Expert Opinion

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Trends in immunoconjugate and ligand-receptor based targeting development for cancer therapy

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Many agents used to treat cancer are toxic to normal tissues. Thus, treatments delivering drug specifically to tumour, while minimising exposure to normal tissue, may be advantageous over non-targeted treatments. The exquisite specificity of the immune system has been used successfully to help develop targeted anticancer agents. The most common (and successful) tissue-specific targeting strategies rely on antibody conjugates, but additional approaches, including targeting through cytokines, peptides and recombinant viruses, have also been used successfully. This review summarises the agents exploiting the immunological principles of target specificity to help maximise delivery to tumour while minimising collateral damage to normal tissues. Such targeted molecules are collectively referred to as immunoconjugates.

Keywords: cancer, cytotoxin, immunoconjugate, targeted drug delivery

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1. Introduction

Paul Ehrlich is credited with developing the concept of targeted therapeutic agents more than a century ago. He coined the term 'magic bullet' to refer to agents that could specifically target and destroy tumour cells as well as microorganisms. Ehrlich's seminal ideas established many of the founding principles of immunology [1,2] and he was the first to lay the conceptual groundwork for the development of immunoconjugates. However, only in the last few decades have targeted therapies become a practical and successful means of treating cancer [3,4]. For the purposes of this review, if a clinical trial demonstrated some positive therapeutic response, the authors have defined it as successful.

2. Definition of an immunoconjugate

Immunoconjugates are molecules consisting of two originally separate components. They are used either therapeutically or diagnostically. In therapeutic immunoconjugates, one component is typically of biological or chemical origin, chosen for its ability to induce cell death. Therapeutic moieties on immunoconjugates include toxins, cytotoxic chemicals, radionuclides, agents blocking gene expression and lytic viruses. In the case of diagnostic immunoconjugates, this component emits a specific detection signal. Diagnostic moieties are primarily radionuclides, but the use of fluorographic or ultrasonographic detection methods may soon replace older scintographic technology. The other component mediates specific binding. This component is typically an antibody in the case of immunoconjugates, but may also be a cytokine, growth factor or a molecule based on immunological principles (such as a ligand-binding peptide derived from a random, combinatorial library).

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3. Therapeutic immunoconjugates

The ideal approach to tumour targeting is to capitalise on a molecule that is uniquely expressed on the surface of cancer cells, but never on normal tissues – a situation that rarely exists. In reality, the best-case scenario usually involves selecting a molecular target that is overexpressed on tumour cells, but is expressed at comparatively low levels in other organs and tissues. The other viable option is to choose a molecular target in which its context is different on tumour cells relative to normal cells. Examples include multipartite receptors that are differentially expressed (with or without one or more of their subunits).

Therapeutic immunoconjugates deliver a toxic payload through a targeting moiety. The prototypical agent is an antibody linked to one of a wide variety of moieties designed to kill the targeted cell, including radionuclides (**Figure 1A**), recombinant cytotoxic proteins [5,6], cytotoxic chemicals [7,8], ribonucleases (RNases) [9,10], or small inhibitory RNAs [11] (**Figure 1B**). Some immunoconjugates use antibody fragments rather than an intact antibody. One class of such immunoconjugates includes the disulfide bond-stabilised Fv portion of an antibody (dsFv) fused to a toxic payload (**Figure 1C**) [5].

A second group involves an unmodified single-chain antibody (scFv) consisting of a linear arrangement of the variable light and heavy chains of an antibody (fused through a short peptide linker) [12] that has been fused to a toxic payload (**Figure 1D**).

In recent years, a large number of immunoconjugates have been designed using selective alternatives to antibodies or their derivatives. The targeting of these newer molecules is mediated by a cell surface receptor-specific binding ligand (**Figure 1E**). These binding ligands are not restricted to signalling molecules such as interleukins, but also include novel binding peptides identified using technologies such as phage display [13] (**Figure 1F, G**). Finally, a plethora of viral delivery vehicles have been designed that use immunoconjugate-based concepts to deliver toxic agents to targeted cells (**Figure 1H – K**) [14].

3.1 Antibody-based immunotherapeutic agents

The first targeted anticancer agents were monoclonal antibodies that selectively targeted and killed cancer cells. These were not true immunoconjugates but rather specific immunological agents that relatively selectively targeted cancer cells. Examples of these drugs include rituximab [15], a chimeric anti-CD20 monoclonal antibody approved by the FDA in 1997 to treat B-cell lymphoma and alemtuzumab [16] – a humanised anti-CD52 monoclonal antibody approved in 2001 to treat chronic lymphocytic leukaemia (**Table 1**). In both these cases, antibody-mediated apoptosis results from a combination of complement-mediated cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC). The former situation arises from the activation of the cascade of complement proteins, leading to the lysis of antibody-bound cells [17]. ADCC arises from the recognition of antibody-bound cells by other immune cells, ultimately leading to the destruction of the targeted cells [17].

3.1.1 Early advances in immunotherapy using conjugated toxins

One of the earliest successful clinical trials of an immunoconjugate was in the treatment of non-Hodgkin's lymphoma using a CD22-targeting monoclonal antibody (RFB4) that was fused chemically to deglycosylated ricin toxin A chain (dgA) [18]. Ricin toxin cleaves ribosomal RNA, thereby abrogating protein synthesis [19,20]. Although these studies showed clinical efficacy, with complete responses in 2 out of 41 patients, and partial responses in 10 additional patients [21,22], vascular leak syndrome arose as a frequent side effect, restricting the clinical development of RTB4-dgA. Modifying the ricin toxin component of RTB4-dgA (**Table 1**) by converting asn-97 of the ricin A chain to alanine resulted in a much lower rate of vascular leak syndrome (relative to RTB4-dgA), and had significantly higher therapeutic efficacy when administered to SCID/Daudi lymphoma mice [23]. No subsequent studies using this immunotoxin in humans have been reported, to the present authors' knowledge.

The next generation of anti-CD22 immunoconjugate, typified by a conjugate termed BL22 (**Table 1**), was constructed by genetically fusing a disulfide-bond-stabilised Fv (dsFv) portion of the RFB4 anti-CD22 antibody to the catalytic and translocation domains of *Pseudomonas aeruginosa* exotoxin A ($PE_{253-613}$; PE38; **Figure 2A**). This manipulation effectively retargeted the PE cytotoxic component to destroy $CD22^+$ cells. Plasmids independently expressing each of the two dsFv chains (one of which is fused to PE) need to be co-expressed in *Escherichia coli* prior to purification in order to link both Fv chains covalently in the BL22 immunoconjugate (see **Figure 1C**).

PE-based immunotoxins are rapidly internalised by receptor-mediated endocytosis prior to being proteolytically cleaved at the junction between the domain responsible for catalytic activity (the ADP ribosyltransferase domain) and the translocation domain. The modular domain organisation of PE is shown schematically in **Figure 2A** [24]. Following proteolytic cleavage in the endosome, the disulfide bridge tethering these two domains is reduced as the endosome is acidified [25]. This, in turn, leads to the ADP ribosyltransferase domain being translocated into the cytoplasm of the target cell [26,27], its ADP ribosylation of elongation factor 2, the halting of protein synthesis and ultimately cell death [28].

BL22 has shown considerable efficacy in treating patients with a variety of leukaemias and lymphomas, demonstrating complete responses in 19 out of 46 recipients and partial responses in an additional 7 patients [29,30]. BL22 has been further modified using antibody phage display, resulting in

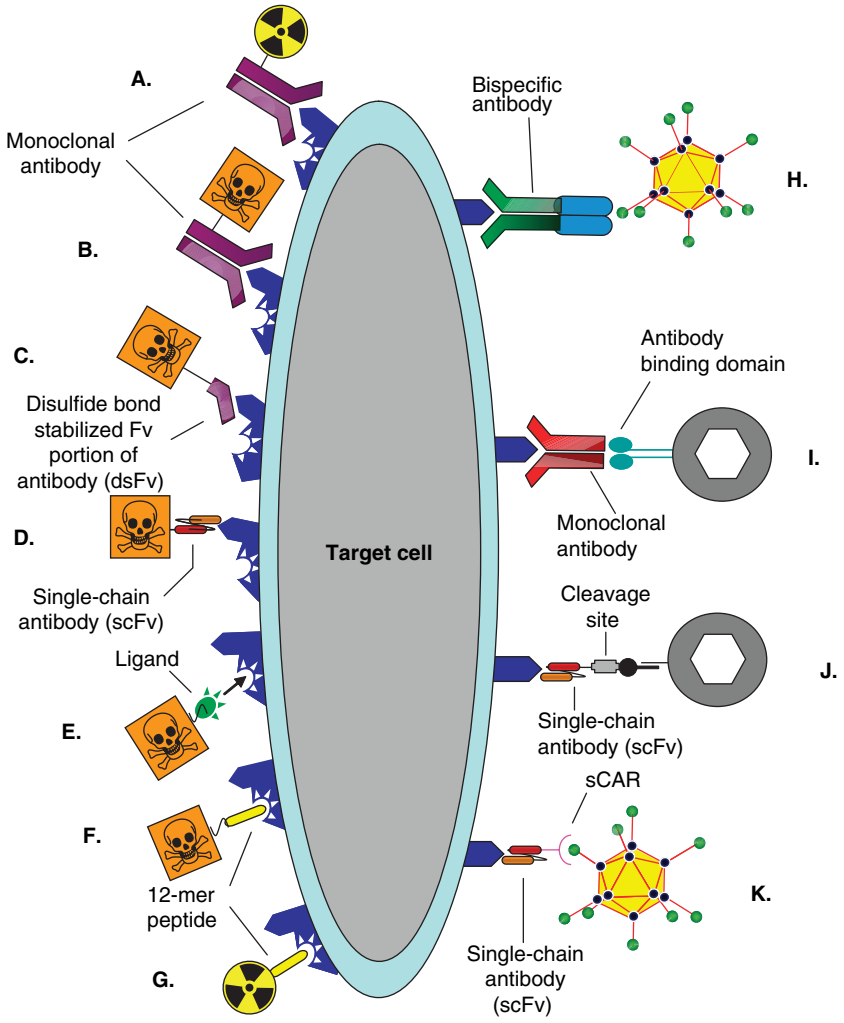

Figure 1. A schematic showing the novel targeting mechanism of immunoconjugates and ligand–receptor-based agents. Antibody or ligand-based agents are shown on the left side of the figure, and viral delivery vehicles are shown on the right side of the figure. Ligands, receptors and antibodies with identical colours indicate that they could theoretically be derived from the same molecule. Black lines represent linker regions.

an immunoconjugate with 10-fold increased affinity for CD22 [31]. This modified BL22 immunoconjugate, RFB4 (THW-Fv)-PE38, which possesses a THW motif in place of SSY within the V_H chain, is undergoing a Phase I clinical trial, and the original drug is the subject of a Phase II clinical trial (**Table 1**). Other immunoconjugates targeting distinct epitopes on CD22 incorporate the monoclonal antibodies HD6 and HD39, each of which has been conjugated to the ribosome-inactivating toxin saporin [32], but these agents have not been clinically developed.

Inotuzumab ozogamicin is a humanised anti-CD22 monoclonal antibody (CMC-544) that has been covalently linked to calicheamicin (a DNA-damaging cytotoxic chemical) and has shown significant therapeutic potential for the treatment of acute lymphoblastic leukaemia and certain B cell lymphomas [33,34]. The drug has been tested in combination with rituximab [35] and is undergoing a Phase I clinical trial (**Table 1**).

3.1.2 The development of antibody-based immunoconjugates targeting a broader array of immunological markers

A number of Phase I clinical trials targeting CD19 have met with limited or no success (**Table 1**). The results are difficult to interpret, as several different monoclonal antibodies have been used (B4, HD37 and B43, respectively), and each of these antibodies were conjugated to a different cytotoxin. For example, B4 was chemically conjugated to blocked ricin (bR), producing anti-B4-bR (**Table 1**), but showed no responses in 16 patients enrolled in a Phase II clinical trial to treat non-Hodgkin's lymphoma [36]. bR toxin refers to the result of modifying the oligosaccharide-binding sites on

Table 1. A list of relevant therapeutic agents that have undergone clinical trials.

Immunotherapeutic agent	Toxin	Site of action	Target	Clinical trial phase	Tumour	Ref.
Alemtuzumab	–	ADCC/CDC	CD52	FDA approved	B cell CLL	[16,133]
Anti-B4-bR	bR	Ribosome	CD19	II	NHL	[36]
Anti-CEA-bR	bR	Ribosome	CEA	I/II	Hepatic metastases	[70]
[^{211}At]-anti-tenascin (81C6)	–	DNA	Tenascin	I	Malignant glioma	[134]
AVE963	DM1	Microtubules	CD33	I	AML	[60]
B-B4-DM1	DM1	Microtubules	CD138	I	MM	[61]
[^{213}Bi]-CHXA-DTPA-HuM195	–	DNA	CD33	I/II	AML	[135]
BL22 (RFB4-PE38)	PE38	Ribosome	CD22	II	Ly, Le	[30]
Ber-H2-sap6	SAP	Ribosome	CD30	I	HD	[47]
BR96scFv-PE40	PE40	Ribosome	Lewis Y	I	Adenocarcinoma	[67]
Cantuzumab mertansine	DM1	Microtubules	CanAg	I	CanAg$^+$ tumours	[59]
CMC-544	CAL	DNA	CD22	I/II	CD22$^+$ B cell Ly	[33]
CR011-vcMMAE	MMAE	Microtubules	gpNMB	I	Melanoma	[57]
Denileukin diftitox	DT_{389}	Ribosome	CD25	FDA approved	CTCL, NHL	[94]
DT_{388}-GMCSF	DT_{388}	Ribosome	GM-CSFR	I	AML	[80]
260F9-rRTA	rRTA	Ribosome	55 kDa	I	brc	[73,74]
Gemtuzumab ozogamicin	CAL	DNA	CD33	FDA approved	AML	[8,136]
HD37-dgA	dgA	Ribosome	CD19	I	NHL	[38,137]
HuM195	–	ADCC/CDC	CD33	III	AML	[138,139]
HuN901-DM1	DM1	Microtubules	CD56	I/II	SCLC, MM	[61,140]
DT_{388}-IL-3	DT_{388}	Ribosome	IL-3R	I	AML	[86]
IL-4(38-37)-PE38KDEL	PE38	Ribosome	IL-4R	I	Glioblastoma	[87]
IL-13-PE38QQR	PE38	Ribosome	IL-13R	III	Malignant glioma	[89]
Inotuzumab ozogamicin	CAL	DNA	CD22	I	ALL, Le, NHL	[33,34]
[^{131}I]-tositumomab	–	DNA	CD20	FDA approved	NHL	[141]
LMB-1	PE38	Ribosome	Lewis Y	I	Adenocarcinoma	[66]
LMB-2	PE38	Ribosome	CD25	I/II	NHL, Le, HCL	[45]
[^{177}Lu]-J591	–	DNA	PSMA	I	Prostate cancer	[142]
Ki-4-dgA	dgA	Ribosome	CD30	I	HD, NHL	[46]
N901-bR	bR	Ribosome	CD56	I	SCLC	[75,76]
OVB3-PE	PE	Ribosome	Ovary	I	Ovarian cancer	[77]
Oregovomab	–	DC priming	CA-125	III	Ovarian cancer	[115,143]
RFB4-dgA	dgA	Ribosome	CD22	I	B cell Ly	[21,22]
RFB4 (THW-Fv)-PE38	PE38	Ribosome	CD22	I	CLL, HCL	[31]
RFT5-dgA	dgA	Ribosome	CD25	I/II	HD, NHL	[40]
Rituximab	–	ADCC/CDC	CD20	FDA approved	Ly, NHL	[15,144]

ADCC: Antibody-dependent cell-mediated cytotoxicity; ALL: Acute lymphoblastic leukaemia; AML: Acute myelogenous leukaemia; bR: Blocked ricin toxin; brc: Breast cancer; CAL: Calicheamicin; CDC; Cellular-dependent cytotoxicity; CLL: Chronic lymphoid leukaemia; crc: Colorectal cancer; CTCL: Cutaneous T cell lymphoma; DC: Dendritic cell; dgA: Deglycosylated ricin A chain; DM: *N*$^{2'}$-deacetyl-*N*$^{2'}$-(3-mercapto-1-oxopropyl) maytansine; DT: Diphtheria toxin; $DT_{388/389}$: C-terminally truncated DT; EGFR: Epidermal growth factor receptor; GM-CSFR: Granulocyte-macrophage colony stimulating factor receptor; gpNMB: Transmembrane glycoprotein NMB precursor; HCL: Hairy-cell leukaemia; HD: Hodgkin's disease; Le: Leukaemia; Ly: Lymphoma; mes: Mesothelioma; MM: Multiple myeloma; MMAE: Monomethyl auristatin E; NHL: Non-Hodgkin's lymphoma; NSCLC: Non-small-cell lung cancer; panc: Pancreatic cancer; PAP: Pokeweed antiviral protein; PE: *Pseudomonas* exotoxin A; PE38/40: *N*-terminally truncated PE; PSMA: Prostate-specific membrane antigen; rRTA: Recombinant ricin toxin A chain; SAP: Saporin; SCLC: Small cell lung carcinoma.

Table 1. A list of relevant therapeutic agents that have undergone clinical trials (continued).

Immunotherapeutic agent	Toxin	Site of action	Target	Clinical trial phase	Tumour	Ref.
ScFv(FRP5)-ETA	PE38	Ribosome	HER2/NEU	I	crc, brc, melanoma	[64]
SGN-35	MMAE	Microtubules	CD30	I	HD	[52]
SS1(dsFv)PE38 (SS1P)	PE38	Ribosome	Mesothelin	I	Ovarian, panc, mes	[5,69]
TP-38	PE38	Ribosome	EGFR	I	Glioblastoma	[91]
TP-40	PE40	Ribosome	EGFR	I	Bladder cancer	[90]
Trastuzumab	–	Receptor blockade	HER2/NEU	FDA approved	brc	[145]
Trastuzumab-SMC-DM1	DM1	Microtubules	HER2/NEU	I	brc	[60]
XomaZyme-791	rRTA	Ribosome	72 kDa	I	crc	[71,72]
[^{90}Y]-epratuzumab	–	DNA	CD22	I/II	NHL	[146,147]
[^{90}Y]-ibritumomab tiuxetan	–	DNA	CD20	FDA approved	NHL	[148]

ADCC: Antibody-dependent cell-mediated cytotoxicity; ALL: Acute lymphoblastic leukaemia; AML: Acute myelogenous leukaemia; bR: Blocked ricin toxin; brc: Breast cancer; CAL: Calicheamicin; CDC; Cellular-dependent cytotoxicity; CLL: Chronic lymphoid leukaemia; crc: Colorectal cancer; CTCL: Cutaneous T cell lymphoma; DC: Dendritic cell; dgA: Deglycosylated ricin A chain; DM: $N^{2'}$-deacetyl-$N^{2'}$-(3-mercapto-1-oxopropyl) maytansine; DT: Diphtheria toxin; $DT_{388/389}$: C-terminally truncated DT; EGFR: Epidermal growth factor receptor; GM-CSFR: Granulocyte-macrophage colony stimulating factor receptor; gpNMB: Transmembrane glycoprotein NMB precursor; HCL: Hairy-cell leukaemia; HD: Hodgkin's disease; Le: Leukaemia; Ly: Lymphoma; mes: Mesothelioma; MM: Multiple myeloma; MMAE: Monomethyl auristatin E; NHL: Non-Hodgkin's lymphoma; NSCLC: Non-small-cell lung cancer; panc: Pancreatic cancer; PAP: Pokeweed antiviral protein; PE: *Pseudomonas* exotoxin A; PE38/40: *N*-terminally truncated PE; PSMA: Prostate-specific membrane antigen; rRTA: Recombinant ricin toxin A chain; SAP: Saporin; SCLC: Small cell lung carcinoma.

the ricin B chain using glycopeptides containing triantennary *N*-linked oligosaccharides [37]. HD37 was chemically conjugated to deglycosylated ricin A chain (H37-dgA; **Table 1**) and tested in the presence or absence of RFB4-dgA. This Phase I clinical trial showed partial responses in 2 out of 22 recipients [38], with vascular leak syndrome being the same serious side effect that has been observed previously for RFB4-dgA [21,22]. Finally, the anti-CD19 monoclonal antibody B43, which was chemically conjugated to pokeweed antiviral protein (PAP), which inactivates ribosomes, showed no therapeutic efficacy [39]. In fact, the administration of B43-PAP to SCID mice challenged with human B-cell precursor acute lymphoblastic leukaemia cells was efficacious only upon co-administration of a cocktail of the three antileukaemic drugs: vincristine, methylprednisolone and L-asparaginase [39].

RFT5-dgA (**Table 1**, **Figure 2A**) is an immunoconjugate covalently linking the Fc portion of the anti-CD25 antibody RFT5 to deglycosylated ricin A chain, and it binds $CD25^+$ cells. RFT5-dgA has been shown to be efficacious in treating some cases of Hodgkin's Disease in a Phase II clinical trial [40]. Anti-Tac(scFv)-PE38 (LMB-2) employs an anti-CD25 single-chain antibody genetically fused with PE38 (**Figure 1D**, **Figure 2A**, **Table 1**). LMB-2 has demonstrated clinical efficacy in some $CD25^+$ lymphomas [41] and leukaemias [42], and also penetrates solid tumours [43]. LMB-2 has been shown to cause fever and liver toxicities [41,42,44]. Additional protein engineering may resolve these issues to allow further development of this immunoconjugate [45].

Chemical conjugation of an anti-CD30 monoclonal antibody to deglycosylated ricin A chain (Ki-4-dgA) effected partial responses in only 1 out of 15 patients in a Phase I clinical trial and, thus, has not been studied further [46]. In addition, the Ber-H2 humanised anti-CD30 monoclonal antibody was chemically fused to saporin (Ber-H2-sap6; **Table 1**) but also failed clinically [47].

Chemical conjugation of an anti-CD33 monoclonal antibody to bR toxin (anti-My9-bR) has similarly met with no therapeutic efficacy [48]. However, of great significance is the fact that gemtuzumab ozogamicin, a humanised anti-CD33 antibody chemically linked to calicheamicin [49,50], has been approved by the FDA for the treatment of relapsed acute myeloid leukaemia [8]. This drug is the first FDA-approved immunoconjugate of a chemical cytotoxin.

3.1.3 Antimicrotubule-based chemical immunoconjugates

Auristatins are cytotoxic chemicals that induce G2-M cell cycle arrest and microtubule disruption, causing apoptosis of targeted cells. Several immunoconjugates containing auristatin analogs have been chemically fused to monoclonal antibodies against CD20 [51], CD30 [52], CD70 [53], prostate-specific membrane antigen [54], p97 [55], E-selectin [56], glycoprotein NMB [57] and Lewis Y antigen [58]. Many of these drugs are still in the very early stages of clinical development as antitumour agents, although auristatin-based immunoconjugates targeting CD30 (SGN-35) and glycoprotein NMB (CR011-vcMMAE) have now completed Phase I clinical trials (**Table 1**).

A solid tumour target is the extracellular domain of the carbohydrate epitope of CanAg (a novel glycoform of Muc1), which is overexpressed in many pancreatic, biliary and colorectal tumours. The humanised monoclonal

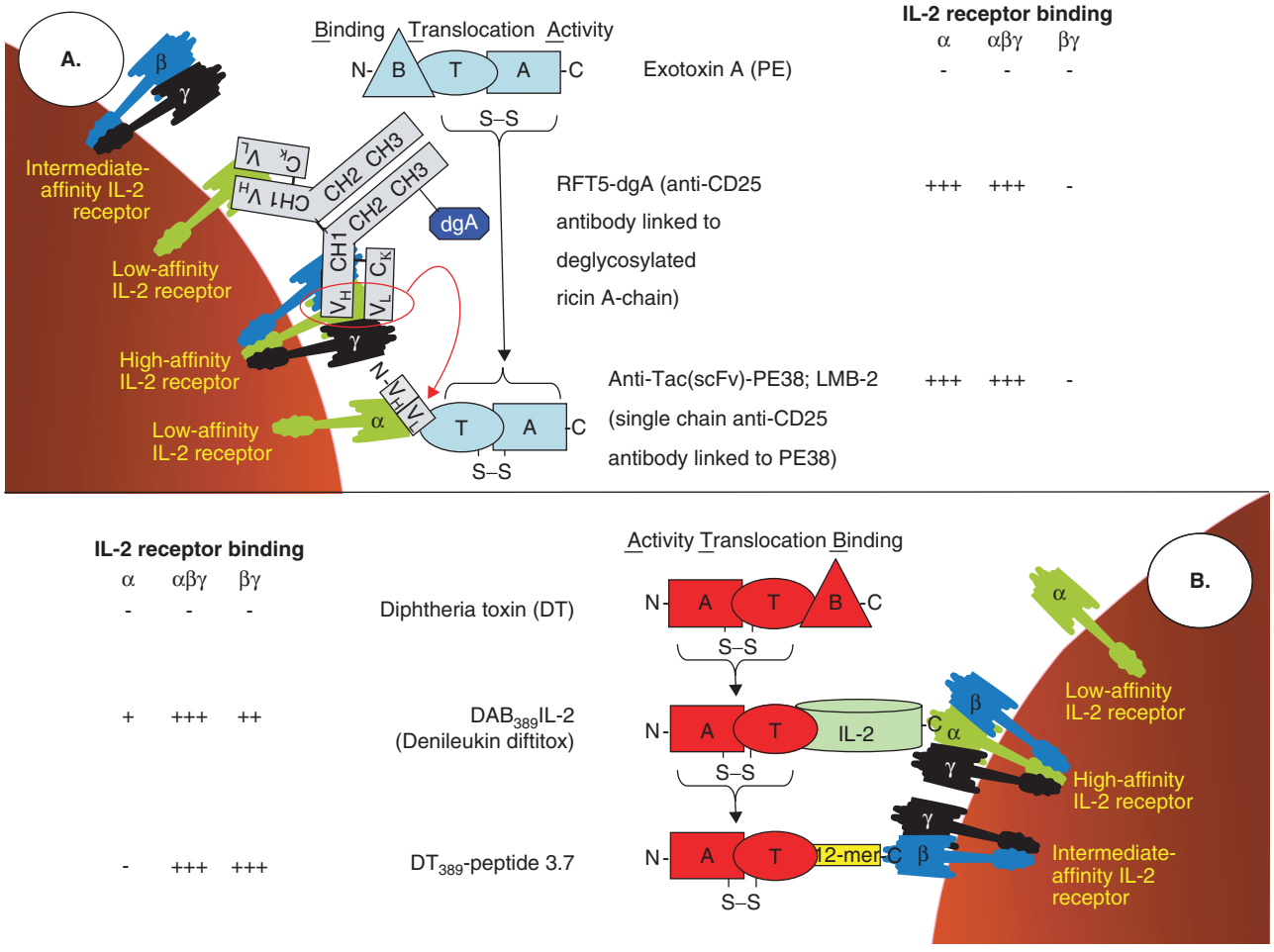

Figure 2. The evolution of immunoconjugates targeting IL-2R with specific emphasis on the modular organisation of the required domains. Two types of modular cytotoxins are shown, one based on *Pseudomonas aeruginosa* exotoxin A (PE; panel **A**) and the other based on diphtheria toxin (DT; panel **B**). The variable heavy (V_H) and light (V_L) chains of the anti-CD25 antibody are shown, along with the constant kappa chain (C_κ). In addition, the CH1 domain of the Fab portion is indicated, along with the CH2 and CH3 domains of the Fc portion of the anti-CD25 antibody. The disulfide bond linking the catalytic and translocation domains of the cytotoxins is also indicated (S–S).

antibody huC242 has been chemically linked to the potent antimicrotubule agent $N^{2'}$-deacetyl-$N^{2'}$-(3-mercapto-1-oxopropyl) maytansine (DM1). This immunoconjugate, designated cantuzumab mertansine (**Table 1**), has effected clinical responses in pancreatic, colorectal and non-small cell lung cancer [59] and is undergoing further evaluation.

Other maytansine-based immunoconjugates undergoing Phase I clinical trials are shown in **Table 1** and include trastuzumab-DM1 (anti-HER2/NEU) [60], AVE9633 (anti-CD33) [60], HuN901-DM1 (anti-CD56) [61] and B-B4-DM1 (anti-CD138) [62].

3.1.4 *Immunoconjugates targeting solid tumours*

As for many potential oncology agents, significant numbers of immunoconjugates have failed clinical trials, the majority of which were targeted to solid tumours (**Table 1**). For example, a Phase I clinical trial testing the efficacy of an anti-HER2/NEU dsFv antibody genetically fused to PE38 (Erb-38) failed due to liver toxicity in addition to lack of clinical efficacy in the five breast cancer patients tested [63]. An anti-HER2/NEU scFv antibody that was genetically fused to truncated PE, designated scFv(FRP5)-ETA (**Table 1**), showed early promise in 11 cancer patients who were being treated for metastatic breast and colorectal cancers, as well as from malignant melanoma. These patients were treated by intratumoural injection with complete regression of the cutaneous tumour lesions in four of these patients, with partial shrinkage being observed in another two [64]. Unfortunately, no objective responses were observed when 18 additional breast cancer patients were evaluated [65].

At least five different immunoconjugates have been designed in which a variety of anti-Lewis Y antibodies

(or antibody fragments) have been linked to truncated PE. All of these molecules have shown little or no efficacy in treating adenocarcinomas [5]. The first published clinical trial examined the efficacy of monoclonal antibody B3 chemically linked to PE38 (LMB-1; **Table 1**). Out of a total of 38 patients, there was one complete response and one partial response, with a significant prevalence of vascular leak syndrome [66]. In a second published clinical trial targeting the Lewis Y antigen, an scFv (BR96) was genetically fused to truncated PE (BR96scFv-PE40) but showed no clinical responses in 38 patients with Lewis Y antigen-positive metastatic carcinoma [67].

Using a combination of antibody phage display and site-directed mutagenesis [68], an immunoconjugate targeting mesothelin, SS1(dsFv)PE38, has been developed. Mesothelin is expressed at high levels in mesothelioma and ovarian and pancreatic cancer [69]. SS1(dsFv)PE38 is comprised of a modified scFv that has been genetically fused to PE38 (**Table 1**). A Phase II clinical trial is scheduled to begin in 2007 [5].

For the sake of completeness, a number immunoconjugates are briefly mentioned that have been designed against poorly characterised or uncharacterised markers on solid tumours, none of which have shown any significant clinical efficacy. These include a Phase I/II clinical trial studying an anticarcinoembryonic antigen (CEA) monoclonal antibody chemically linked to bR toxin (anti-CEA-bR) to treat colorectal cancer [70]. An attempt to target a 72 kDa glycoprotein using a monoclonal antibody chemically liked to rRTA (XomaZyme-791; **Table 1**) showed no clinical responses in a Phase I clinical trial of colorectal cancer [71,72]. A monoclonal antibody against a 55-kDa breast cancer antigen chemically linked to rRTA was tested in a Phase I clinical trial (260F9-rRTA; **Table 1**); dose-limiting sensorimotor neuropathies terminated the trial [73,74]. N901 is a murine monoclonal antibody binding CD56 that is chemically fused to bR, to treat small-cell lung carcinoma (N901-bR; **Table 1**). In this case, vascular leak syndrome was the dose-limiting toxicity [75,76]. Murine anti-OVB3 monoclonal antibody reacting against a common epitope on human ovarian cancers was chemically linked to full-length PE (OVB3-PE; **Table 1**). This drug showed significant central nervous system toxicity [77].

3.1.5 *Therapeutic radioimmunoconjugates*

Antibody targeting has also been used to guide radionuclides to tumour to mediate their destruction. Examples of such radionuclides include, ^{90}Y ($t_{½}$ = 64 h; 2.3 MeV), ^{131}I ($t_{½}$ = 8 days; 0.1 – 0.8 MeV), ^{177}Lu ($t_{½}$ = 6.7 days; 0.5 MeV), ^{211}At ($t_{½}$ = 7.2 h; 6.0 MeV), ^{213}Bi ($t_{½}$ = 46 min; 6.0 MeV), and ^{225}Ac ($t_{½}$ = 10 days; 8.8 – 24.8 MeV) [17,78]. Only two radioimmunoconjugates have been FDA approved at this time: [^{90}Y]-ibritumomab tiuxetan (murine anti-CD20 monoclonal antibody) and [^{131}I]-tositumomab (murine anti-CD20 monoclonal antibody), and both are used to treat B cell lymphoma. However, a large number of additional radioimmunoconjugates are described in **Table 1** that are being studied in preclinical and clinical trials.

The use of ^{225}Ac-based immunoconjugates has some significant advantages over the other radioisotopes mentioned in this section – namely the fact that it generates four high-energy alpha particles during its decay. Relative to the other radioimmunoconjugates listed above, ^{225}Ac delivers a comparatively massive dose of radiation to an extremely small area (2 – 4 cell diameters), which is ideal for targeting tumour cells while minimising collateral damage [79]. Chelating agents such as 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) have been developed to stably link ^{225}Ac to a wide variety of immunoconjugates, such as HuM195, J591 and B4, (**Table 1**) in tumour-bearing nude mice [78]. Results from a clinical trial examining the efficacy of [^{225}Ac]-DOTA-HuM195 are anticipated shortly.

3.2 Cytokine-containing immunoconjugates

Some immunoconjugates have entirely replaced the antibody (or antibody fragment) component of the molecule with targeting ligands such as cytokines or peptides. For example, human granulocyte-macrophage colony-stimulating factor (GM-CSF) has been fused to the C-terminally truncated form of diphtheria toxin (DT_{1-388}; DT_{388}) to treat GM-CSF$^+$ acute myeloid leukaemia (DT_{388}-GMCSF; **Table 1**) [80]. By eliminating the 147 amino acids at the C-terminus of the wild-type diphtheria toxin (DT_{1-388}), the endogenous binding domain was completely replaced by GM-CSF, redirecting this cytotoxin against cells expressing the GM-CSF receptor, such as acute myeloid leukaemic cells [80]. DT_{1-388} is proteolytically cleaved within a disulfide loop formed by cys-186 and cys-201 prior to binding to its target [81], which in this case is GM-CSF receptor. Following its internalisation by receptor-mediated endocytosis, the DT_{388} component undergoes a conformational change, the disulfide bond is reduced and the ADP ribosyltransferase domain is translocated into the cytoplasm. From this compartment the toxin is able to ADP ribosylate elongation factor 2, thereby halting translation and inducing cell death [82].

Although severe hepatotoxicity terminated further clinical studies involving DT_{388}-GMCSF [3], others have successfully targeted GM-CSF to the tumour microenvironment by fusing it to scFv(L19). In this case the targeting moiety is a single-chain human antibody fragment targeting the extra-domain B domain of fibronectin, a marker of angiogenesis [83]. Human IL-15 has also been fused to scFv(L19) and has shown to be efficacious in targeting CD8$^+$ T cells *in vitro* and in animal models [83,84]. Human clinical trials of IL-15 or GM-CSF fused to scFv(L19) are likely to be conducted shortly.

IL-3 fused to the C-terminus of DT_{388} (DT_{388}-IL-3; **Table 1**) has been developed to target IL-3R$^+$ haematological malignancies [85]. Large-scale production of this

immunoconjugate has been achieved [86], and a Phase I clinical trial to treat acute myeloid leukaemia is underway.

A fusion protein combining PE38 with IL-4, IL-4(38-37)-PE38KDEL (**Table 1**), has been used to treat IL-4R$^+$ glioblastoma [87]. Liver toxicity with this agent has halted its further clinical development. In contrast, IL-13 fused to PE38 (IL13-PE38QQR; **Table 1**) is efficacious in treating malignant glioma, with manageable side effects [88]. Therefore, this immunoconjugate is undergoing a Phase III clinical trial [89]. A fusion of transforming growth factor-α to truncated PE (TP-40; **Table 1**), has been tested following intravesical injection to treat superficial bladder cancer, with 8 out of 43 patients responding in a Phase I clinical trial [90]. A similar immunoconjugate (TP-38; **Table 1**) was more recently given by intracerebral injection to treat bifrontal recurrent glioblastoma. During the course of a Phase I clinical trial, 3 patients out of 15 showed a radiographic response. In addition, one patient showed complete remission, surviving for over 83 weeks at the time that the data from the clinical trial was published [91]. Although transforming growth factor-α fused to PE38 has shown no signs of toxicity when locally administered, further studies of this immunoconjugate have not yet been undertaken, to the present authors' knowledge.

3.2.1 *Denileukin diftitox to treat CD25$^+$ haematological malignancies*

Denileukin diftitox (DAB_{389}IL-2; **Table 1**, **Figure 2B**) is an immunoconjugate in which the catalytic and translocation domains of diphtheria toxin ($DT_{1\text{-}389}$) have been genetically fused to human IL-2 [92]. Following a series of successful clinical trials [93,94], denileukin diftitox was approved in 1999 by the FDA to treat CD25$^+$ cutaneous T-cell leukaemia/lymphoma. Denileukin diftitox was also shown to be effective in additional IL-2R$^+$ haematological malignancies of T- or B-cell origin [93], including panniculitic lymphoma [95], chronic lymphocytic leukaemia [96], and B-cell non-Hodgkin's lymphomas [97]. The efficacy of denileukin diftitox in treating haematological malignancies was enhanced by administering arginine butyrate [98] or RXR rexinoids [99,100] thought to be due to upregulated expression of the private alpha subunit (CD25; IL-2Rα) and shared beta subunit (CD122; IL-2Rβ) of IL-2R [101,102]. These results suggest that the efficacy or selectivity of an immunoconjugate can be further enhanced by manipulation of its cognate receptor. This point has received little attention in targeted drug strategies so far.

3.2.2 *Denileukin diftitox to deplete regulatory T cells*

Regulatory T cells (Tregs) are CD4$^+$ T cells expressing CD25 (in addition to other phenotypic features). These immune cells are elevated in many patients with epithelial carcinomas where they defeat host immunity [103,104]. Animal models demonstrate that depleting Tregs can boost endogenous and therapeutically-induced antitumour immunity with improved tumour rejection [104]. As Tregs express IL-2R, it was predicted that denileukin diftitox would deplete them in patients with epithelial carcinomas, which the present authors [105], and others [106,107], have since demonstrated in clinical trials. Additional trials of denileukin diftitox to deplete Tregs in cancer are ongoing. Anti-CD25 scFv coupled to PE38 (LMB-2; **Table 1**, **Figure 2A**) also depletes Tregs *in vitro* [108], and could be tested in a human trial.

3.3 IL-2R targeting using conjugated peptides

The vast diversity of the T-cell receptor and B-cell receptor repertoire results from random genetic recombination events [109-111]. As a result of random scrambling of small segments of antigen recognition sites, T-cell receptors and immunoglobulin antigen binding sites are created that are able to recognise an essentially infinite number of antigens. This natural immunological strategy has also been used in the laboratory to create libraries of molecules with nearly infinite capacities to recognise antigens. Strategies include making random scFv libraries, combinatorial chemical libraries and phage display libraries, among others. Phage display libraries such as Ph.D. (New England BioLabs) have a theoretical diversity of 4×10^{15} unique peptides fused to the minor coat protein of bacteriophage M13 and are expressed on the phage's surface [13]. These 12-mer peptides have the potential to bind to essentially any particular target ligand or receptor. The present authors' laboratory has used phage display to identify small 12-mer peptides targeting cell surface epitopes that specifically bind dendritic cells [112].

With regard to IL-2R, none of its subunits are exclusively expressed on Tregs, making it impossible to deliver a toxic payload without causing some degree of bystander injury – a common problem in targeted cancer immunotherapy. The authors of this review selected mouse CD122 (IL-2Rβ) as an alternative target on Tregs, as it was slightly less broadly distributed on the surface of other types of immune cells compared with CD25, and as CD122 is expressed on some epithelial carcinomas. CD122 is not known to be glycosylated, and phosphorylation only occurs within its C-terminal cytoplasmic domain in response to IL-2 binding, making the extracellular *N*-terminal domain amenable to overexpression and purification from *E. coli*. Using phage display, peptides that bind to mouse CD122 were identified. The authors are now testing the efficacy of one particular immunoconjugate made from the fusion of one 12-mer peptide fused to $DT_{1\text{-}389}$ (DT_{389}-peptide 3.7; **Figure 2B**) in depleting Tregs or killing CD122$^+$ tumours in mice. If the efficacy of toxin delivery using conjugated peptides is demonstrated, the same peptide can also be directly conjugated to other therapeutic agents as alternative approaches to present Treg or CD122$^+$ tumour targeting (compare **Figure 1G** with **Figure 1F**).

3.4 Targeting of tumour-associated antigens to dendritic cells

The tumour-associated antigen CA-125, which is used to monitor treatment responses in ovarian cancer, can be targeted *in vivo* by administering the murine monoclonal antibody oregovomab (**Table 1**). This antigen–antibody complex is speculated to prime dendritic cells [113], leading to T cell activation [114], and is undergoing a Phase III clinical trial [115].

3.5 Viral immunoconjugates

Frequently, the tropism of a viral vector does not match the cells that need to be transduced. Viral immunoconjugates can help to target the vector to the cells of choice. Different antibody molecule components have been used to target viral vectors to the cells of choice: bispecific antibodies, monoclonal antibodies and single-chain antibodies.

Bispecific antibodies consist of two coupled antibody molecules: one specific for the vector and the other specific for the targeted cell (**Figure 1H**). Bispecific antibodies have the advantage that the vector molecule does not need to be genetically modified, facilitating the use of existing vectors. An example is the use of a bispecific antibody to target an adenoviral vector to the pulmonary endothelium, which is usually refractory to adenovirus infection. An example of a bispecific conjugate is the linking of a monoclonal antibody against a membrane-bound ectozyme (angiotensin-converting enzyme, which is highly expressed on the pulmonary endothelium) to the Fab fragment of a neutralising monoclonal antibody to the adenovirus vector. This construct leads to a 20-fold higher lung transduction compared with an unmodified control vector. At the same time, liver transduction has been shown to be decreased, reflecting a change in the natural adenovirus vector tropism. The latter frequently hampers systemic administration, as unwanted liver transduction can lead to significant side effects [116,117].

A different way to couple antibody to a vector has been facilitated by genetic incorporation of an immunoglobulin-binding domain into the vector (**Figure 1I**). Unlike the previous approach, here the viral vector is modified specifically to recognise the Fc antibody domain. To this end, the Z-domain of the *Staphylococcus aureus* protein A has successfully been inserted into the viral attachment protein of several different types of vectors [118-121]. A recent example is the introduction of an immunoglobulin-binding domain into the envelope protein (Env) of a lentiviral vector, which allowed coupling to a monoclonal antibody against P-glycoprotein that is expressed on melanoma cells. Targeting to metastatic melanoma cells was achieved in SCID mice [122]. Although the great pool of available monoclonal antibodies makes this approach very attractive for the easy screening of targeting moieties, *in vivo* applications might be hampered by interference by normally circulating antibodies that could compete with coupled antibody.

To target the viral vector specifically, genetic incorporation of an scFv into the vector can be used (**Figure 1J**). This technique was recently exploited to target a retroviral vector to CEA-expressing tumour cells. An scFv against CEA was genetically coupled with a matrix metalloproteinase cleavage site and the Env of the vector. This construction facilitates binding to the tumour via the scFv and then cleavage of the scFv from the vector by tumour expressed matrix metalloproteinase [123]. This separation of scFv from the Env prior to transduction of the target cell is important because: i) transduction depends on conformational changes of the Env, which may be inhibited by the fused scFv; and ii) it exposes the receptor Env binding domain, enabling interaction with the ubiquitously expressed Pit-2 receptor on the tumour cell. This strategy has effected selective tumour transduction *in vivo* [123]. For adenovirus vectors, such separating of scFv from the capsid is not necessary because vector entry is not dependent on complex conformational changes of the viral attachment protein.

However, a different hurdle had to be overcome before scFv was genetically fused to the capsid. Although adenovirus proteins are synthesised in the cytosol, scFvs require the rough endoplasmic reticulum for the formation of important disulfide bridges. This problem was overcome using cytosolically stabilised scFvs (intrabodies), which could be coupled to an artificial adenovirus fibre protein [124]. Regarding the scFv moiety, it was quickly recognised that the strategic placement of a pair of cysteine residues within the V_L and V_H domains generated a disulfide-bond-stabilised single-chain antibody (dsFv), with greatly enhanced half-life and stability [125].

Antibodies can also be coupled to adenovirus by exploiting the natural virus receptor (coxsackie and adenovirus receptor; **Figure 1K**), the ectodomain (sCAR) of which can be fused to an scFv. Such a construct using an antibody against CEA has effected specific transduction of hepatic tumour grafts while reducing liver tropism [126].

Many other possibilities of vector targeting, including those not involving immunoconjugates, have recently been reviewed [14]. Some have been used to target cells of the immune system, such as dendritic cells. A fusion of the coxsackie and adenovirus receptor ectodomain with CD40 ligand (CD40L), using a trimerisation motif that facilitates a natural configuration of the components, has been used to target the CD40 receptor on murine dendritic cells. This resulted in improved dendritic cell transduction in mice by > 10,000-fold [127]. Alternatively, human dendritic cells have been successfully targeted by coupling antibodies to DC-SIGN or CD40 to the vector via an immunoglobulin-binding domain [128]. This latter study illustrates another example of how vector targeting with immunoconjugates can lead to the transduction of cells previously refractory to transduction with the vector.

The ability to target specific cells is potentially vast, as many antibodies are available and can easily be coupled to a

suitable vector. Monoclonal antibodies coupled to the viral receptor, and especially single-chain antibodies genetically fused to the vector, are highly promising tools for therapeutic *in vivo* application of viral immunoconjugates. Targeting motifs, such as peptides identified through phage display can also be used in this manner.

4. Lessons learned from the design of immunoconjugates that have received FDA-approval

Among the drugs listed in **Table 1** that are either *bona fide* immunoconjugates or ligand-receptor-based therapeutic agents, only four of them have been FDA-approved at this time: i) gemtuzumab ozogamicin; ii) denileukin diftitox; iii) [^{90}Y]-ibritumomab tiuxetan; and iv) [^{131}I]-tositumomab. These drugs have a number of significant similarities and differences that are briefly discussed in this section.

4.1 Pretargeting of immunoconjugates

As circulating CD20$^+$ lymphoma cells are often outnumbered by the large number of normal CD20$^+$ B cells in the circulatory system, the administration of either [^{90}Y]-ibritumomab tiuxetan or [^{131}I]-tositumomab often leading to significant bystander injury in the liver, kidney, spleen and bone marrow. To circumvent this drawback, unlabelled ('cold') antibodies can be administered prior to treatment with the radioimmunoconjugate of interest [129]. Pretargeting of gemtuzumab ozogamicin can likewise be performed using unconjugated gemtuzumab prior to treating patients with the calicheamicin-conjugated drug. On the other hand, in the case of denileukin diftitox, pretargeting with the unconjugated ligand (IL-2) in the absence of the DT_{389} payload is undesirable as this increases the population of Tregs in cancer patients [130].

4.2 Optimisation of drug targeting through the design of both synthetic and natural linkers

The Achilles' heel of many immunoconjugates is the engineered linker connecting the cytotoxic payload to the delivery vehicle. If these two moieties readily separate in the bloodstream, the result could be disastrous. On the other hand, once an immunoconjugate reaches its target and is internalised, lysosomal degradation of the linker is often essential for the toxic payload to enter the cytoplasm in order to kill the target cell. Examples of such linkers include peptide linkers capable of enzymatic proteolysis, *N*-succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1 carboxylate linkers, disulfide linkages containing moieties that create steric hindrance thereby resisting plasma degradation, or non-disulfide linkers that are stable in plasma, but nonetheless are subject to lysosomal degradation [131].

In the case of gemtuzumab ozogamicin, the antibody component is covalently linked to calicheamicin by means of an acid-labile hydrazone linker. Therefore, once the immunoconjugate has been internalised by its target cell via receptor-mediated endocytosis, the acidification of the lysosomal vacuole will result in the cleavage of this bond [60]. Separation of the antibody from its toxic payload (calicheamicin) is essential to ensure the unencumbered passage of the calicheamicin across both cytoplasmic and nuclear membranes, eventually binding to the minor groove of DNA in the nucleus, causing double-stranded breaks and cell death.

Likewise, the DT_{389} component of denileukin diftitox possesses an equally critical protease cleavage site between the ADP ribosyltransferase domain and the translocation domains that is often nicked prior to the internalisation of the drug [81]. Once denileukin diftitox is internalised, the disulfide bond that stabilises the toxin and translocation domains (**Figure 2B**) is reduced, leading to a conformational change in the DT_{389} component that results in the exclusive translocation of the catalytic domain into the cytoplasm of its target cell, where it eventually causes apoptosis.

In the case of [^{90}Y]-ibritumomab tiuxetan, the highly stable metal chelator tiuxetan is responsible for linking the radioactive yttrium atom to the antibody. Upon intravenous administration of this drug to patients, little detectable free yttrium can be detected elsewhere in the body. In contrast, the radioactive iodine atom in [^{131}I]-tositumomab is covalently linked to a tyrosine residue on the antibody – a bond that is comparatively labile – leading to the separation of the radionuclide from the antibody [60]. This demonstrates the importance of carefully choosing radionuclides as well as their linkers. By replacing either of the abovementioned radionuclides with ^{225}Ac-DOTA, it might potentially be possible to enhance the potency of these immunoconjugates [79], while at the same time creating a much more stable therapeutic agent with a longer biological half-life.

4.3 Receptor-mediated endocytosis

Radioimmunoconjugates inherently possess a distinct advantage over other classes of immunoconjugates in that they do not need to be internalised to irradiate and destroy their target cell. Another advantage of this type of immunotherapy is that neighboring tumour cells can nonetheless be destroyed even if they fail to express the targeting marker, if radiation from a neighboring cell incorporating the immunoconjugate reaches it. Conversely, healthy tissues can also be inadvertently irradiated – a situation that is described as collateral damage [60].

In contrast to radioimmunoconjugates, other types of immunoconjugates (such as gemtuzumab ozogamicin) or ligand–receptor-based agents (such as denileukin diftitox) must first be internalised to exert their cytotoxic effect. As antibodies can bind to a receptor through a diverse number of different epitopes, it is not guaranteed that such an interaction will necessarily lead to receptor-mediated endocytosis. Thus, numerous monoclonal antibodies must

be tested against a given receptor to ensure that at least one is capable of being internalised. In contrast, the internalisation of ligand-based therapeutic agents such as denileukin diftitox is mediated by the comparatively natural interaction between the IL-2 moiety and its cognate receptor (IL-2R). Therefore, as demonstrated in **Table 1**, DT_{389} can be effectively internalised by a large number of potential receptors by fusing it to an endogenous ligand of choice.

4.4 Immunogenicity

Gemtuzumab ozogamicin was developed using a fully humanised monoclonal antibody. It is conjugated to a chemical moiety (calicheamicin) that has shown no evidence of being immunogenic. Howeber, in contrast, [^{90}Y]-ibritumomab tiuxetan and [^{131}I]-tositumomab are mouse antibodies and, thus, will elicit an immune response when administered to humans. Truncated DT, which is the cytotoxic component of denileukin diftitox is highly immunogenic, which can hamper further treatment cycles, following initial treatment [4]. It is often found that the antibodies raised by a patient against a particular immunoconjugate are not neutralising, in which case it is generally considered reasonable to continue with the immunotherapy.

Modification of immunoconjugates using polyethylene glycol (PEGylation) is a typical method by which immunogenicity can be reduced while also prolonging the serum half-life of the immunoconjugate [132]. There is always a risk that PEGylation may disturb important residues on the toxin, but this approach can be worth evaluating on a case-by-case basis. Problems with immunogenicity might also be eliminated by utilising a cytotoxic payload such as human RNase [10] or small inhibitory RNA [11], which might not elicit an important immune response.

Finally, increasing the potency or binding affinity of a given immunoconjugate would circumvent the problem of its inherent immunogenicity, as repeated administration might be better tolerated and fewer cycles of drug treatment might be necessary. For example, improvement in binding affinity has previously been demonstrated for the anti-CD22 antibody BL22 [31]. Conjugating ^{225}Ac to several existing immunoconjugates is anticipated to significantly increase their potency [79].

5. Expert opinion

Many anticancer agents are limited by their induction of collateral damage in normal tissues. Specific targeting to tumour can help reduce this collateral damage. The exquisite selectivity of the immune system has been exploited to help guide anticancer drugs specifically to their intended targets, while avoiding normal tissues. The most successful class of immunoconjugates so far are the antibody conjugates. Nonetheless, technological advances have spawned newer and novel agents including cytokine, chemokine, peptide and viral conjugates as well.

Aside from their therapeutic potential, the diagnostic power of tumour-specific targeting has also been exploited in immunoconjugates. Although progress has been made in tissue-specific targeting, one of the present limitations of most immunoconjugate-based anticancer drugs appears to be their inability to access and penetrate solid tumours efficiently. This problem may not be easily overcome even though the present molecules being engineered are typically much smaller than those from the previous eras (newer immunoconjugates tend not to rely on either whole antibodies or antibody fragments for targeting).

Although antibodies remain the most successful means of specific targeting, in many instances, their mode of tumour killing remains incompletely understood. A better understanding of cytotoxic mechanisms will likely help with the development of more potent agents, as selectively is also enhanced with other technological advances. Likewise, additional levels of control of targeted killing can be effected by placing the gene for a cytotoxic agent under the control of a tissue-specific promoter, or by placing a tissue-specific activating motif upstream of an inactive prodrug that will be activated only in the proper cell or tissue environment. Molecules employing such additional levels of tissue-specific targeting have already been tested in limited studies.

Problems with the immunogenicity of most immunoconjugates persist, which can diminish the efficacy of multiple cycles of treatment with the same drug. One approach to solving this issue is to remove immunodominant regions on particular molecules. A second approach involves increasing immunoconjugate target affinity, with minimal toxicity from nonspecific tissue targeting. Aside from the obvious benefit of improved specificity, any reduction in the number of treatment cycles could possibly decrease the likelihood of an unwanted immune response against the immunoconjugate. Immunogenicity usually results from a protein epitope. Thus, development of small molecule chemical combinatorial libraries that allow selective tissue targeting might also help overcome immunogenicity issues, but this area of investigation remains in its infancy. Finally, the development of novel cytotoxic payloads such as human RNases, small interfering RNAs, or other similarly cytotoxic molecules that are not as immunogenic as proteins and peptides may help reduce immunogenicity. Modification of any particular immunoconjugate may also be considered on a case-by-case basis in order to improve its pharmacokinetic properties.

In conclusion, immunoconjugates harness the power of the immune system specificity to help target anticancer drugs to the intended target (the tumour), while helping reduce collateral damage to normal tissue. Recent advances in technology have allowed the development of many novel classes of immunoconjugates, although the therapeutic utility of most remains to be firmly established. Conjugated antibodies have been the most successful molecules in this class so far. Haematological malignancies have been the most

successful targets. Nonetheless, the landscape is rapidly evolving. Thus, the near future will present many new immunoconjugates targeting wider spectra of tumours, including more agents against epithelial carcinomas, where drug successes for advanced-stage disease have remained modest. Certain viral immunoconjugates may help overcome the issue of poor tumour penetration, but much work remains to be done in this regard.

Declaration of interest

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