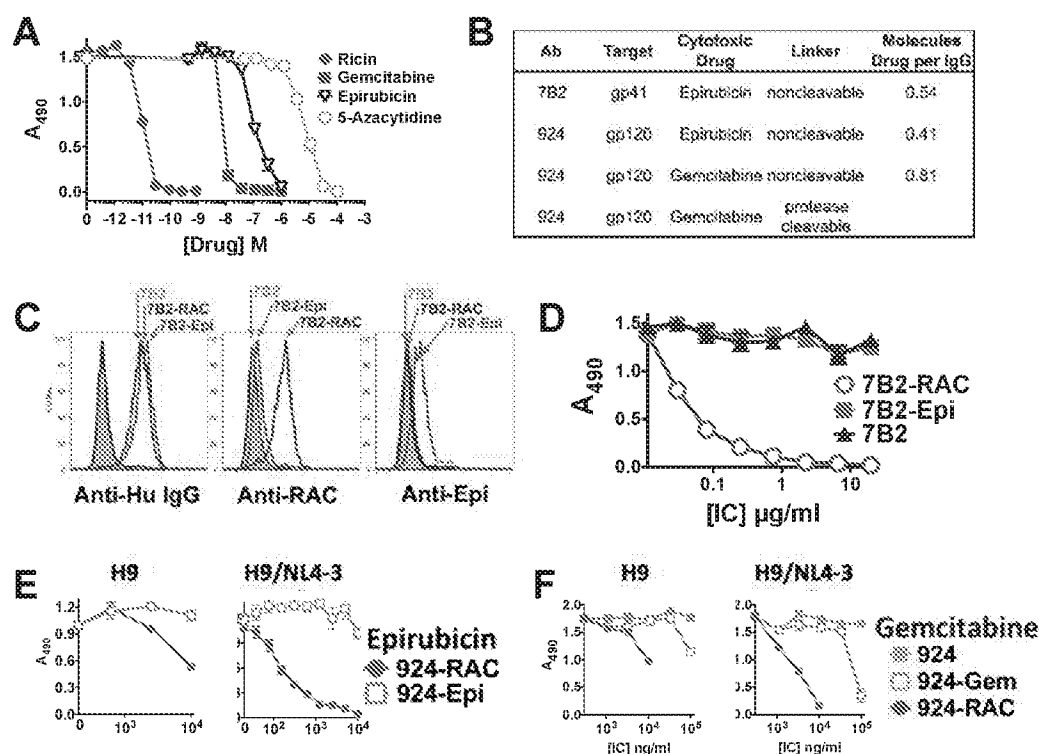


Fig. 5. Testing of ADCs utilizing the chemotherapeutic agents epirubicin and gemcitabine. (A) Cytotoxicity of unconjugated small drugs used in ADCs, compared to ricin. H9 cells were incubated with serial dilutions of conventional chemotherapeutic agents used to treat lymphoma or with ricin. Toxicity was tested in a 3 day MTS dye-reduction assay, and the results converted to % viability. Representative of 2-3 experiments. (B) Characterization of ADCs. (C) Binding of 7B2-RAC and 7B2-epirubicin to H9/NL4-3 cells. Cells were first incubated with unconjugated 7B2 or ADC, washed, and detected using the indicated secondary Abs. MAb and ADC and IT were detected equally with anti-Hu IgG, whereas only the appropriate conjugate gave a signal with RAC or epirubicin specific MAbs. (D) Cytotoxicity of ICs on H9/NL4-3 cells. A 3 day MTS assay was performed in the presence of CD4-IgG2. Results are $A_{490} - \text{Bkgd}$, mean and SEM of triplicate samples. Representative of 5 experiments. (E and F). Cytotoxicity of anti-gp120 MAb 924 to epirubicin (E), gemcitabine (F), compared to RAC. The indicated ICs were incubated with uninfected H9 cells, or persistently-infected H9/NL4-3 for 3 days, and cell viability measured by MTS dye reduction. Mean and SEM of triplicate values. Representative of at least two experiments.

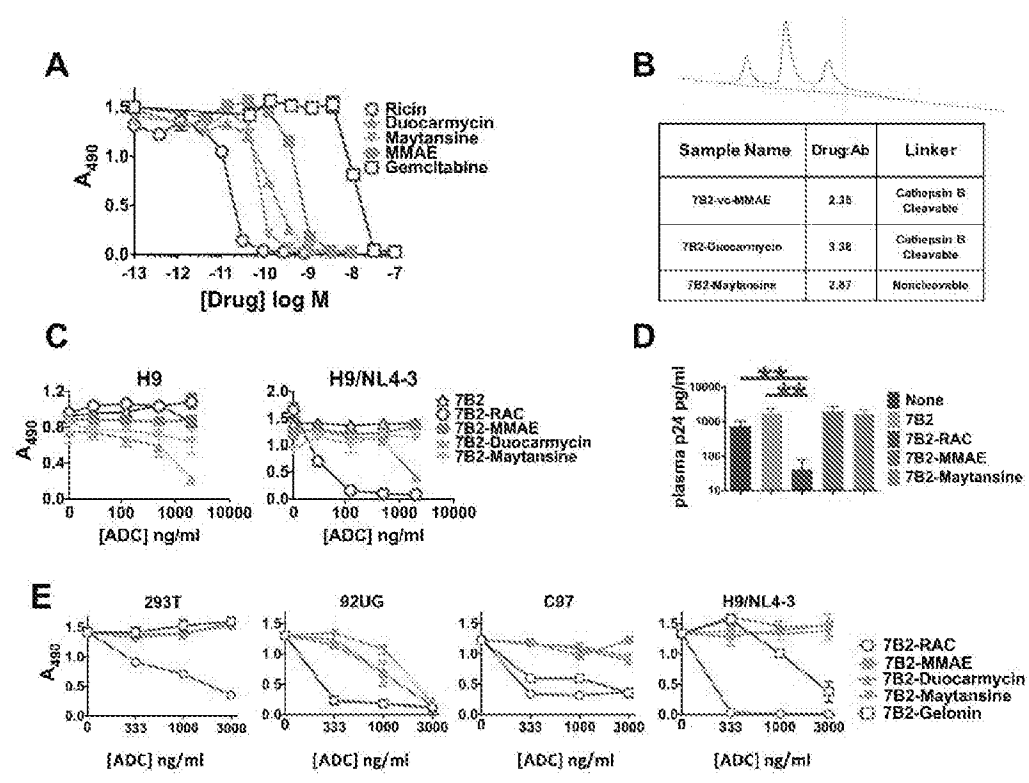


Fig. 6. Preparation of ADCs using newer cytotoxic agents. (A) Cytotoxicity of unconjugated small drugs used in ADCs, compared to ricin, as performed in Fig. 5A. Representative of 2 experiments. (B) At the top, a tracing from hydrophobic interaction chromatography demonstrates an ADC consisting of a mixture of 0, 2, 4, or 6 small drugs per Ab. The table shows the average drug:MAB ratio calculated from such tracings, as well as the linker type. (C) Cytotoxicity of ADCs and a RAC-conjugate on uninfected H9 and H9/NL4-3, tested in the presence of CD4-IgG2, using MTS dye reduction assay, mean and SEM of triplicate samples. Representative of 2-4 experiments. (D) In vivo efficacy of anti-HIV ICs. Mice received the indicated conjugate in the presence of CD4-IgG2. The first 3 columns on the left are replicated from Fig. 1, and all were part of the same experiment. One of 2 experiments. (E) ADCs were tested on 293T cells stably expressing native gp120/gp41 trimers of the clinical isolates 92UG and C97, on non-transfected 293T, and on H9/NL4-3. ADCs were tested in the presence of sCD4 (300ng/ml). Representative of 3 experiments.

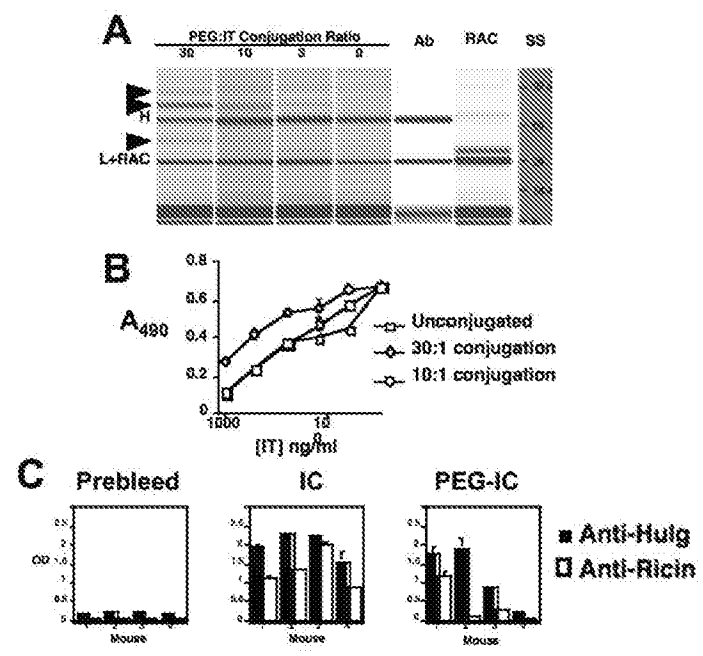
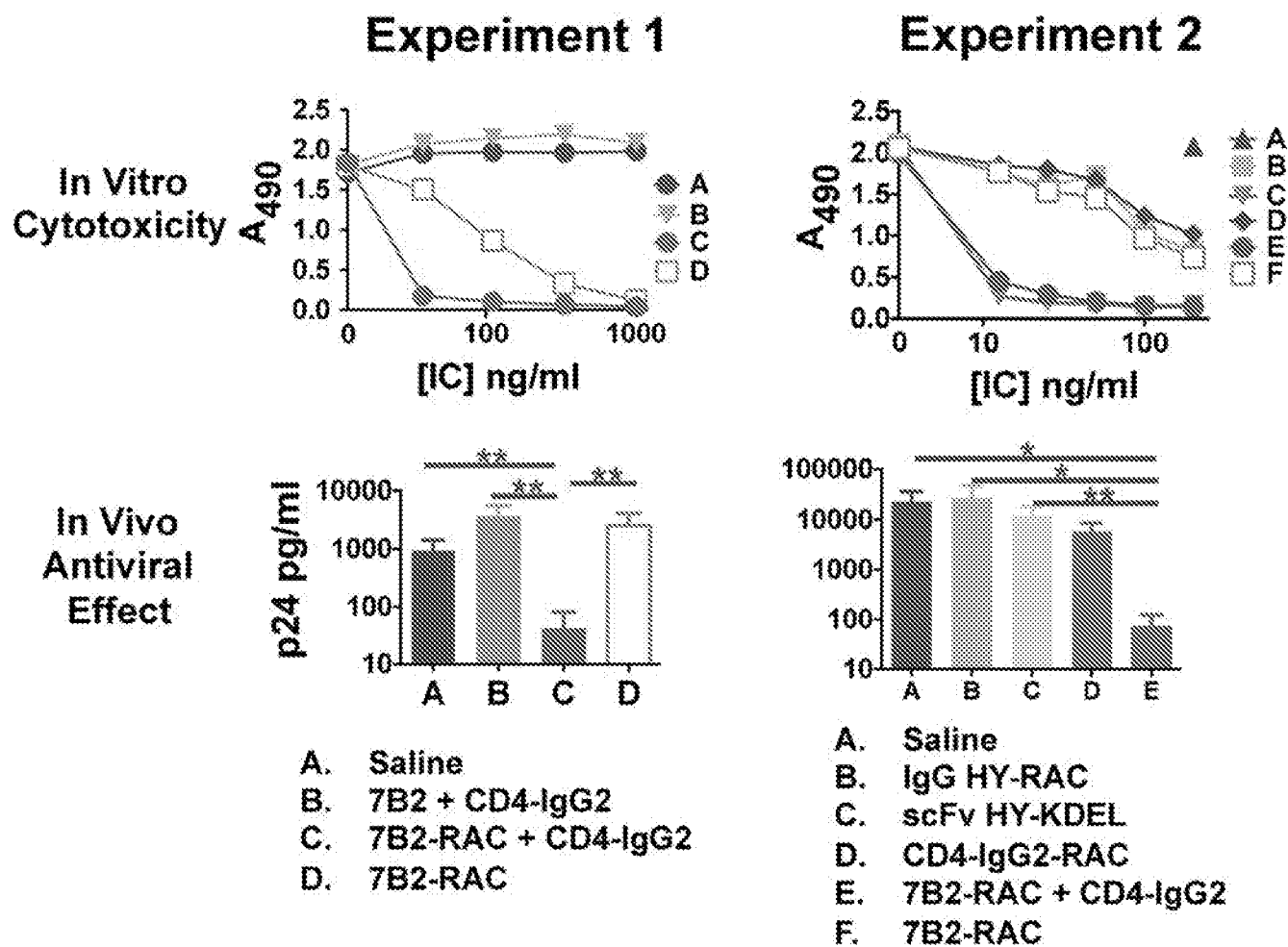
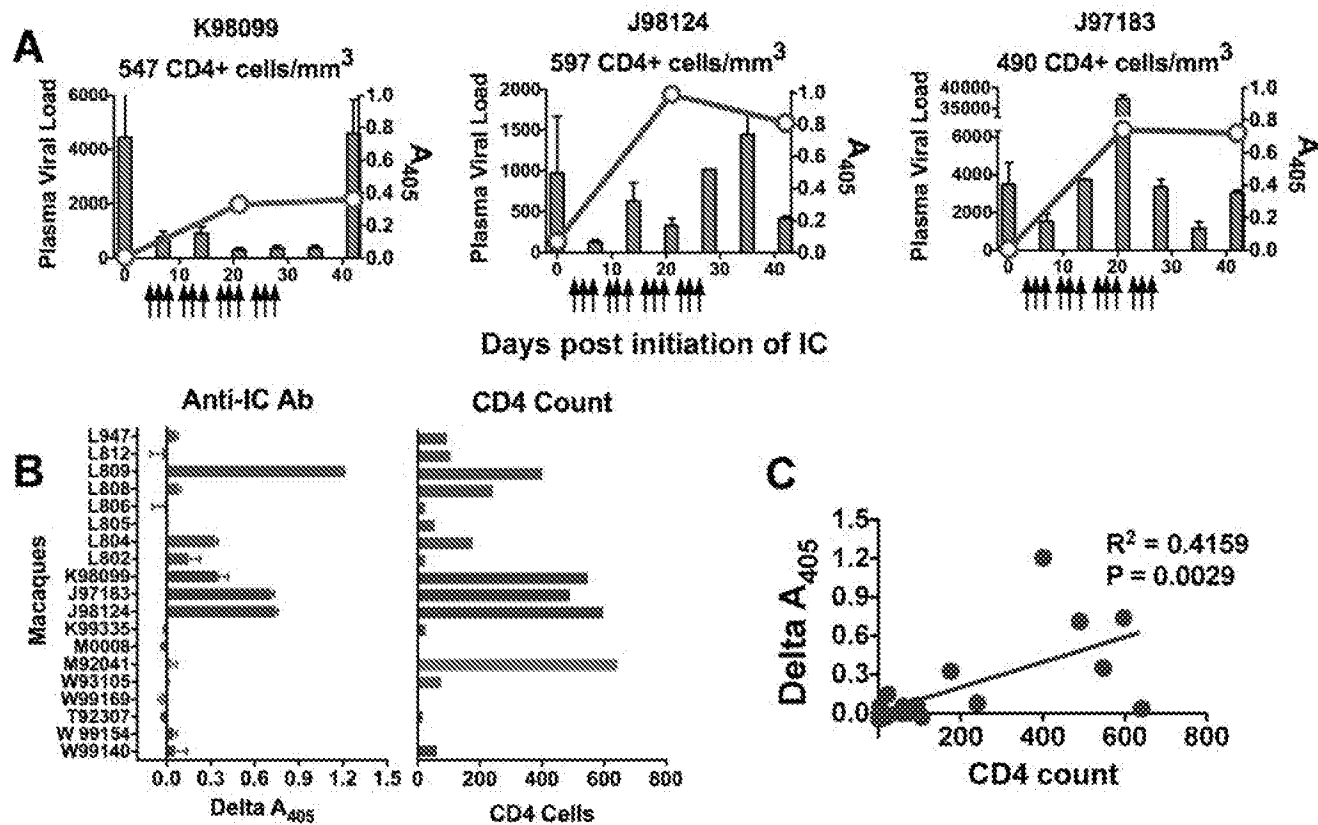
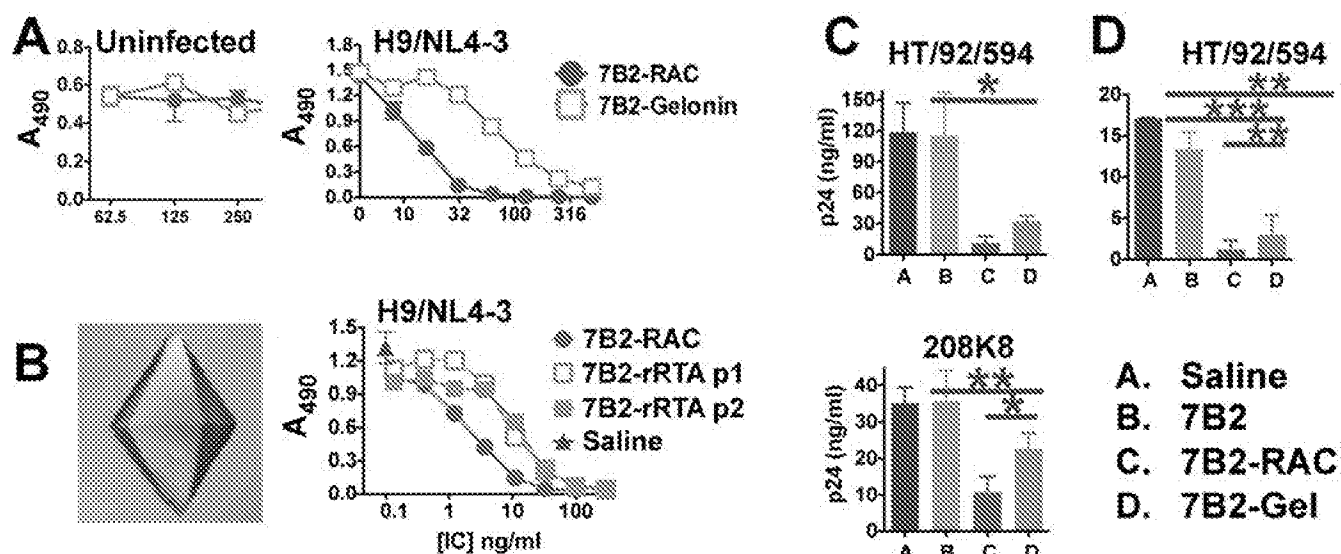


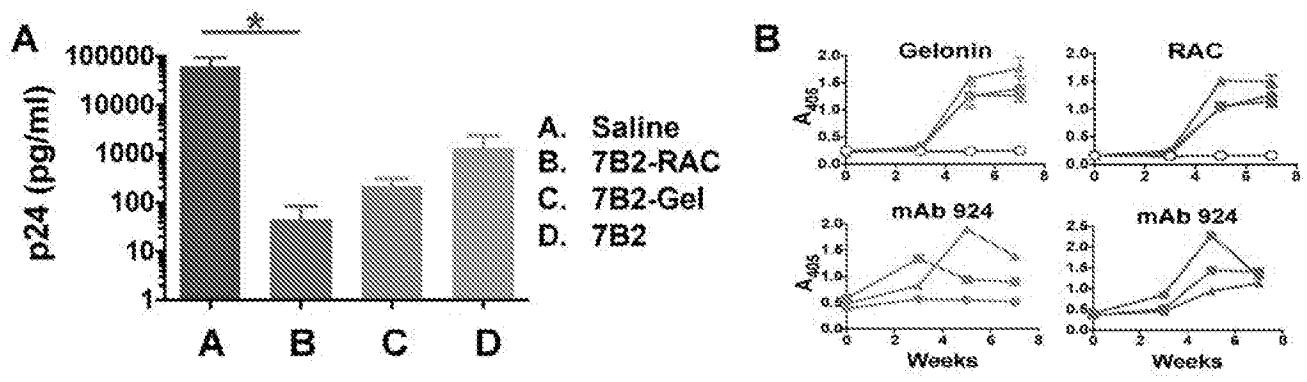
Fig. 7. Effects of PEGylating 7B2-RAC. PEG₅₀₀₀ was conjugated to 7B2-RAC at 30, 10, or 3X molar ratios. (A) Microcapillary electrophoresis of reduced protein preparations. Results show incomplete PEGylation (arrow heads). Antibody L chain and RAC co-migrate at MW ~28,000. (B) Cytotoxicity of PEGylated ITs. Killing by the 30:1 conjugate was inhibited in MTS assay on H9/NL4-3 cells. (C) Immunogenicity of PEGylated ITs. IT alone or the 10:1 PEG-conjugate was administered to mice (4 mice/group, 2 weekly injections, and bled 1 week later). Murine antibodies against human Ig and to RAC were measured by ELISA.

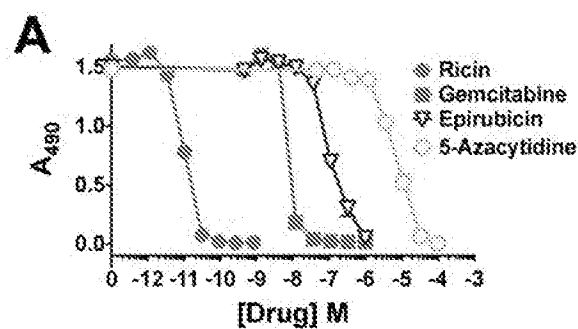
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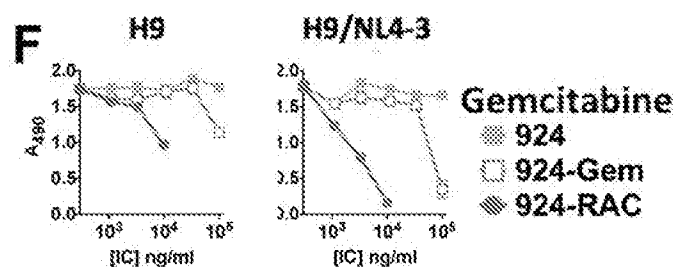
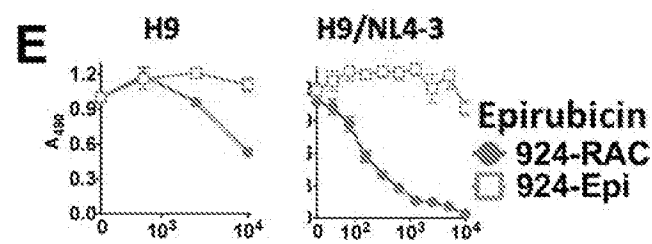
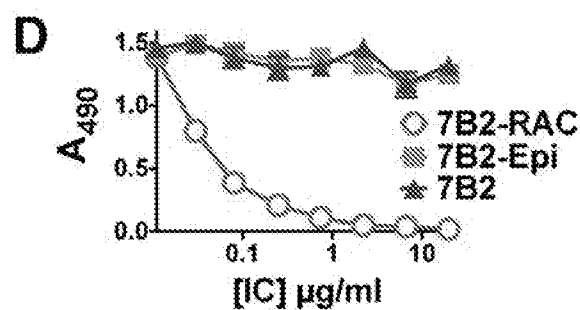
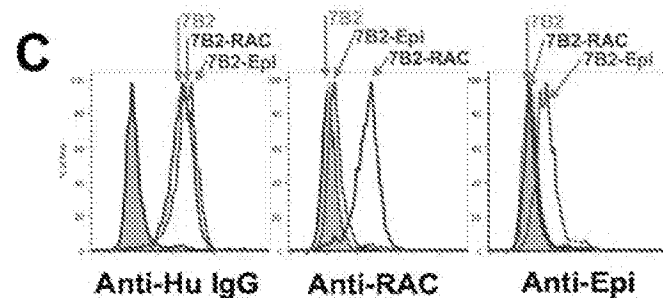




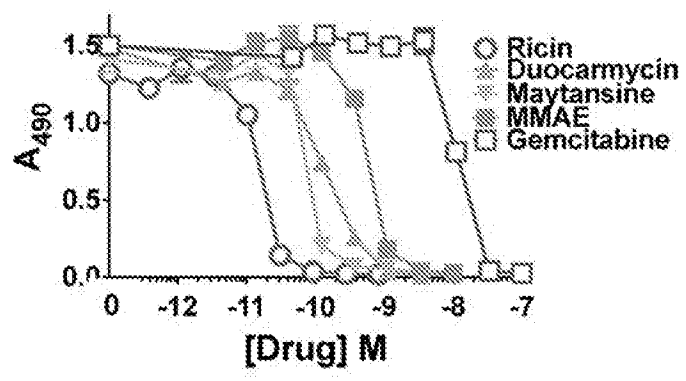


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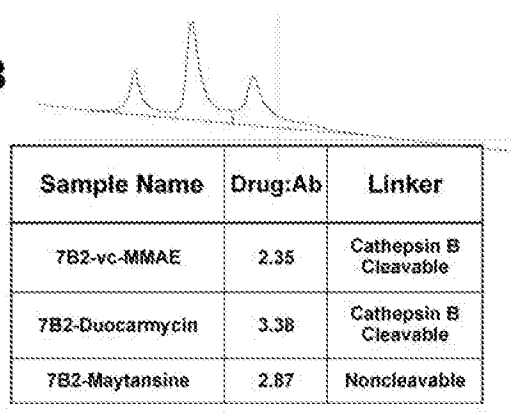
Ab	Target	Cytotoxic Drug	Linker	Molecules Drug per IgG
7B2	gp41	Epirubicin	noncleavable	0.54
924	gp120	Epirubicin	noncleavable	0.41
924	gp120	Gemcitabine	noncleavable	0.81
924	gp120	Gemcitabine	protease cleavable	



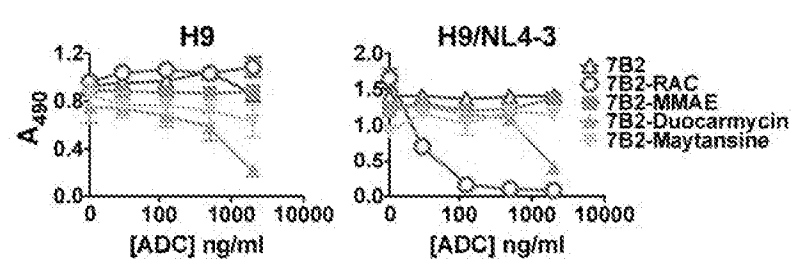
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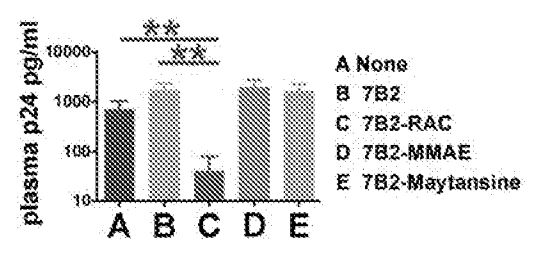
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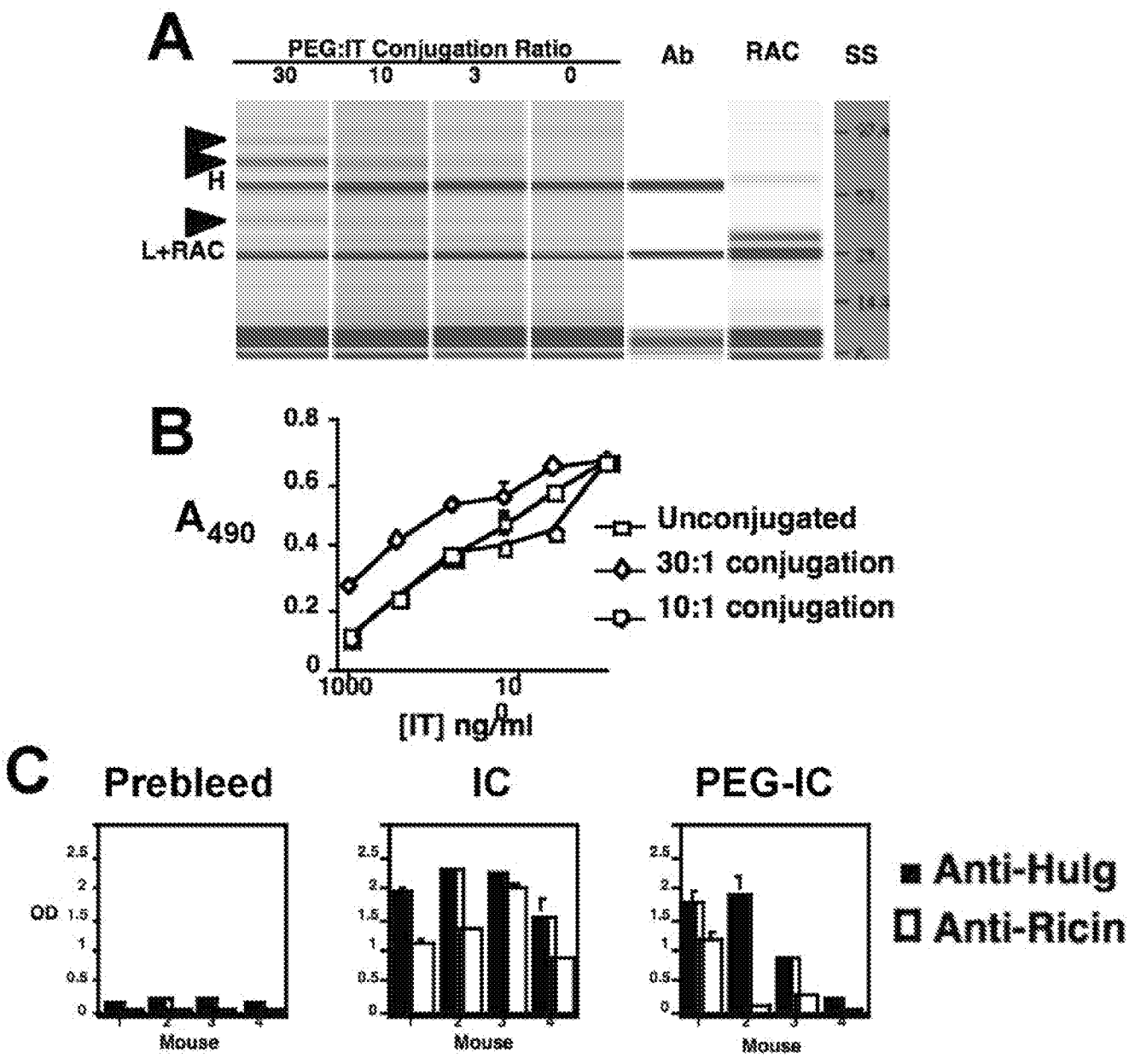


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Novel antibody–antibiotic conjugate eliminates intracellular *S. aureus*

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Staphylococcus aureus is considered to be an extracellular pathogen. However, survival of *S. aureus* within host cells may provide a reservoir relatively protected from antibiotics, thus enabling long-term colonization of the host and explaining clinical failures and relapses after antibiotic therapy. Here we confirm that intracellular reservoirs of *S. aureus* in mice comprise a virulent subset of bacteria that can establish infection even in the presence of vancomycin, and we introduce a novel therapeutic that effectively kills intracellular *S. aureus*. This antibody–antibiotic conjugate consists of an anti-*S. aureus* antibody conjugated to a highly efficacious antibiotic that is activated only after it is released in the proteolytic environment of the phagolysosome. The antibody–antibiotic conjugate is superior to vancomycin for treatment of bacteraemia and provides direct evidence that intracellular *S. aureus* represents an important component of invasive infections.

S. aureus is the leading cause of bacterial infections in humans worldwide and represents a major health problem in both hospital and community settings¹. However, *S. aureus* is not exclusively a pathogen and commonly colonizes the anterior nares and skin of healthy individuals. When infection does occur, the most serious infections such as endocarditis, osteomyelitis, necrotizing pneumonia and sepsis take hold after dissemination of the bacteria into the bloodstream². Over the last several decades, infection with *S. aureus* has become increasingly difficult to treat due to the emergence and rapid spread of methicillin-resistant *S. aureus* (MRSA), which is resistant to all known β -lactam antibiotics³. Alarming, reduced susceptibility to vancomycin and resistance to linezolid and daptomycin have already been reported in MRSA clinical strains⁴.

Investigations going back at least 50 years have revealed that *S. aureus* is able to invade and survive inside mammalian cells, including the phagocytic cells that are responsible for bacterial clearance^{5–11}. *S. aureus* is taken up by host phagocytic cells, primarily neutrophils and macrophages, within minutes after intravenous infection¹². While the majority of the bacteria are effectively killed by these cells, incomplete clearance of *S. aureus* inside blood-borne phagocytes can allow these infected cells to act as ‘Trojan horses’ for dissemination of the bacteria away from the initial site of infection. Indeed, patients with normal neutrophil counts may be more prone to disseminated disease than those with reduced neutrophil counts^{5,13,14}. Once delivered to the tissues, *S. aureus* can invade various non-phagocytic cell types, and intracellular *S. aureus* in tissues is associated with chronic or recurrent infections including osteomyelitis¹⁵, recurrent rhinosinusitis¹⁶, pulmonary infections¹⁷ and endocarditis¹⁸. In addition, intracellular *S. aureus* has been shown to undermine innate immune responses and induce subsequent destruction of neutrophils^{19,20}. Together, these data suggest that ablating intracellular *S. aureus* is key to clinical success, but until now there has been no way to test this hypothesis directly.

Intracellular MRSA are protected from antibiotics

To confirm the hypothesis that mammalian cells provide a protective niche for *S. aureus* in the presence of antibiotic therapy, we compared the efficacy of three major antibiotics that are currently used as the standard of care for invasive MRSA infections (vancomycin, daptomycin and linezolid) against extracellular planktonic bacteria versus bacteria sequestered inside murine macrophages (Table 1). All three antibiotics failed to kill a highly virulent community-acquired MRSA strain, USA300, sequestered inside macrophages exposed to clinically achievable concentrations of the antibiotics, consistent with other studies showing that the majority of existing antibiotics are inefficient at killing intracellular *S. aureus* both *in vitro*²¹ and *in vivo*²².

Intracellular *S. aureus* spreads infection

To compare directly the virulence of intracellular bacteria versus free-living planktonic bacteria and to determine whether the intracellular bacteria are able to establish infection in the presence of vancomycin *in vivo*, mice were infected with equivalent doses of *S. aureus* taken directly from broth culture or bacteria sequestered inside host peritoneal

Table 1 | Antibiotic minimum inhibitory concentrations for MRSA

Antibiotics	Extracellular MRSA (MIC ($\mu\text{g ml}^{-1}$))	Intracellular MRSA (MIC ($\mu\text{g ml}^{-1}$))	Serum C_{max} ($\mu\text{g ml}^{-1}$)
Vancomycin	1	>100	50
Daptomycin	4	>100	60
Linezolid	0.3	>20	20
Rifampicin	0.004	50	20

Extracellular MIC is the minimum antibiotic dose that prevented growth of MRSA seeded in trypticase soy broth. Intracellular MIC is the minimum dose that prevented survival of MRSA sequestered inside murine macrophages (> indicates continued growth at the highest antibiotic dose tested). Data are summarized from three different experiments. Serum C_{max} , the expected serum concentrations for clinically relevant antibiotics²⁷.

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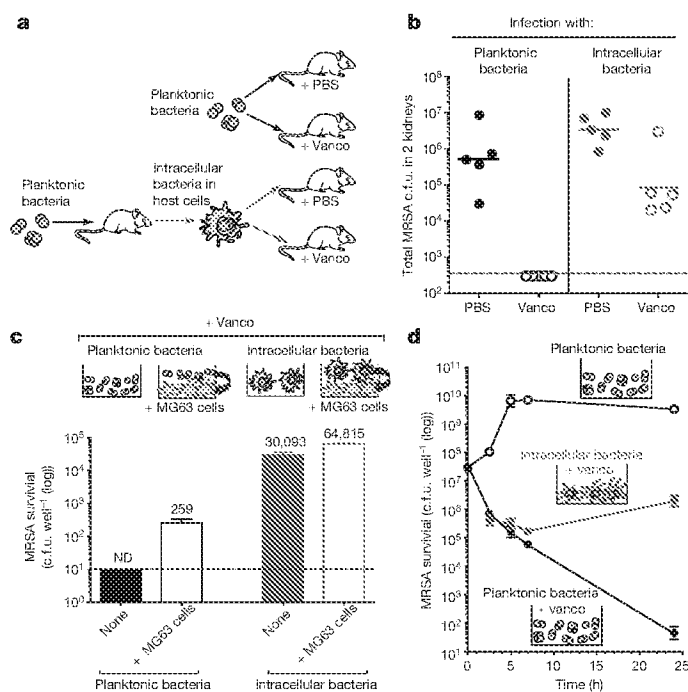


Figure 1 | Intracellular MRSA are protected from vancomycin.

a, Experimental design for generating planktonic versus intracellular bacteria for infection and treatment with vancomycin (vanco). **b**, Bacterial loads in kidney, 4 days after infection. c.f.u., colony-forming units. Each point represents data from a single mouse ($n = 5$ mice per group). **c**, Planktonic or intracellular bacteria were suspended in media containing vancomycin and cultured alone or on a monolayer of MG63, and surviving bacteria were enumerated 1 day later. Error bars represent means $\pm$ standard deviation (s.d.). **d**, Survival of MRSA cultured with vancomycin with or without MG63 cells. Error bars show s.d. ND, none detected. Dashed lines indicate the limit of detection. Representative of three independent experiments.

macrophages and neutrophils (Fig. 1a). Mice infected with intracellular bacteria had equivalent or higher bacterial burdens in the kidneys 4 days after infection compared with those infected with planktonic bacteria (Fig. 1b). Infection with intracellular bacteria also resulted in more consistent colonization of the brain (Extended Data Fig. 1). To characterize this observation further, we infected MG63 osteoblasts with either planktonic MRSA or an equivalent number of intracellular MRSA, in the presence of vancomycin. Planktonic bacteria exposed to vancomycin alone were efficiently killed, (Fig. 1c). However, we recovered a small number of surviving bacteria (approximately 0.06% of input) associated with the MG63 cells 1 day after infection, which had been protected from vancomycin by invasion into osteoblasts. MRSA that were sequestered inside peritoneal cells showed a significant increase in both survival and efficiency of MG63 infection in the presence of vancomycin. About 15% of intracellular MRSA in the leukocytes survived under vancomycin exposure identical to that which sterilized cultures of planktonic bacteria. Intracellular *S. aureus* that were sequestered in MG63 cells (Fig. 1d), primary human brain endothelial cells and A549 bronchial epithelial cells (Extended Data Fig. 2) were also able to increase by almost tenfold over a 24 h period under constant exposure to a concentration of vancomycin that killed free-living bacteria. Together, these data suggest that intracellular reservoirs of MRSA in myeloid cells can promote dissemination of infection to new sites in the presence of active antibiotic treatment, and that intracellular growth can occur in a variety of cell types despite constant antibiotic therapy.

Designing the antibody–antibiotic conjugate

These findings suggested that therapies aimed at eliminating intracellular bacteria may improve clinical success. To test this conclusively,

we developed a novel therapeutic that is activated specifically inside mammalian cells that have taken up *S. aureus*. The antibody–antibiotic conjugate (AAC) consists of an anti-*S. aureus* antibody (THIOMAB, engineered to contain unpaired cysteines) covalently linked via the introduced cysteines to a highly efficacious antibiotic using a cathepsin-cleavable linker containing a novel quaternary ammonium salt (Fig. 2a). The resulting AAC has no direct antibacterial activity when bound to planktonic *S. aureus* and does not diffuse into mammalian cells, owing to the size of the antibody. However, when AAC-opsonized bacteria are taken up by host cells, intracellular proteases cleave the linker and readily release the antibiotic in its active form²³ (Fig. 2b). Depending on the antibody specificity, we found that thousands of AACs can bind to a single bacterium and deliver a sufficient concentration of free antibiotic within the phagosome to result in bacterial killing.

The antibody and antibiotic components were carefully chosen and optimized for maximal efficacy. A panel of more than 40 anti-*S. aureus* antibodies were cloned and purified from B cells derived from the peripheral blood of patients recovering from various *S. aureus* infections and screened for binding to a panel of clinically relevant *S. aureus* strains and to USA300 isolated directly *ex vivo* from the kidneys of infected mice. We found the greatest extent of binding with antibodies directed against wall-teichoic acids (WTAs), pathogen-specific polyanionic glycopolymers that are connected to the thick peptidoglycan layers of Gram-positive bacteria (Fig. 2c). *S. aureus* produces WTAs composed of phospho-ribitol repeating units that are further modified by either α - or β -O-linked *N*-acetylglucosamine (GlcNAc) sugars mediated by TarM or TarS glycosyltransferases, respectively²⁴. A human immunoglobulin G₁ (IgG₁) that recognizes β -O-linked GlcNAc sugar modifications on WTA bound to all *S. aureus* strains tested. Monoclonal antibodies recognizing the α -O-linked GlcNAc bound well to *S. aureus* strains cultured *in vitro*; however, expression of the α -O-linked GlcNAc was absent on some *S. aureus* isolates. On those strains that co-expressed the α - and β -O-linked GlcNAc, β -specific antibodies yielded consistently higher binding to *in vivo*-derived MRSA (Fig. 2c). Antigen specificity of the antibodies was confirmed by genetic means, such that antibodies against α - or β -GlcNAc sugar modifications on WTAs failed to bind to *S. aureus* strains lacking the TarM or TarS glycosyltransferase, respectively (Fig. 2d). We estimate that $\sim 15,000$ β -WTA-specific antibodies can bind to protein-A-deficient *S. aureus* USA300 by measuring binding of radiolabelled antibodies (data not shown).

To characterize antibody binding to WTA at the molecular level further, we co-crystallized the Fab fragment from an anti- β -WTA antibody in complex with a synthetic form of the minimal repeating β -WTA unit (C2 carbon of ribitol-1-phosphate linked to the C1 position in GlcNAc via a β -glycosidic bond) and determined a crystal structure of the complex at 1.7 Å resolution (Extended Data Table 1). The structure reveals how the minimal β -WTA epitope binds to the complementarity determining region (CDR) of the Fab antibody fragment (Fig. 2e). The heavy chain H1 and H3 CDR loops form a binding site for the GlcNAc, with the GlcNAc hexose ring stacking against the Trp 33 indole side chain. Importantly, the antibody has an extended L1 loop containing two arginine residues (Arg 27d and 28) that interact with the phosphate group on the ribitol backbone. This arginine ‘tweezers’ motif triangulates the ribitol phosphodiester backbone in relation to the GlcNAc moiety, and is probably a key reason for the β -anomer-specific recognition of GlcNAc-WTA by the antibody.

An extensive set of criteria was used to choose the optimal antibiotic for the AAC platform. We chose the rifamycin class of antibiotics for their high potency, unaltered bactericidal activity in low phagolysosomal pH, and their ability to withstand intracellular insults. A rifamycin derivative (a rifalogue) that allowed connection to the linker antibody through a tertiary amine (T. Pillow, manuscript in preparation) had optimum physicochemical properties to allow for efficient bacterial killing. The chemical groups known to make direct contact with

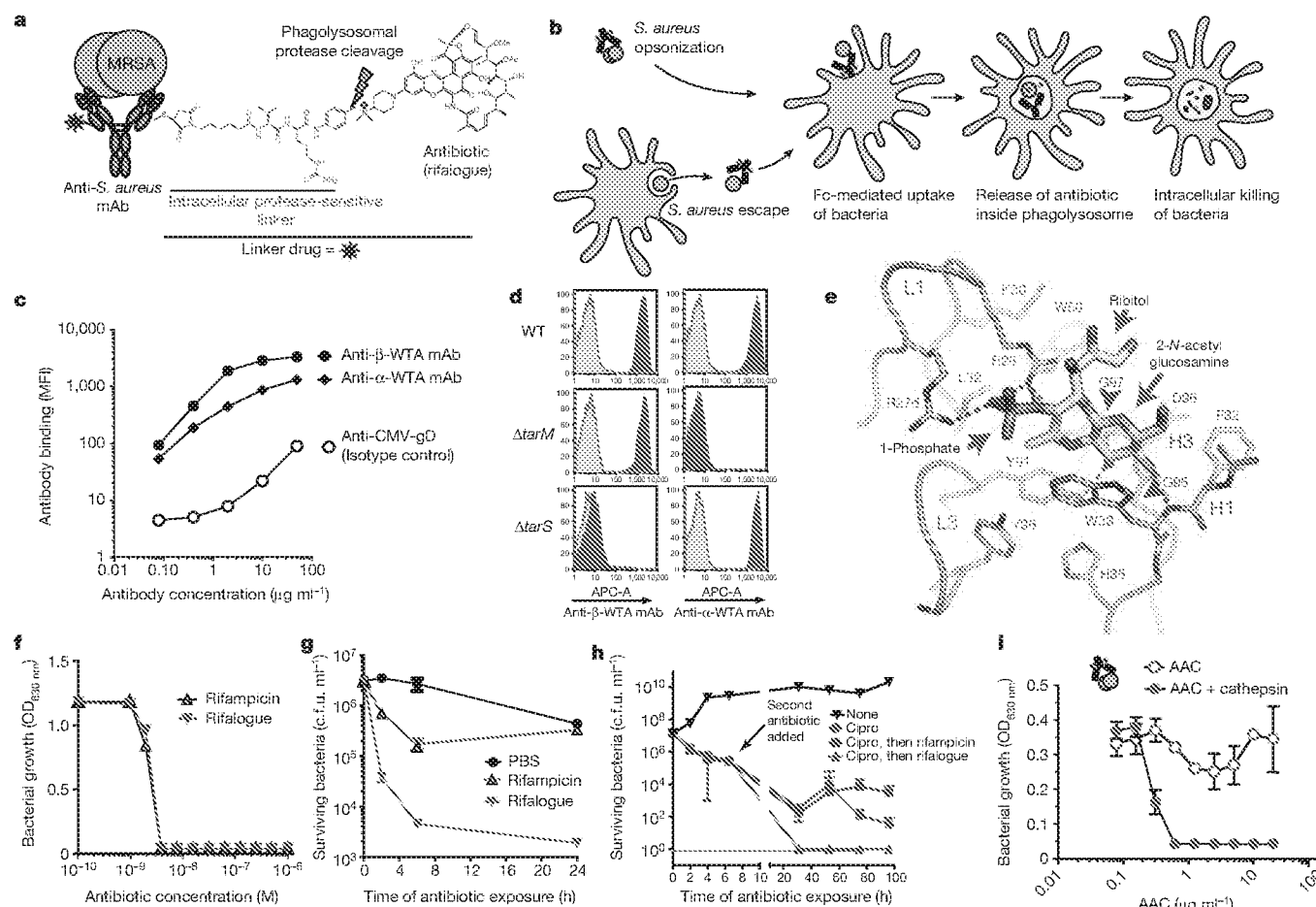


Figure 2 | AAC design. **a**, Model of AAC (not drawn to scale). **b**, Mechanism of AAC action. **c**, Binding of Alexa-488 anti- β -GlcNAc WTA monoclonal antibody (mAb) or anti- α -GlcNAc WTA monoclonal antibody, or isotype control antibody, anti-cytomegalovirus glycoprotein-D (gD) to USA300 isolated from infected kidneys ($n = 3$). MFI, mean fluorescence intensity. **d**, Binding of anti-GlcNAc WTA antibodies (red) or isotype control (grey) to protein-A-deficient USA300 lacking *tarM* or *tarS* ($n = 3$). WT, wild type. **e**, Crystal structure of anti- β -GlcNAc WTA Fab bound to a synthetic minimal β -WTA unit.

bacterial RNA polymerase are conserved between rifampicin and the rifalogue²⁵. Consistent with this, the minimum inhibitory concentration (MIC) for the rifalogue is identical to rifampicin (4×10^{-9} M), as measured in a conventional growth inhibition assay using planktonic bacteria (Fig. 2f). The rifalogue, but not rifampicin, resulted in a more than 1,000-fold decrease in the number of viable, but non-replicating, bacteria after overnight incubation in minimal PBS buffer (Fig. 2g). The rifalogue was also able to kill classically defined persister cells, bacteria that presumably enter a dormant state to survive antibiotic treatment (for example, ciprofloxacin) of growing cultures²⁶ (Fig. 2h), while treatment with rifampicin had no effect on recovery of persister cells, in agreement with previous observations²⁶. Additionally, rifalogue, but not rifampicin, was able to kill non-dividing MRSA sequestered inside murine macrophages, in part due to its capacity to accumulate rapidly in mammalian cells (Extended Data Fig. 3). As predicted, the rifalogue had no ability to kill extracellular bacteria when linked to the anti- β -WTA monoclonal antibody in the AAC format, but was fully bactericidal after the AAC was treated with cathepsin B to release the active antibiotic (Fig. 2i).

To confirm that the linker used in the AAC is cleaved after internalization of the AAC inside cells, we examined cleavage of the linker with a fluorescence resonance energy transfer (FRET)-based probe consisting of the same anti-MRSA antibody conjugated to two dye molecules that were separated by the same linker used in the AAC²⁷. When the

Antibody light chain (pink) and heavy chain (blue) are shown. **f**, MIC determination for rifampicin and rifalogue on USA300 ($n = 5$). **g**, Survival of stationary phase USA300 incubated with 1×10^{-6} M rifampicin or rifalogue ($n = 4$). **h**, USA300 bacteria were incubated without antibiotic (black) or with $3 \mu\text{g ml}^{-1}$ ciprofloxacin (Cipro; green, red and grey). $1 \mu\text{g ml}^{-1}$ of rifalogue (red) or rifampicin (grey) was added as indicated ($n = 3$). **i**, Intact AAC does not kill planktonic bacteria but does after pre-treatment with cathepsin-B ($n = 3$). **g–i**, Error bars show s.d. for triplicate samples ($n = \text{biological repeats}$).

fluorescent molecules are covalently associated, fluorescence at 488 nm is quenched; upon cleavage of the linker, quenching is lost. Video microscopy demonstrated that the linker was cleaved within minutes of uptake of the MRSA opsonized with the FRET conjugate (Fig. 3a). We confirmed by mass spectrometry analysis that free rifalogue was released inside macrophages after uptake of MRSA opsonized with active AACs, but not when opsonized with a non-cleavable version of the AAC, which was prepared by replacement of the natural amino acid L-citrulline with D-citrulline at the P1 position, making the linker uncleavable by cathepsins (Fig. 3b).

AACs eradicate intracellular *S. aureus* infections

When the AAC was used to opsonize MRSA, it readily killed bacteria inside the macrophages, whereas the same bacteria opsonized with non-cleavable β -WTA AAC or monoclonal antibody alone survived (Fig. 3c). AAC-opsonized MRSA were killed inside every cell type tested, including human macrophages, endothelial and epithelial cell lines (Fig. 3d). These data demonstrate that the AAC releases active antibiotic and kills *S. aureus* only after internalization inside host cells, providing a unique tool to assess the importance of intracellular *S. aureus* in various settings. To determine whether specifically targeting intracellular *S. aureus* could prevent cell-to-cell transfer of bacteria, infected peritoneal cells were incubated with MG63 osteoblast cells in the presence of vancomycin (Fig. 3e). Addition of AAC to the

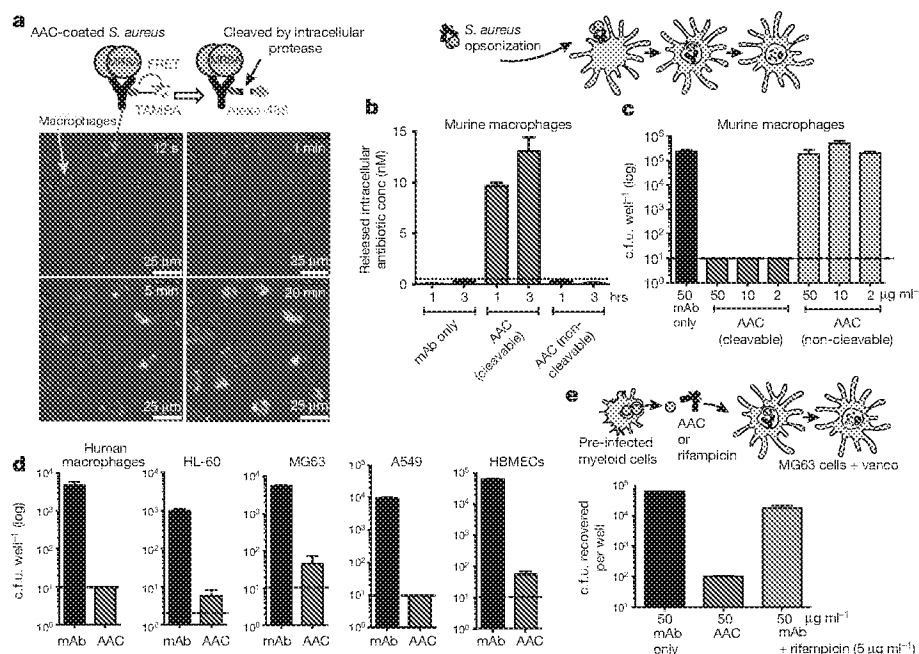


Figure 3 | AAC linker is cleaved after internalization of bacteria.

a, Live cell imaging monitoring cleavage of AAC linker in macrophages with FRET-based antibody conjugate (representative of three fields). TAMRA, tetramethylrhodamine. **b**, Mass spectrometric quantification of released antibiotic inside macrophages from AAC made with standard (cleavable) or non-cleavable linker. mAb, monoclonal antibody. **c**, **d**, USA300, opsonized with antibody, AAC, or non-cleavable AAC,

culture media resulted in a significant reduction in the number of viable intracellular bacteria recovered 1 day after infection. Similar treatment with the unconjugated anti-MRSA antibody plus a tenfold higher molar concentration of rifampicin, purported to be one of the best-known antibiotics for treatment of intracellular bacteria²⁸, had very marginal efficacy. To confirm that targeting intracellular *S. aureus* could also prevent transfer of infection from intracellular reservoirs *in vivo*, mice were infected by intravenous injection with equivalent doses of planktonic or intracellular MRSA and then treated with PBS or vancomycin, with or without a single dose of AAC. In the presence of vancomycin only intracellular bacteria were able to efficiently colonize the brain (Extended Data Fig. 1); however, treatment with a single dose of AAC effectively eliminated the bacteria that had escaped vancomycin treatment (Extended Data Fig. 4)

AAC is superior to vancomycin *in vivo*

To determine the role of intracellular infection in bacteraemia, AACs were tested in an intravenous infection model. First, we tested the efficacy of unconjugated anti-MRSA antibodies by treating mice with either anti- β -WTA antibodies, anti- α -WTA antibodies or intravenous immunoglobulin (IGIV), a pooled immunoglobulin preparation from ~10,000 humans, 1 h before intravenous infection with wild-type USA300 MRSA. *S. aureus* is a common colonizer of human skin and mucosal surfaces and preliminary analysis of multiple sources of human serum, including IGIV, demonstrated that human serum contains approximately 300 $\mu\text{g ml}^{-1}$ of anti-*S. aureus* antibodies, of which ~70% are directed towards the GlcNAc modifications of WTA (Extended Data Fig. 5). Even in this prophylactic treatment setting, unconjugated antibodies did not have any efficacy in preventing infection, whereas treatment with vancomycin eliminated detectable bacteria (Fig. 4a). However, the efficacy of vancomycin was limited when treatment was initiated several hours after infection was established (Fig. 4b). In at least one model of bacteraemia, 95% of the bacteria in blood are found to be associated with neutrophils within 15 min (ref. 6), suggesting that failure of vancomycin in this model could be

enumerated 48 h after incubation with indicated cells lines. HBMECs, human brain microvascular endothelial cells. Horizontal line indicates the limit of detection. **e**, Intracellular USA300 as in Fig. 1a were added to a monolayer of MG63 with antibody, AAC, or a mixture of antibody plus rifampicin in media containing vancomycin (vanco). Surviving bacteria were enumerated 24 h later. **b–e**, Error bars represent means $\pm$ s.d. from triplicate wells. Data are representative of 2–4 biological replicates.

due to antibiotic escape through survival inside host cells. To test this directly, mice were treated with a single dose of AAC 24 h after infection. A single dose of AAC was efficacious and proved superior to twice daily vancomycin treatment initiated at the same time (Fig. 4c and Extended Data Fig. 6a).

Mouse serum has no appreciable anti-*S. aureus* antibody. To determine whether endogenous anti-WTA antibodies found in normal human serum might compete for binding with the AAC, SCID mice were reconstituted with physiological levels of human IgG by daily treatment with IGIV. The resulting SCID-huIgG mice had sustained levels of at least 10 mg ml^{-1} of human IgG in the serum and were equally susceptible to infection with MRSA compared to untreated controls (data not shown). Despite the presence of potentially competing antibodies, a single dose of anti-MRSA AAC administered at 24 h after infection with MRSA was still more efficacious than vancomycin (Fig. 4d). An AAC made with an irrelevant antibody (anti-cytomegalovirus glycoprotein D antibody)—which could still bind to protein A on MRSA—had much less efficacy. AACs made with anti- β -GlcNAc WTA antibodies appeared to be more efficacious than those made with anti- α -GlcNAc WTA antibodies (Extended Data Fig. 6b), suggesting that the extent of antibody binding determines antibiotic delivery. Treatment with the AAC was more effective than treatment with an equivalent dose or repeated dosing of unconjugated rifampicin, and release of the antibiotic from the AAC was essential for efficacy, as an AAC generated with the non-cleavable linker was not efficacious *in vivo* (Fig. 4e).

Discussion

Treatment of patients with invasive MRSA infections with conventional antibiotics results in failure rates of up to 50% (refs 29–32), in most cases without measurable outgrowth of antibiotic-resistant strains. Survival of antibiotic-susceptible subpopulations of *S. aureus* in the presence of antibiotic treatment is well documented and is probably due to multiple factors, including survival of persister bacteria that are not actively dividing and poor antibiotic exposure of

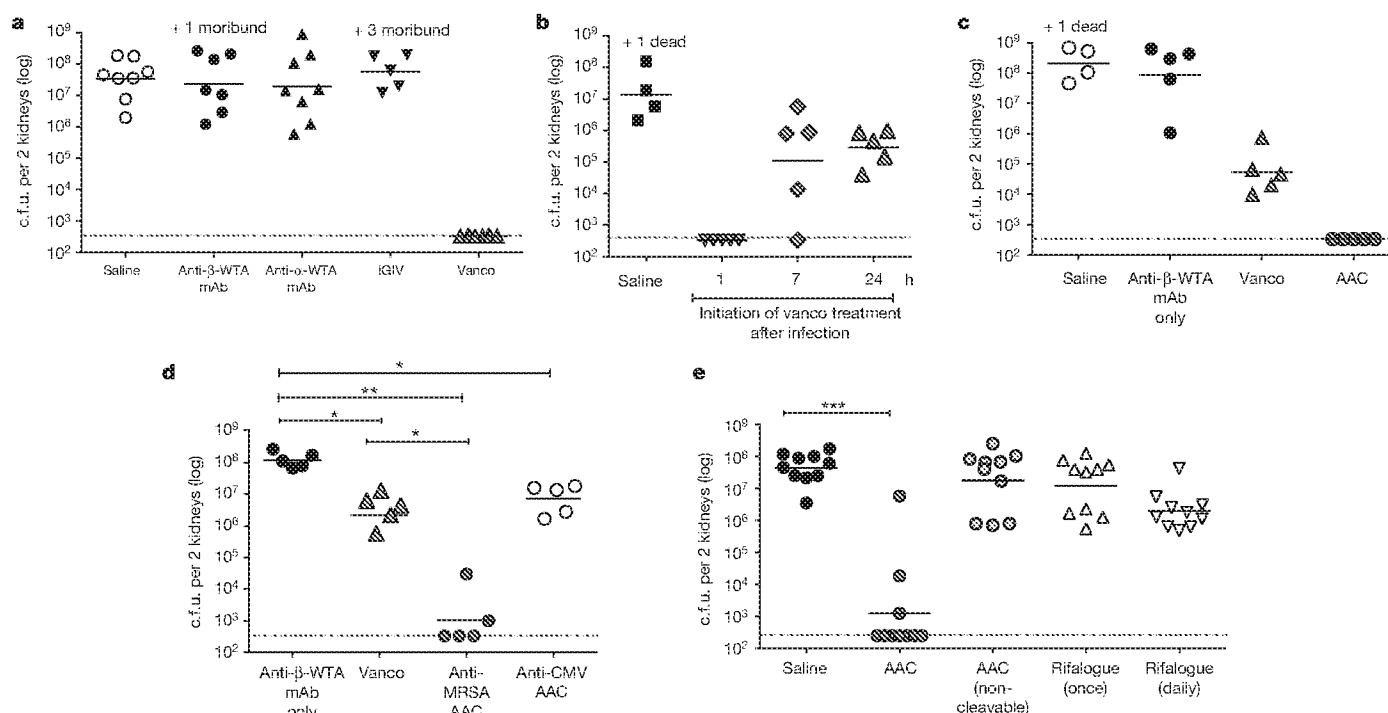


Figure 4 | AAC is a more effective treatment than vancomycin after intravenous infection. **a**, Wild-type (WT) mice ($n = 8$ per group) were treated with 50 mg kg^{-1} of the indicated anti-MRSA antibodies 1 h before MRSA infection or twice daily with 110 mg kg^{-1} vancomycin (Vanco). **b**, Treatment of wild-type mice ($n = 5$ per group) with 110 mg kg^{-1} vancomycin (twice daily) was initiated either at 1 h, 7 h or 24 h after infection. **c**, Wild-type mice ($n = 5$ per group) were treated with saline, anti- β -WTA antibody used in the AAC (monoclonal antibody (mAb)), vancomycin (twice daily), or anti-MRSA AAC (a single dose of 50 mg kg^{-1}) starting 24 h after infection. **d**, **e**, SCID mice (**d**, 5 mice per

group; **e**, 10 mice per group) were injected with human IgG to achieve a concentration of 10 mg ml^{-1} , then infected with MRSA. Treatment as indicated was begun 24 h after infection. Vancomycin, 110 mg kg^{-1} twice daily; AAC, 50 mg kg^{-1} a single dose on day 1; rifalogue, 0.5 mg kg^{-1} either once on day 1, or once daily (days 1–3). **a–e**, Bacteria in kidneys were determined 4 days after infection. Schematic of each experimental design is shown in Extended Data Fig. 7. Each point shows data from a single animal. Bars show geometric mean. Dashed lines indicate limit of detection. Mann–Whitney U -test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$.

bacteria in protected niches^{33–35}. However, it has been difficult to determine conclusively what role intracellular *S. aureus* plays in disease pathogenesis and antibiotic failure. To address directly the extent to which this ability to survive and replicate inside cells contributes to the failure of conventional antibiotics, we developed a novel therapeutic in the form of an AAC that is specifically designed to be activated only within host cells. We found that a single dose of AAC treatment is effective in a murine model of bacteraemia, in which vancomycin, the current standard of care for MRSA infection, failed (Fig. 4). This suggests that ablation of the intracellular pool of pathogens is key for clinical success in the treatment of potential fatal MRSA infection.

In designing the AAC to test this hypothesis, we used an antibiotic that kills intracellular bacteria. This rifalogue antibiotic also ablates non-replicating bacteria, and antibiotic-resistant persister cells. We posit that this characteristic, along with its ability to accumulate within the intracellular milieu, is essential for AAC success. When the closely related rifampicin, which does not have these characteristics, was conjugated to the same anti-WTA antibody it did not kill intracellular bacteria and exhibited poor efficacy *in vivo* (Extended Data Fig. 8). These studies show that arming antibodies with unique bactericidal antibiotics can result in promising new therapies. Many potent antibiotic-like compounds with the desired characteristics to kill difficult-to-treat intracellular pathogens fail in clinical practice owing to poor pharmacokinetic properties or undesired host toxicity. The use of an AAC might be able to overcome these problems, making it a novel platform for delivering potent antibacterial compounds that may not have a suitable profile as unconjugated drugs. Antibiotic failure has been associated with the ability of a variety of bacterial pathogens to survive within host phagocytic cells³⁶. The

extent to which other common human pathogens beyond *S. aureus* depend on this intracellular protected niche merits investigation; the AAC platform promises to enhance antibiotic efficacy against these infectious diseases.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions S.M.L. designed and executed the *in vitro* and *in vivo* analysis of the AAC mechanism of action. T.P., L.S. and J.A.F. designed and synthesized antibiotics and linker drugs. M.X., J.K., S.P. and D.Y. designed and analysed *in vivo* models for intravenous infection. H.R., L.D., M.D. and R.V. designed and conjugated linker antibiotic to antibodies. K.K.K., W.L.H., J.H.M. and S.M. characterized the anti-MRSA antibodies. Y.K., H.H., K.M.L., E.P. and J.C. did mass spectrometry analysis of the rifalogs during *in vitro* efficacy studies. P.L. and R.F. performed X-ray crystallography of anti- β -WTA monoclonal antibody. J.P.L. designed the synthesis of β -phospho-ribitol. B.-C.L. and C.C. characterized FRET constructs and helped with video microscopy. E.L. determined the number of antibody-binding sites on MRSA. M.S., K.K. and P.S.A. isolated anti-MRSA antibodies from patients. M.W.T. contributed to bacterial genetics and data analysis. E.J.B. and S.M. initiated the project and S.M. led the project. S.M.L., E.J.B. and S.M. composed the paper with input from all authors.

Additional Information The structure of the anti- β -WTA Fab bound to the synthetic WTA fragment (β -GlcNAc anomer) has been deposited in the Protein Data Bank under accession number 5D6C. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to E.J.B. (brown.eric@gene.com) or S.M. (sanj@gene.com).

METHODS

Ethics statement. All animal procedures were conducted under a protocol (#08–1990) approved by the Genentech Institutional Animal Care and Use Committee in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility in accordance with the Guide for the Care and Use of Laboratory Animals and applicable laws and regulations. For cloning of antibodies from human B cells, informed written consent was obtained from all donors and was provided in accordance with the Declaration of Helsinki. Approval was obtained from the health research ethics committee of Denmark through the regional committee for the Capital Region of Denmark.

Bacterial strains. All *in vivo* experiments were done with MRSA-USA300 NRS384 obtained from NARSA (<https://www.beiresources.org>) unless noted otherwise. The generation of protein-A-deficient strain Δmcr USA300 NRS384, as well as protein-A-deficient USA300 lacking *tarM* or *tarS* has been described previously^{38,39}. The protein-A-deficient strains were used only in some *in vitro* experiments to determine antibody specificity.

MIC determinations for extracellular bacteria. The MIC for extracellular bacteria was determined by preparing serial twofold dilutions of the antibiotic in tryptic soy broth. Dilutions of the antibiotic were made in quadruplicate in 96-well culture dishes. MRSA (NRS384 strain of USA300) was taken from an exponentially growing culture and diluted to 1×10^4 c.f.u. ml⁻¹. The bacteria were cultured in the presence of antibiotic for 18–24 h with shaking at 37°C and bacterial growth was determined by reading the optical density (OD) at 630 nm. The MIC was determined to be the dose of antibiotic that inhibited bacterial growth by >90%.

MIC determinations for intracellular bacteria. Intracellular MIC was determined on bacteria that were sequestered inside mouse peritoneal macrophages (see later for generation of murine peritoneal macrophages). Macrophages were plated at a density of 4×10^5 cells ml⁻¹ and infected with MRSA at a ratio of 10–20 bacteria per macrophage. Macrophage cultures were maintained in growth media supplemented with 50 µg ml⁻¹ of gentamycin to inhibit the growth of extracellular bacteria and test antibiotics were added to the growth media 1 day after infection. The survival of intracellular bacteria was assessed 24 h after addition of the antibiotics. Macrophages were lysed with Hanks buffered saline solution supplemented with 0.1% bovine serum albumin (BSA) and 0.1% Triton-X, and serial dilutions of the lysate were made in PBS solution containing 0.05% Tween-20. The number of surviving intracellular bacteria was determined by plating on tryptic soy agar plates with 5% defibrinated sheep blood.

***In vivo* transfer of infection model.** USA300 stocks were prepared for infection from actively growing cultures in tryptic soy broth. Bacteria were washed three times in PBS and aliquots were frozen at –80°C in PBS 25% glycerol.

Intracellular bacteria infections. Seven-week-old female A/J mice (Stock 000646) were obtained from Jackson Labs and infected by peritoneal injection with 5×10^7 c.f.u. of USA300. Mice were killed 1 day after infection and the peritoneum was flushed with 5 ml of cold PBS. Peritoneal washes were centrifuged for 5 min at 1,000 r.p.m. at 4°C in a table-top centrifuge. The cell pellet containing peritoneal cells was collected and cells were treated with 50 µg ml⁻¹ of lysostaphin (Cell Sciences, CRL 309C) for 20 min at 37°C to kill contaminating extracellular bacteria. Peritoneal cells were washed three times in ice-cold PBS to remove the lysostaphin. Peritoneal cells from donor mice were pooled, and recipient mice were injected with cells derived from five donors per each recipient by intravenous injection into the tail vein. To determine the number of live intracellular colony-forming units, a sample of the peritoneal cells were lysed in HB (Hanks balanced salt solution supplemented with 10 mM HEPES and 0.1% BSA) with 0.1% Triton-X, and serial dilutions of the lysate were made in PBS with 0.05% Tween-20.

Free bacteria infections. A/J mice were infected with various doses of free bacteria using a fresh aliquot of the glycerol stocks used for the peritoneal injections. Actual infection doses were confirmed by c.f.u. plating. For the data shown in Fig. 1a the actual infection dose for intracellular bacteria was 1.8×10^6 c.f.u. per mouse, and the actual infection dose for free bacteria was 2.9×10^6 c.f.u. per mouse. Selected mice were treated with a single dose of 110 mg kg⁻¹ of vancomycin by intravenous injection immediately after infection.

***In vitro* transfer of infection to non-phagocytic cells.** **Generation of MRSA-infected peritoneal cells.** Six-to-eight-week-old female A/J mice (see earlier) were infected with 1×10^8 c.f.u. of the NRS384 strain of USA300 by peritoneal injection. The peritoneal wash was harvested 1 day after infection, and the infected peritoneal cells were treated with 50 µg ml⁻¹ of lysostaphin diluted in HEPES buffer supplemented with 0.1% BSA (HB buffer) for 20 min at 37°C. Peritoneal cells were then washed twice in ice-cold HB buffer. The peritoneal cells were diluted to 1×10^6 cells ml⁻¹ in RPMI 1640 tissue culture media supplemented with 10 mM HEPES and 10% fetal calf serum, and 5 µg ml⁻¹ vancomycin. Free MRSA from the primary infection was stored overnight at 4°C in PBS

solution as a control for extracellular bacteria that were not subject to neutrophil killing.

Infection of osteoblasts, HBMEC and A549 cells. MG63 cell line (CRL-1427) and A549 cells (CCL185) were obtained from ATCC and maintained in RPMI 1640 tissue culture media supplemented with 10 mM HEPES and 10% fetal calf serum (RPMI-10). HBMEC cells (catalogue #1000) and ECM media (catalogue #1001) were obtained from ScienceCell Research Labs. The cells were used without further authentication or testing for mycoplasma contamination. Cells were plated in 24-well tissue culture plates and cultured to obtain a confluent layer. On the day of the experiment, the cells were washed once in RPMI (without supplements). MRSA or infected peritoneal cells were diluted in complete RPMI-10 and vancomycin was added at 5 µg ml⁻¹ immediately before infection. Peritoneal cells were added to the osteoblasts at 1×10^6 peritoneal cells per ml. A sample of the cells was lysed with 0.1% Triton-X to determine the actual concentration of live intracellular bacteria at the time of infection. The actual titre for all infections was determined by plating serial dilutions of the bacteria on tryptic soy agar with 5% defibrinated sheep blood.

Generation of the anti-*S. aureus* antibodies. The human IgG antibodies against anti-β-GlcNAc WTA monoclonal antibody (mAb) and anti-α-GlcNAc WTA mAb were cloned from peripheral B cells from patients after *S. aureus* infection using a monoclonal antibody discovery technology that conserves the cognate pairing of antibody heavy and light chains⁴⁰. Antibodies were expressed by transfection of mammalian cells⁴¹. Supernatants containing full-length IgG1 antibodies were harvested after 7 days and used to screen for antigen binding by enzyme-linked immunosorbent assay (ELISA). These antibodies were positive for binding to cell wall preparations from USA300. Antibodies were subsequently produced in 200-ml transient transfections and purified with protein A chromatography (MabSelect SuRe, GE Life Sciences) for further testing.

Synthesis of the linker drug. Synthesis of the rifalogue linker drug was performed as follows. Protease cleavable linker MC-VC-PAB-OH²³ (1.009 g, 1.762 mmol, 1.000, 1.009 mg) was taken up in *N,N*-dimethylformamide (6 ml, 77 mmol, 44, 5,700 mg). To this was added a solution of thionyl chloride (1.1 equiv., 1.938 mmol, 1.100, 231 mg) in dichloromethane (DCM) (1 ml, 15.44 mmol, 8.765, 1,325 mg) in portions dropwise (half was added over 1 h, stirred for 1 h at room temperature, then the other half was added over another hour). The solution remained a yellow colour. Another 0.6 equiv. of thionyl chloride was added as a solution in 0.5 ml DCM dropwise, carefully. The reaction remained yellow and was stirred sealed overnight at room temperature. The reaction was monitored by liquid chromatography mass spectrometry (LC/MS), indicating 88% conversion to benzyl chloride. Another 0.22 equiv. of thionyl chloride was added dropwise as a solution in 0.3 ml DCM. When the reaction approached 92% benzyl chloride, the reaction was bubbled with N₂. The concentration was increased from 0.3 M to 0.6 M.

MC-VC-PAB-Cl (0.9 mmol) was cooled to 0°C and rifalogue (dimethyl piperazinebenzoxazinorifamycin⁴² (0.75 g, 0.81 mmol, 0.46, 750 mg)) was added. The mixture was diluted with another 1.5 ml of DMF to reach 0.3 M. Stirred open to air for 30 min. *N,N*-diisopropylethylamine (3.5 mmol, 3.5 mmol, 2.0, 460 mg) was added and the reaction stirred overnight open to air. Over the course of 4 days, four additions of 0.2 equiv. *N,N*-diisopropylethylamine base were added while the reaction stirred open to air, until the reaction appeared to stop progressing. The reaction was diluted with DMF and purified on high-performance liquid chromatography (HPLC; 20–60% ACN/FA-H₂O) in several batches to give MC-VC-PAB-rifalogue (0.38 g, 32% yield) *m/z* = 1,482.8.

The non-cleavable rifalogue linker drug was synthesized using the exact same method, but replacing MC-VC-PAB-OH with MC-V-D-Cit-PAB-OH.

Conjugation of the linker drug to antibody. Construction and production of the THIOMAB variant of anti-WTA antibody was done as reported previously⁴³. Briefly, a cysteine residue was engineered at the Val 205 position of the anti-WTA light chain to produce its THIOMAB variant. The thio anti-WTA was conjugated to MC-vc-PAB-rifalogue. The antibody was reduced in the presence of 50-fold molar excess dithiothreitol (DTT) overnight. The reducing agent and the cysteine and glutathione blocks were purified away using HiTrap SP-HP column (GE Healthcare). The antibody was re-oxidized in the presence of 15-fold molar excess dehydroascorbic acid (MP Biomedial) for 2.5 h. The formation of interchain disulfide bonds was monitored by LC/MS. A threefold molar excess of the linker drug (MC-VC-PAB-rifalogue) over protein was incubated with the THIOMAB for 1 h. The AAC was purified by filtration through a 0.2 µm SFCA filter (Millipore). Excess-free linker drug was removed by filtration. The conjugate was buffer exchanged into 20 mM histidine acetate pH 5.5/240 mM sucrose by dialysis. The number of conjugated MC-VC-PAB-rifalogue molecules per mAb was quantified by LC/MS analysis. Purity was also assessed by size-exclusion chromatography.

Mass spectrometric analysis. LC/MS analysis was performed on a 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS (Agilent Technologies). Samples were chromatographed on a PRLP-S column, 1,000 Å, 8 µm (50 mm × 2.1 mm, Agilent Technologies) heated to 80 °C. A linear gradient from 30–60% B in 4.3 min (solvent A, 0.05% TFA in water; solvent B, 0.04% TFA in acetonitrile) was used and the eluent was directly ionized using the electrospray source. Data were collected and deconvoluted using the Agilent Mass Hunter qualitative analysis software. Before LC/MS analysis, AAC was treated with lysyl endopeptidase (Wako) for 30 min at 1:100 w/w enzyme to antibody ratio, pH 8.0, and 37 °C to produce the Fab and the Fc portion for ease of analysis. The drug-to-antibody ratio (DAR) was calculated using the abundance of Fab and Fab+1 calculated by the MassHunter software.

Flow cytometry to compare expression of anti-MRSA antibodies. Analysis of bacteria isolated from infected mice. Balb/c mice were infected with 1×10^7 c.f.u. of MRSA (USA300) by intravenous injection and kidneys were harvested on day 3 after infection. Kidneys were homogenized using a GentleMACS dissociator in 5 ml volume per two kidneys using M-Tubes and the program RNA01.01 (Miltenyi Biotec). Homogenization buffer was: PBS plus 0.1% Triton-X-100, $10 \mu\text{g ml}^{-1}$ DNAase (bovine pancreas grade II, Roche) and protease inhibitors (complete protease inhibitor cocktail, Roche 11-836-153001). After homogenization, the samples were incubated at room temperature for 10 min and then diluted with ice-cold PBS and filtered through a $40 \mu\text{m}$ cell strainer. Tissue homogenates were washed twice in ice-cold PBS and then suspended in a volume of 0.5 ml per two kidneys in HB buffer (Hanks balanced salt solution supplemented with 10 mM HEPES and 0.1% BSA). The cell suspension was filtered again and $25 \mu\text{l}$ of the bacterial suspension was taken for each staining reaction (Fig. 2c).

Antibody staining for flow cytometry. Bacteria (1×10^7 of *in vitro* grown bacteria (Fig. 2d), or $25 \mu\text{l}$ of tissue homogenate described earlier (Fig. 2c) were suspended in HB buffer and blocked by incubation with $400 \mu\text{g ml}^{-1}$ of mouse IgG (Sigma, I5381) for 1 h. Fluorescently labelled antibodies were added directly to the blocking reaction and incubated at room temperature for an additional 10–20 min. Bacteria were washed three times in HB buffer and then fixed in PBS 2% paraformaldehyde before FACS analysis. Test antibodies (anti-β-WTA, anti-α-WTA or isotype control-anti CMV-gD) were conjugated with Alexa-488 using amine reactive reagents (Invitrogen, succinimidyl-ester of Alexa Fluor 488, NHS-A488). Antibodies in 50 mM sodium phosphate were reacted with a 5–10-fold molar excess of NHS-A488 in the dark for 2–3 h at room temperature. The labelling mixture was applied to a GE Sepharose S200 column equilibrated in PBS to remove excess reactants from the conjugated antibody. The number of A488 molecules per antibody was determined using the ultraviolet method as described by the manufacturer.

For analysis of bacteria in tissue homogenates a non-competing anti-*S. aureus* antibody (rF1 (ref. 38)) was conjugated to Alexa-647 to distinguish *S. aureus* from similar sized particles. Test antibodies were examined at a range of doses from 80 ng ml^{-1} to $50 \mu\text{g ml}^{-1}$. Flow cytometry was performed using a Beckton Dickinson FACS ARIA (BD Biosciences) and analysis was performed using FlowJo analysis software (Flow Jo LLC).

WTA-antibody complex purification and crystallization. The anti-β-WTA antibody Fab fragment was expressed in *Escherichia coli* and purified on Protein G Sepharose followed by SP sepharose cation exchange and size-exclusion chromatography. Antibody was concentrated to 30 mg ml^{-1} in MES buffer (20 mM MES pH 5.5, 150 mM NaCl) and mixed with a 2:1 mol/mol ratio of the WTA analogue (diluted in water) for crystallization trials. Sparse matrix crystallization screening provided initial hits in PEG-8000 based conditions, which were further optimized to provide diffraction quality crystals. Ultimately, data were collected on a crystal grown by the vapour diffusion method in a sitting drop containing $0.5 \mu\text{l}$ protein and $0.5 \mu\text{l}$ 0.08 M sodium cacodylate pH 6.5, 0.16 M calcium acetate, 14.4% PEG-8000, and 20% glycerol. Crystals were cryo-protected in mother liquor, flash frozen in liquid nitrogen, and stored for data collection at 100 K.

Data collection and structure determination. Data were collected to 1.7 Å at beamline 22ID at the Advanced Photon Source (APS) under cryo-cooled conditions (100 K) at a wavelength of 1.0 Å. Data were reduced using HKL2000 and SCALEPACK in the space group $P2_12_12_1$, with unit cell parameters of $a = 63.7 \text{ Å}$, $b = 111.4 \text{ Å}$, $c = 158.4 \text{ Å}$ (see Extended Data Table 1 for processing statistics). The structure was solved by sequential molecular replacement searches using Fab constant and variable regions (Protein Data Bank accession 4I77) as individual search models. Iterative rounds of manual model adjustment with COOT followed by simulated annealing, coordinate, and b -factor refinement with Phenix and BUSTER (Global Phasing) gave a final model with R/R_{free} values of 20.6% and 23.7% respectively. Ramachandran statistics calculated by MolProbity indicate that 97.2% of the model residues lie in favoured regions, with 0.5% outliers.

Generation of a synthetic WTA fragment. *Synthesis of dibenzyl phosphorochloridate.* A mixture of NCS (3.5 g, 26.6 mmol) was suspended in toluene (80 ml). Then dibenzyl phosphonate (2.0 g, 7.6 mmol) was added. The mixture was stirred at room temperature overnight. The white solid was filtered off and the organic phase was evaporated to give dibenzyl phosphorochloridate (**1**; 2.1 g, 96%) as light yellow oil. $^1\text{H NMR}$ (300 MHz, CDCl_3 , 25 °C) δ 7.36 (s, 10H), 5.20 (m, 4H).

Synthesis of 4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-1-O-acetyl-D-ribitol-5-dibenzylphosphate. A mixture of **2** (described in ref. 44) (500 mg, 0.95 mmol) dissolved in pyridine (12 ml) was cooled to -30 °C and **1** (described ref. 44) (595 mg, 2.0 mmol) was added, stirring for 2 h at -30 °C and warmed to room temperature for 4 h. The mixture was added to H_2O , and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel: 200 to ~300 mesh; dichloromethane: methanol in a 30:1 as eluent) to give 4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-1-O-acetyl-D-ribitol-5-dibenzylphosphate (**3**; 190 mg, 24%) as light yellow solid. $^1\text{H NMR}$ (300 MHz, Acetone- d_6 , 25 °C) δ 7.29–7.23 (m, 10H), 7.08 (d, 1H), 5.08 (t, 1H), 4.99–4.78 (m, 6H), 4.31–3.97 (m, 8H), 3.82–3.63 (m, 3H), 1.88 (s, 3H), 1.86 (s, 6H), 1.79 (s, 3H), 1.69 (s, 3H). LC/MS (m/z) ES+ 784 $[\text{M} + \text{H}]^+$.

Synthesis of 4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-1-O-acetyl-D-ribitol-5-phosphate. A mixture of **3** (150 mg, 0.19 mmol) dissolved in MeOH (6 ml) was hydrogenated over 10% Pd/C (20 mg) for 2 h at room temperature. Then the mixture was filtered, and the filtrate was evaporated to give 4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-1-O-acetyl-D-ribitol-5-phosphate (**4**; 100 mg) as light yellow oil. LC/MS (m/z) ES+ 604 $[\text{M} + \text{H}]^+$.

Synthesis of 4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-D-ribitol-5-phosphate (5). A mixture of **4** (80 mg, 0.16 mmol) dissolved in MeOH (10 ml) was cooled to 5 °C and K_2CO_3 (30 mg, 0.21 mmol) was added and stirred at 5 °C for 3 h. The reaction was then quenched with 1 N HCl, and concentrated *in vacuo*. The crude product was purified by gel filtration (LH-20, MeOH) to give 4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-D-ribitol-5-phosphate (**5**; 13.3 mg, 23%) as a white solid $^1\text{H NMR}$ (300 MHz, MeOH- d_4 , 25 °C) δ 4.62 (d, 1H), 4.30–4.02 (m, 3H), 3.92–3.32 (m, 9H), 2.03 (s, 3H). LC/MS (m/z) ES+ 436 $[\text{M} + \text{H}]^+$.

Time of kill for free antibiotics on non-replicating bacteria. *S. aureus* (USA300) was taken from an overnight stationary phase culture, washed once in PBS and suspended at 1×10^7 c.f.u. ml^{-1} in PBS with no antibiotic or with 1×10^{-6} M antibiotic in a 10 ml volume in 50 ml polypropylene centrifuge tubes. The bacteria were incubated at 37 °C overnight with shaking. At each time point, three 1 ml samples were removed from each culture and centrifuged to collect the bacteria. Bacteria were washed once with PBS to remove the antibiotic and the total number of surviving bacteria was determined by plating serial dilutions of the bacteria on agar plates.

Killing of persister cells by free antibiotics. *S. aureus* (USA300) was taken from an overnight stationary phase culture, washed once in tryptic soy broth (TSB) and then adjusted to a final concentration of 1×10^7 c.f.u. ml^{-1} in a total volume of 10 ml of either TSB or TSB with ciprofloxacin (0.05 mM). Cultures were incubated with shaking at 37 °C for 6 h and then the second antibiotic, either rifampicin ($1 \mu\text{g ml}^{-1}$) or the rifalogue ($1 \mu\text{g ml}^{-1}$) was added. At the indicated times, samples were removed from each culture, washed once with PBS to remove the antibiotic and re-suspended in PBS. The total number of surviving bacteria was determined by plating serial dilutions of the bacteria on agar plates. At the final time point the remainder of each culture was collected and plated.

Cathepsin release assay for AAC. To quantify the amount of active antibiotic released from AACs after treatment with cathepsin B, AACs were diluted to $200 \mu\text{g ml}^{-1}$ in cathepsin buffer (20 mM sodium acetate, 1 mM EDTA, 5 mM L-cysteine, pH 5). Cathepsin-B (from bovine spleen, Sigma C7800) was added at $10 \mu\text{g ml}^{-1}$ and the samples were incubated for 1 h at 37 °C. As a control, AACs were incubated in buffer alone. The reaction was stopped by addition of 9 volumes of bacterial growth media, TSB pH 7.4. To estimate the total release of active antibiotic, serial dilutions of the reaction mixture were made in quadruplicate in TSB in 96-well plates and MRSA (USA300) was added to each well at a final density of 2×10^3 c.f.u. ml^{-1} . The cultures were incubated overnight at 37 °C with shaking and bacterial growth was measured by reading absorbance at 630 nm using a plate reader.

Synthesis of anti-β-WTA antibody FRET conjugate. We synthesized and conjugated a maleimide FRET peptide to the anti-β-WTA TH10MAB antibody. We used a FRET pair of tetramethylrhodamine (TAMRA) and fluorescein. The maleimide FRET peptide was synthesized by standard Fmoc solid-phase chemistry using a PS3 peptide synthesizer (Protein Technologies; B.-C.L., M.D. and R.V., manuscript in preparation)²⁷. Briefly, 0.1 mmol of Rink amide resin was used to generate C-terminal carboxamide. We used a Fmoc-Lys(Mtt)-OH at the N- and C-terminal residues in order to remove the Mtt group on the resin and carry out additional

side-chain chemistry to attach TAMRA and fluorescein. The sequence of Val-Cit-Leu was added between the FRET pair as a cathepsin-cleavable spacer. The crude maleimide FRET peptide or maleimidocarbonyl-K(TAMRA)-G-V-Cit-L-K (fluorescein) cleaved off from the resin was subjected to further purification by reverse-phase HPLC with a Jupiter 5u C4 column (5 μ m, 10 mm $\times$ 250 mm; Phenomenex). Our FRET probe allows monitoring not only of the intracellular trafficking of the antibody conjugate, but also the processing of the linker in the phagolysosome. The intact antibody conjugate fluoresces only in red due to the fluorescence resonance energy transfer from the donor. However, upon the substrate cleavage of the FRET peptide in the phagolysosome, the green fluorescence from the donor is expected to appear.

Video microscopy to detect cleavage of the linker inside macrophages. Murine peritoneal macrophages were plated on chamber slides (Ibidi, catalogue 80826) in complete media as described for the macrophage intracellular killing assay. USA300 was labelled with Cell Tracker Violet (Invitrogen C10094) at 100 μ g ml⁻¹ in PBS 0.1% BSA by incubation for 30 min at 37 °C. The labelled bacteria were opsonized with the anti- β -WTA-FRET probe by incubation for 1 h in HB buffer. Macrophages were washed once immediately before addition of the opsonized bacteria, and bacteria were added to cells at 1 $\times$ 10⁷ bacteria per ml. For non-phagocytosis controls, the macrophages were pre-treated with 60 nM Latrunculin A (Calbiochem) for 30 min before and during phagocytosis. The slides were placed on the microscope immediately after addition of bacteria to the cells and movies were acquired with a Leica SP5 confocal microscope equipped with an environmental chamber with CO₂ and temperature controllers from Ludin. The images were captured every minute for a total time of 30 min using a Plan APO CS $\times$ 40, N.A: 1.25, oil immersion lens, and the 488 nm and 543 nm laser lines to excite Alexa-488 and TAMRA, respectively. Phase images were also recorded using the 543 nm laser line.

Quantification of released antibiotic inside macrophages. Primary murine peritoneal macrophages or RAW 264.7 cells (purchased from ATCC) were infected in 24-well tissue culture dishes as described later for the intracellular killing assay with MRSA opsonized with AAC at 100 μ g ml⁻¹ in HB. The RAW 264.7 cells were used without further authentication or testing for mycoplasma contamination. After phagocytosis was complete, the cells were washed and 250 μ l of complete media plus gentamycin was added to wells and the cells were incubated for the indicated time points. At each time point, the supernatant and cellular fractions were collected followed by acetonitrile (ACN) addition to 75% final concentration and incubated for 30 min. Cell and supernatant extracts were lyophilized by evaporation under N₂ (TurboVap; Biotage) and reconstituted in 100 μ l of 50% ACN, filtered using a 0.45 glass fibre filter plate (Phenomenex) and analysed by LC/MS/MS as follows.

The rifalogue was separated on an Acquity UPLC (Waters Corporation) under gradient elution using a Phenomenex Kinetex XB-C18 column (100 Å, 50 $\times$ 2.1 mm internal diameter, 2.6 μ m particle size). The column was maintained at room temperature. The mobile phase was a mixture of 10 mM ammonium acetate in water containing 0.1% formic acid (A) and 90% acetonitrile (B) at a flow rate of 1 ml min⁻¹. The rifalogue was eluted with a gradient of 3–98% B over 1 min, followed by 0.8 min at 98% B, then 0.7 min of 3% B to re-equilibrate the column. The injection volume was 10 μ l.

The Triple Quad 6500 mass spectrometer (Ab Sciex) was operated in a positive ion multiple reaction-monitoring (MRM) mode. The rifalogue precursor (Q1) ion monitored was 927.6 m/z and the product (Q3) ion monitored was 895.2 m/z with collision energy at 27 eV and declustering potential at 191 V. The MS/MS setting parameters were as follows: ion spray voltage, 5,500 V; curtain gas, 40 psi; nebulizer gas (GS1), 35 psi, (GS2), 50 psi; temperature, 600 °C; and dwell time, 150 ms.

Linear calibration curves were obtained for 0.41–100 nM concentration range by spiking rifalogue into cell or supernatant fractions (lacking MRSA or AAC) that were treated similarly to samples. Concentrations of rifalogue were calculated with MultiQuant software (Ab Sciex).

In vitro intracellular killing assay. Non-phagocytic cell types. MG63 (CRL-1427) and A549 (CCL185) cell lines were obtained from ATCC and maintained in RPMI 1640 tissue culture media supplemented with 10 mM HEPES and 10% fetal calf serum (RPMI-10). HUVEC cells were obtained from Lonza and maintained in EGM endothelial cell complete media (Lonza). HBMEC cells (catalogue #1000) and ECM media (catalogue #1001) were obtained from ScienceCell Research Labs. The cells were used without further authentication or testing for mycoplasma contamination.

Murine macrophages. Peritoneal macrophages were isolated from the peritoneum of 6–8-week-old Balb/c mice (Charles River Laboratories). To increase the yield of macrophages, mice were pre-treated by intraperitoneal injection with 1 ml of thioglycolate media (Becton Dickinson). The thioglycolate media was prepared at

a concentration of 4% in water, sterilized by autoclaving, and aged for 20 days to 6 months before use. Peritoneal macrophages were harvested 4 days after treatment with thioglycolate by washing the peritoneal cavity with cold PBS. Macrophages were plated in DMEM supplemented with 10% fetal calf serum, and 10 mM HEPES, without antibiotics, at a density of 4 $\times$ 10⁵ cells well⁻¹ in 24-well culture dishes. Macrophages were cultured overnight to permit adherence to the plate.

Human M2 macrophages. CD14⁺ monocytes were purified from normal human blood using a Monocyte Isolation Kit II (Miltenyi, catalogue 130-091-153) and plated at 1.5 $\times$ 10⁵ cells cm⁻² on tissue culture dishes pre-coated with fetal calf serum (FCS) and cultured in RPMI 1640 media with 20% FCS plus 100 ng ml⁻¹ rhM-CSF. Media was refreshed on day 1 and on day 7, the media was changed to 5% serum plus 20 ng ml⁻¹ IL-4. Macrophages were used 18 h later.

Assay protocol. In all experiments bacteria were cultured in TSB. To assess intracellular killing with AACs, USA300 was taken from an exponentially growing culture and washed in HB. AACs or antibodies were diluted in HB (Hanks balanced salt solution supplemented with 10 mM HEPES and 0.1% BSA) and incubated with the bacteria for 1 h to permit antibody binding to the bacteria (opsonization), and the opsonized bacteria were used to infect macrophages at a ratio of 10–20 bacteria per macrophage (4 $\times$ 10⁶ bacteria in 250 μ l of HB per well). Macrophages were pre-washed with serum-free DMEM media immediately before infection, and infected by incubation at 37 °C in a humidified tissue culture incubator with 5% CO₂ to permit phagocytosis of the bacteria. After 2 h, the infection mix was removed and replaced with normal growth media (DMEM supplemented with 10% FCS, 10 mM HEPES) and gentamycin was added at 50 μ g ml⁻¹ to prevent growth of extracellular bacteria⁴⁵. At the end of the incubation period, the macrophages were washed with serum-free media, and the cells were lysed in HB supplemented with 0.1% Triton-X (lyses the macrophages without damaging the intracellular bacteria). Serial dilutions of the lysate were made in PBS solution supplemented with 0.05% Tween-20 (to disrupt aggregates of bacteria) and the total number of surviving intracellular bacteria was determined by plating on tryptic soy agar with 5% defibrinated sheep blood.

ELISA for quantification of anti-MRSA antibodies in human serum. Cell wall preparations (CWPs) were generated from protein-A-deficient *S. aureus* by incubating 40 mg of pelleted bacteria per ml of 10 mM Tris-HCl (pH 7.4) supplemented with 30% raffinose, 100 μ g ml⁻¹ of lysostaphin (Cell Sciences), and EDTA-free protease inhibitor cocktail (Roche), for 30 min at 37 °C. The lysates were centrifuged at 11,600g for 5 min, and the supernatants containing cell wall components were collected.

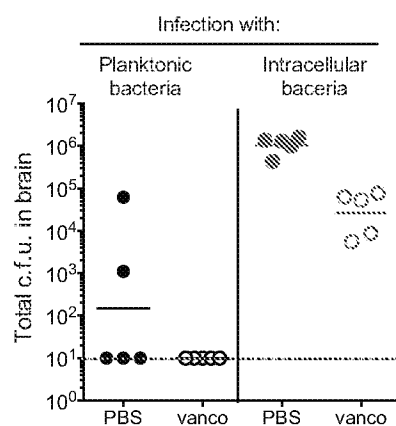
ELISA experiments were performed using standard protocols. Briefly, plates were pre-coated with CWP and then incubated with human IgG preparations: purified human IGIV Immune Globulin (ASD Healthcare), pooled serum from healthy donors or from MRSA patients. The concentrations of anti-staphylococcal IgG present in the serum or purified IgG were calculated by using a calibration curve that was generated with known concentrations of anti-peptidoglycan mAb (4479) against peptidoglycan.

Intravenous infection model for testing efficacy of the AAC. Seven-week-old female mice, Balb/c, were obtained from Jackson West, or SCID mice were obtained from Charles River Laboratories. Infections were carried out by intravenous injection into the tail vein. SCID-huIgG model: CB17.SCID mice were reconstituted with IGIV Immune Globulin (ASD Healthcare) using a dosing regimen optimized to achieve constant serum levels of >10 mg ml⁻¹ of human IgG. IGIV was administered with an initial intravenous dose of 30 mg per mouse followed by a second dose of 15 mg per mouse by intraperitoneal injection after 6 h, and subsequent daily dosings of 15 mg per mouse by intraperitoneal injection for 3 consecutive days. Mice were infected 4 h after the first dose of IGIV with 2 $\times$ 10⁷ c.f.u. of MRSA diluted in PBS by intravenous injection. The wild-type USA300, protein-A-sufficient strain was used for all *in vivo* experiments. Mice that received vancomycin were treated with twice daily intraperitoneal injections of 110 mg kg⁻¹ of vancomycin starting between 6 and 24 h after infection for the duration of the study. Experimental therapeutics (AAC, anti-MRSA antibodies or free rifalogue antibiotic) were diluted in PBS and administered with a single intravenous injection 30 min to 24 h after infection. All mice were killed on day 4 after infection, and kidneys were harvested in 5 ml of PBS. The tissue samples were homogenized using a GentleMACS dissociator (Miltenyi Biotec). The total number of bacteria recovered per mouse (two kidneys) was determined by plating serial dilutions of the tissue homogenate in PBS 0.05% Tween on tryptic soy agar with 5% defibrinated sheep blood.

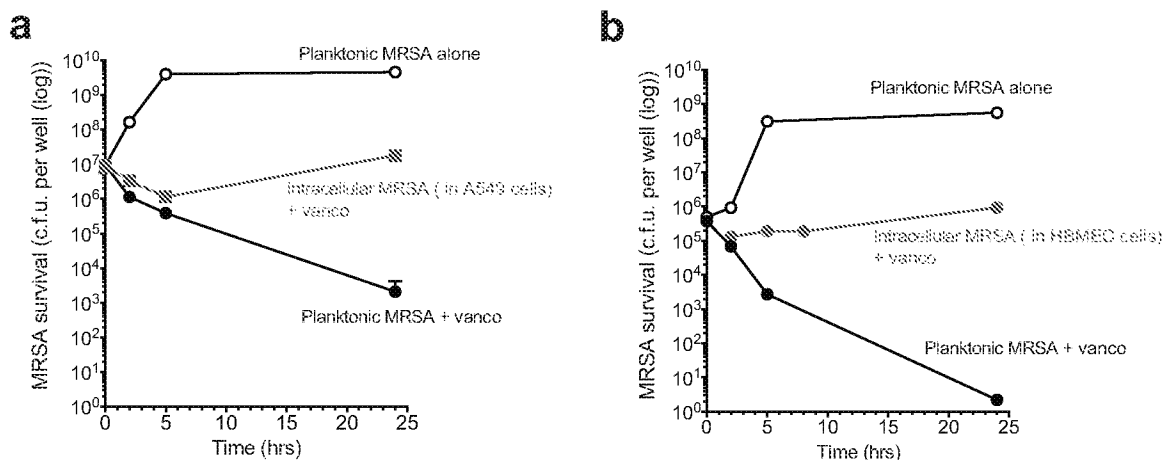
Statistical analysis. All experiments were performed on biological replicates. Sample size for each experimental group per condition is reported in appropriate figure legends and Methods. For cell culture experiments, sample size was not predetermined, and all samples were included in the analysis. In animal experiments no statistical methods were used to predetermine sample size (n = number

of mice per group), and all animals were used for analysis unless the mice died or had to be euthanized when found moribund. These cases are annotated in the figures. The mice were not randomized after infection, and the investigators were not blinded to outcome assessment. When appropriate, statistically significant differences between control and experimental groups were determined using Mann–Whitney tests.

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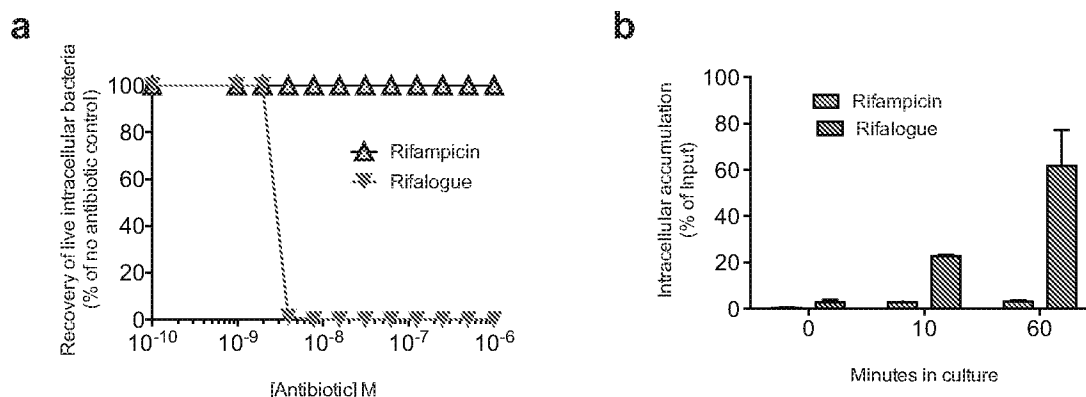
Extended Data Figure 1 | Intracellular MRSA can infect the brain even in the presence of vancomycin. *In vivo* infection of mice shown in Fig. 1a. Mice ($n = 5$) were infected with equivalent doses of free bacteria or intracellular bacteria and treated with vancomycin at 110 mg kg^{-1} 10 min after infection and then once per day. Bacterial burden was monitored in the brain 4 days after infection. Bars show geometric mean.



Extended Data Figure 2 | MRSA is able to grow in the presence of vancomycin when cultured on a monolayer of infectable cells.

a, b, Similar to the set up in Fig. 1d, planktonic MRSA were either seeded in media alone, or in the presence of vancomycin. Intracellular bacteria were generated by infecting a monolayer of either A549 bronchial epithelial cells (**a**) or HBMECs (**b**) in the presence of vancomycin (vanco). In these experiments plates were centrifuged to promote contact of the bacteria with the monolayer to enhance intracellular infection. At each time point, the culture supernatant was collected to recover extracellular

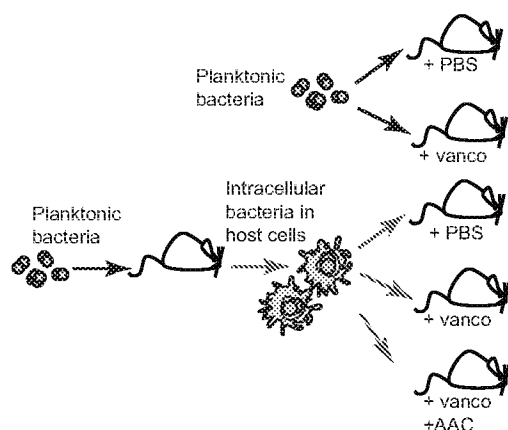
bacteria and adherent cells were lysed to release intracellular bacteria. Extracellular bacteria (planktonic bacteria) grew well in media alone, but were killed by vancomycin. In wells containing a monolayer of mammalian cells (intracellular MRSA + vanco) a fraction of the bacteria were protected from vancomycin during the first 5 h after infection and were able to expand within the intracellular compartment over 24 h. Error bars show s.d. from triplicate wells. Representative of three independent experiments.



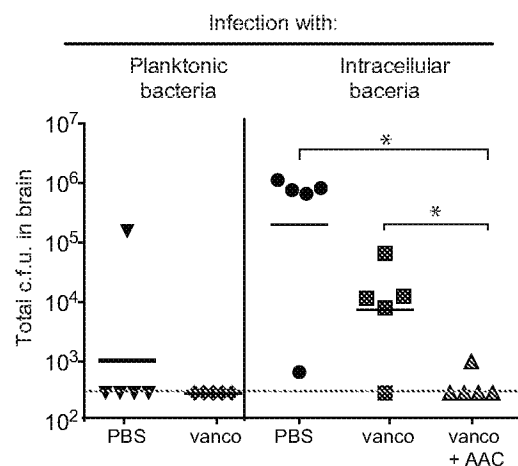
Extended Data Figure 3 | Rifalogue can also kill intracellular bacteria.

a, Determining the intracellular MIC for rifalogue and rifampicin. MRSA was allowed to infect peritoneal macrophages and macrophages were cultured overnight in gentamycin to kill extracellular bacteria. Various doses of rifalogue (red) or rifampicin (grey) were added to the culture medium 1 day after infection and the number of viable intracellular bacteria was determined 24 h later by spotting macrophage lysates onto agar plates. Data shown are representative of more than three independent experiments. **b**, Diffusion of rifalogue versus rifampicin into murine macrophages. Murine peritoneal macrophages were incubated with rifalogue or rifampicin in the culture media. Wells were harvested at 10 and 60 min and the total amount of antibiotic associated with the cells was determined by quantitative mass spectrometry. Results are shown as percentage of input. Error bars show s.d. from triplicate

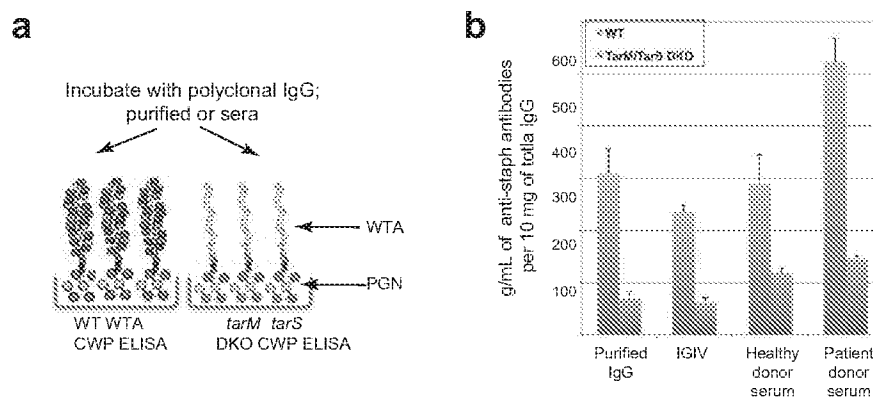
wells. Representative of two experiments. Rifalogue is more lipophilic than rifampicin as a result of its two additional fused aromatic rings, determined by measuring logDs at pH 7, with rifalogue at 3.4, being 100-fold higher than rifampicin (logD 1.3). Additionally, rifalogue has a more basic amine (pK_a 9.7) compared to that found in rifampicin (pK_a 8.2). This balance of lipophilicity and basicity in rifalogue allows it to localize preferentially in lysosomes. It is challenging to develop antibiotics with these properties for systemic administration due to poor pharmacokinetic (PK) properties and toxicity profiles associated with indiscriminate accumulation of these molecules in all host cells. However, appending such antibiotics to an anti-MRSA specific antibody both extends its half-life in the circulation and converts it into an inactive pro-drug whose properties are manifest only after it has been released in phagolysosomes of cells infected with MRSA.



Extended Data Figure 4 | AAC kills MRSA that survive treatment with vancomycin. *In vivo* infection of mice as shown in Fig. 1a. Mice were infected with equivalent doses of free bacteria or intracellular bacteria and treated with either saline (PBS) or vancomycin (vanco) at 110 mg kg^{-1} , 10 min after infection and then once per day. Selected mice were given



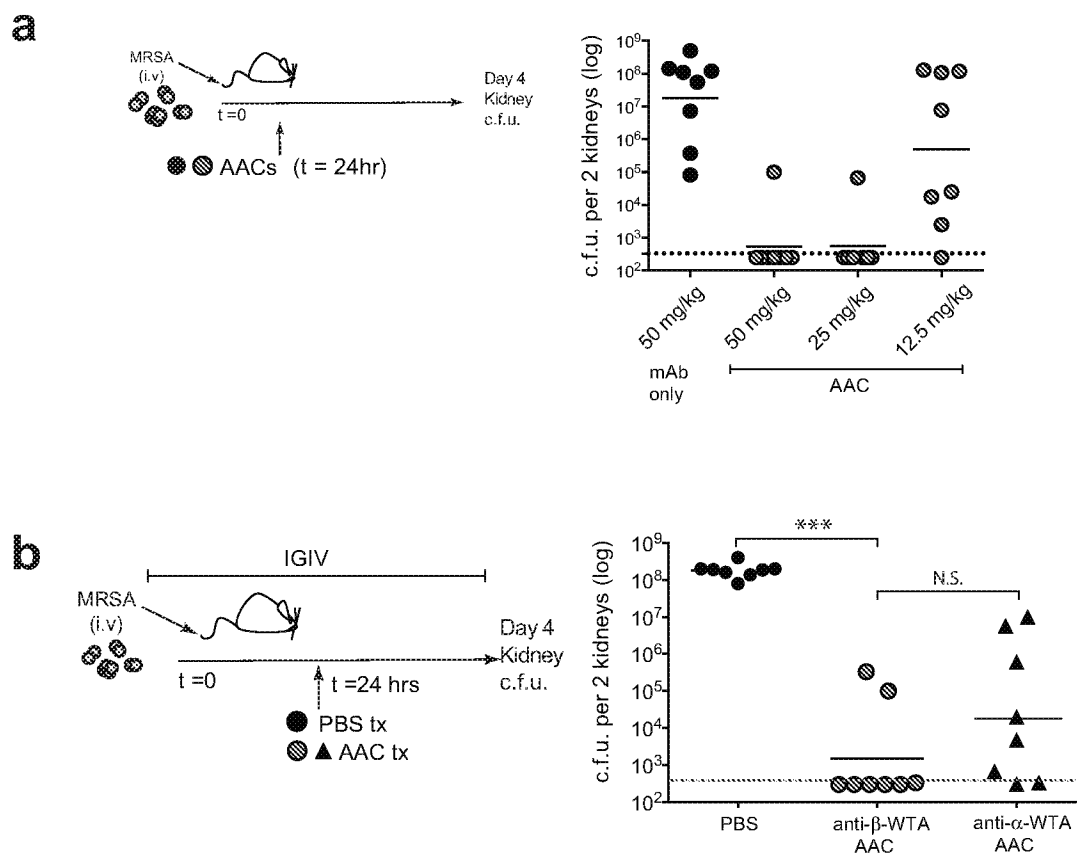
vancomycin as described earlier and also treated with a single dose of AAC at 50 mg kg^{-1} 10 min after infection. Four days after infection, bacterial burden was monitored in the brain. Each point represents data from a single mouse ($n = 5$). Bars show geometric mean. * $P < 0.05$, Mann-Whitney U-test.



Extended Data Figure 5 | Human serum contains high levels of anti-*S. aureus* antibodies that can compete with the AAC for binding.

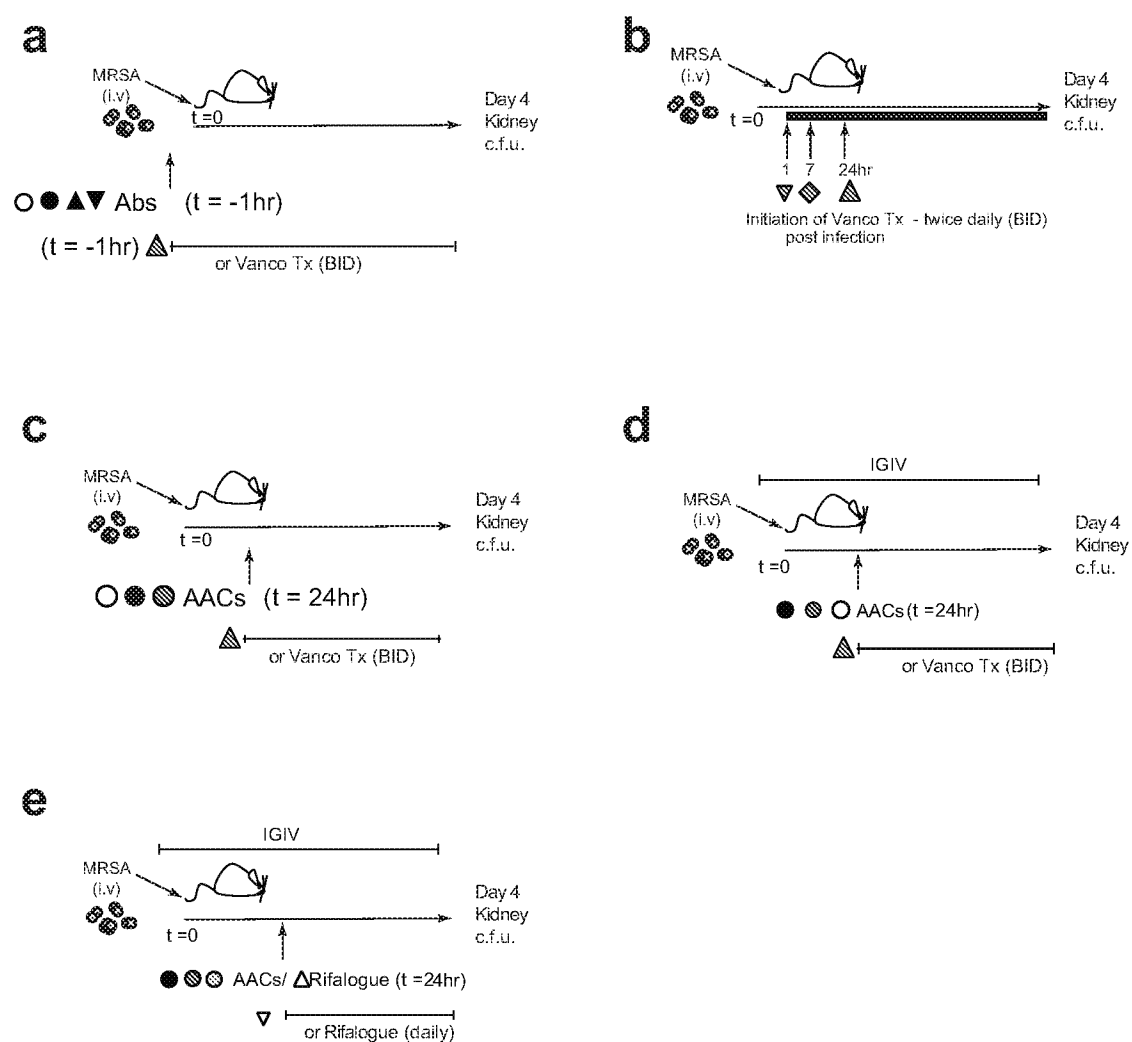
a, To estimate the concentration of antibodies that could potentially compete for binding with the anti- β -WTA antibody used in the AAC, human IgG from various sources (normal human serum, serum from MRSA infected patients, purified human IgG (Sigma) or IGIV derived from pooled normal donors) was tested for binding to various bacterial cell wall preparations (CWPs) by enzyme-linked immunosorbent assay (ELISA). CWPs were made from either USA300 (wild type (WT)) or $\Delta tarM \Delta tarS$ (DKO) USA300; the latter strain is deficient in the WTA-GlcNAc antigen recognized by the anti-WTA antibodies. For these studies protein-A-deficient USA300 background strains were used to

minimize non-specific antibody binding. A standard curve was generated by titrating known amounts of an anti-MRSA antibody directed against peptidoglycan on both cell wall extracts. **b**, Estimated concentration of anti-*S. aureus* antibodies in human serum. The amount of anti-MRSA antibodies in each sample was estimated by comparing the signal obtained for each sample with the standard curve. In the absence of WTA GlcNAc antigens, ~60–70% less serum IgG binding was observed (DKO ELISA; red bars). This indicates the high prevalence of natural antibodies against WTA in adult human serum. Results are reported as $\mu\text{g ml}^{-1}$ of antibody per 10 mg ml^{-1} of total IgG. Error bars show mean $\pm$ s.d. from triplicate wells. Data are representative of two independent experiments.

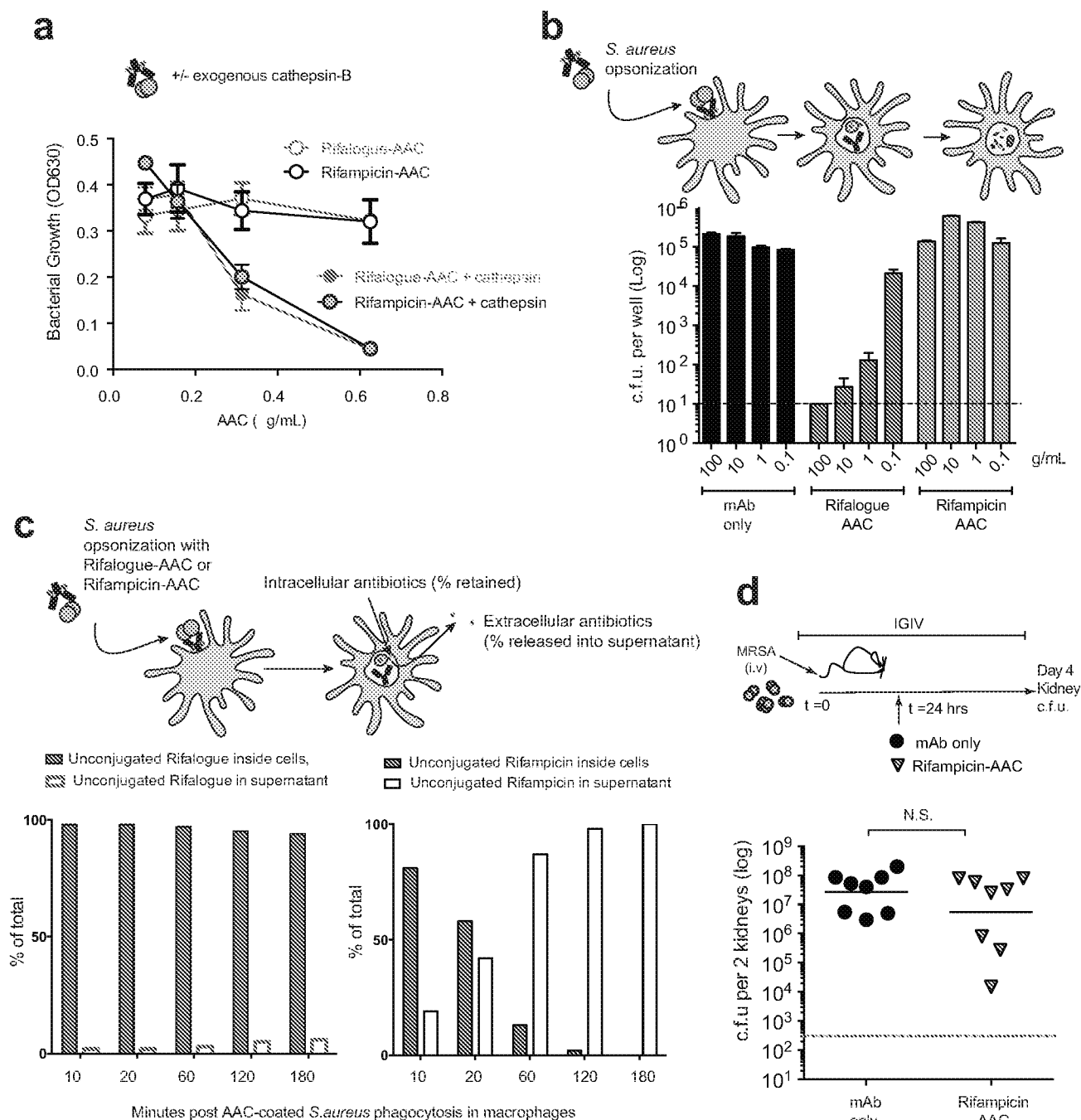


Extended Data Figure 6 | Optimization of the *in vivo* model of bacteraemia. **a**, Titration of AAC in intravenous infection model shown in Fig. 4c ($n = 8$ mice per group). **b**, Efficacy of AACs specific for β -WTA or α -WTA in SCID-IGIV model. SCID mice ($n = 8$ mice per group) were reconstituted with IGIV Immune Globulin using a dosing regimen optimized to achieve constant serum levels of $>10 \text{ mg ml}^{-1}$ of human

IgG and infected with MRSA. Mice were treated with 60 mg kg^{-1} of the indicated AACs in a single intravenous injection 1 day after infection and bacterial burden was monitored in kidneys 4 days after infection. Each point represents data from a single animal. Bars show geometric mean. Mann-Whitney test: *** $P < 0.005$, not significant (NS) $P > 0.05$.



Extended Data Figure 7 | Schematic of *in vivo* experiments presented in Fig. 4.



Extended Data Figure 8 | Comparison of anti- β -WTA AACs made with rifalogue and rifampicin. **a**, Rifampicin-AAC and rifalogue-AAC release equivalent amounts of free antibiotic after treatment with exogenous cathepsin-B. Released antibiotics from AACs made with rifalogue (red) and rifampicin (grey) are equally active as they can kill USA300 grown in broth culture. Error bars show s.d. from triplicate wells. **b**, Intracellular killing assay in primary mouse macrophages as shown in Fig. 3c indicates that the rifalogue-AAC, but not rifampicin-AAC is able to kill intracellular *S. aureus*. Error bars show s.d. from triplicate wells. **c**, Greater intracellular retention of unconjugated rifalogue compared with rifampicin after release from AAC inside macrophage cells. MRSA

was opsonized with AACs and incubated with macrophages (RAW 264.7 cells) to permit phagocytosis. The macrophages were washed to remove extracellular bacteria and samples of cell lysates or supernatants were collected in triplicate at indicated time points and the total amount of released antibiotic was determined by quantitative mass spectrometry. Error bars represent means $\pm$ s.d. from triplicate wells. **a–c**, Representative of two or more independent experiments. **d**, Rifampicin-AAC is not efficacious in the SCID-IGIV intravenous infection model as shown in Fig. 4d. Each point represents data from a single mouse ($n = 8$ mice per group). Bars show geometric mean. Mann-Whitney *U*-test: not significant (NS), $P > 0.05$.

Extended Data Table 1 | Data collection and refinement statistics for anti- β -WTA-WTA complex

	anti- β -WTA Fab-- WTA complex
Data collection	APS SER-CAT 22ID
Space group	P2 ₁ 2 ₁ 2 ₁
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	63.71, 111.47, 158.41
α , β , γ (°)	90, 90, 90
Resolution (Å)	50-1.72 (1.78-1.72)
<i>R</i> _{work} or <i>R</i> _{free}	0.056 (0.839)
<i>I</i> / σ <i>I</i>	29.4 (2.2)
Completeness (%)	99.9 (99.9)
Redundancy	6.0 (5.9)
Refinement	
Resolution (Å)	33.64-1.72
No. reflections (total/test)	120147/6101
<i>R</i> _{work} / <i>R</i> _{free}	20.6/23.7%
No. atoms	
Protein	6695
WTA	56
Glycerol	12
Calcium	4
Water	694
<i>B</i> -factors	
Protein	35.9
WTA	56.4
Glycerol	49.4
Calcium	38.7
Water	43.3
R.m.s. deviations	
Bond lengths (Å)	0.010
Bond angles (°)	1.13

*Values in parentheses are for highest-resolution shell.