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Therapeutic potential of anticancer immunotoxins

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Immunotoxins are chimeric proteins consisting of a tumor-specific ligand (antibody, growth factor or peptide) linked to a modified toxin. These molecules bind to cell surface receptors and are subsequently internalized by endocytosis, resulting in cell death. Advances in protein engineering and phage display have enabled the selection of high-affinity targeting moieties. Denileukin diftitox is the only FDA-approved immunotoxin, although others such as BL22 are currently in different phases of development. This review elaborates the key findings of the important clinical studies relating to various chimeric toxins.

The introduction of the 'magic bullet' concept by Paul Ehlrich, over 100 years ago, led to the search for agents that can selectively target cancer cells. Immunotoxins are a class of antineoplastic agents comprising a modified toxin linked to a cell-selective agent, such as a growth factor or antibody, for specifically targeting cancer cells [1]. The generation of an immunotoxin involves the chemical coupling or genetic fusion of a cell-selective ligand with a complete toxin or a modified form of the toxin. The ligand provides the cell binding and internalizing property, whereas the toxin inhibits crucial cell function and causes cell death upon translocation to the cytosol. The ligand can be a recombinant antibody or an antibody fragment, growth factor, carbohydrate antigen or tumor-associated antigen. Bacterial toxins, such as Pseudomonas exotoxin (PE) and diphtheria toxin (DT), and plant toxins, such as ricin, saporin, gelonin and poke weed antiviral protein, are used in immunotoxin constructs [2]. The advantages of this approach over chemotherapy are the selective delivery of drugs to tumors, thereby reducing systemic toxicity and increasing potency.

The first generation of immunotoxins, developed 35 years ago, employed chemical conjugates of antibodies with intact toxins, or toxins with attenuated cell-binding properties. Although they showed tumor regression in some lymphoma patients, they were typically ineffective because the constructs were heterogeneous, nonspecific and were too large to infiltrate solid tumors [3]. The next generation also employed chemical conjugation with deletions in native cell-binding domains, generating many more target-specific immunotoxins.

Recombinant DNA techniques were applied in the production of third-generation immunotoxins to promote tumor specificity and penetration, and to reduce the cost and complexity of production. The cell-binding domain of the toxin is genetically removed and the modified toxin fused with a ligand or with DNA elements encoding the Fv portion of an antibody in these constructs. The light- and heavy-chain variable fragments are either genetically linked (scFv) [4,5] or held together by a disulfide bond (dsFv) [6]. Compared with the single-chain toxins (scFv), disulfide-stabilized Fv (dsFv) molecules did not aggregate, were stable and also overcame the major obstacle of poor penetration into bulky tumor masses. These targeted toxins posed problems such as immunogenicity, nonspecific toxicity and instability [6]. The identification and mutation of B-cell epitopes in PE and their incorporation into immunotoxins reduced the immunogenicity of the toxin [7]. A new field of immunotoxins called bispecific immunotoxins (DT2219ARL) has also emerged recently – involving the simultaneous specific targeting of two targets on cancers for increased efficacy and reduced nonspecific toxicity [8]. Recent endeavors involve a new generation of immunotoxins in which the cytotoxic moiety is an endogenous protein of human origin, for example proapoptotic protein or RNase [9].

At present, only one agent, denileukin diftitox, which is a fusion protein of truncated DT and interleukin 2 (IL2), has been approved

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by the FDA [10]. Several similar fusion proteins are currently in clinical trials. This review elaborates on the toxins that are exploited in immunotoxin constructs and the immunotoxins in clinical trials now.

Immunotoxin development

A variety of toxins, mainly from plants, fungi or bacteria, have been characterized, structurally optimized for *in vitro* stability, activity and safety, and evaluated *in vivo* in animal studies and clinical trials. Among them, ricin, PE and DT are the most frequently used. Immunotoxins with RNases as the cytotoxic moiety are recent examples aimed at reducing the immunogenicity. These toxins generally consist of several domains – the cell-binding or cell-recognition domain, the translocation domain, which enables release of the toxin into the cytosol, and the activity domain responsible for cytotoxicity. In immunotoxin development the binding domain of these toxins is replaced by the cancer-cell-specific ligands. The cancer-cell-specific ligands direct the internalization of the toxins via receptor-mediated endocytosis. Upon internalization the catalytic domain of the toxin is cleaved in the late endosome and translocates to the cytosol leading to cell death by various mechanisms (Fig. 1). Table 1 represents the classification of clinically used toxins based on their mechanism of action. Many modifications in the toxin fragments have enabled improvements in cytotoxic activity and reduced immunogenicity over the years. The modified form of the toxins has also been identified in Table 1.

Production and clinical testing of immunotoxins

Immunotoxins are usually produced in bacterial or yeast expression systems. The design strategy for an immunotoxin includes: confirmation of the type and stage at which cancer cells will be the best targets, analysis of the tumor specificity of the antigen that is targeted by the recombinant antibody, as well as the affinity of immunotoxins for the cell type and the ability of the immunotoxins to penetrate and enter normal tissues and tumor cells. The stability, immunogenicity and side-effects of immunotoxins should be monitored during pilot studies. Anti-immunotoxin antibodies can reduce the effectiveness of immunotoxins by accelerating their clearance from the circulation or by blocking the functional domains of the targeting module or toxin.

Preclinical development

In vitro cytotoxicity studies of the immunotoxin are performed in tumor cell lines overexpressing the receptors where the IC_{50} (half maximal inhibitory concentration) value of the immunotoxin is evaluated. *In vivo* antitumor activity is tested in mouse xenograft models bearing receptor-positive tumor cells. Additionally, specific killing in tumor samples from patients further warrants the future development of the immunotoxin.

Clinical development

A Phase I study is mainly a dose escalation study carried out in cohorts of 3–6 healthy patients to determine the maximum tolerated dose (MTD), side effects and pharmacokinetics of the drug. Patient choice largely depends on the tumor stage, the resistance to other treatments or poor previous treatment response. The response rate, safety and efficacy of the drug for future development are all assessed. Response to a tumor is a single point-in-time measurement of a patient's CT (computerized tomography) scan to show how much tumor shrinkage occurred. The response rate is the percentage of the treated patients who responded to a particular treatment during the period under study. The responses generated are classified based on the effects of treatment on tumor size – CR (complete remission, disappearance of the tumor), PR (partial remission, >50% reduction), MR (minor response, 25–50% reduction), SD (stable disease, neither increasing nor decreasing) or progressive disease (increase in tumor size by 20%). A Phase II study is performed on larger groups (20–300 patients) and is designed to assess how well the drug works. Phase III studies are randomized controlled multicenter trials on large patient groups (300–3000, or more) and are aimed at being the definitive assessment of how effective the drug is when compared with the current 'gold standard' treatment.

Toxins in clinical trials

Several clinical trials have been conducted with immunotoxins and fusion toxins over the past 35 years. These studies defined several pharmacologic and toxicologic barriers that had to be overcome. Tables 2 and 3 give an overview of these clinical studies. Targeted toxins with 30% or more CR and PR rates include Ontak™ (denileukin diftitox), for refractory cutaneous T-cell lymphomas, LMB-2 and BL22, for refractory hairy cell leukemia (HCL), as well as IL4(38-37)-PE38KDEL and IL13-PE38QQR for interstitial therapy of high-grade gliomas.

Targeting hematologic tumors

Denileukin diftitox (Ontak™)

DAB_{389}IL2 (Denileukin diftitox, Ontak™) is the first FDA-approved immunotoxin. It is used in the treatment of recurrent cutaneous T-cell lymphoma (CTCL) [11]. It is the fusion protein of human IL2 and a truncated form of DT (DAB_{389}). It targets the high-affinity IL2 receptor (IL2R), consisting of CD25 (IL2Rα), CD122 (IL2Rβ) and CD132 (IL2Rγ), which is overexpressed in various malignancies such as CTCL, adult T-cell leukemia (ATL), Hodgkin's disease (HD), and other B- and T-cell leukemias and lymphomas, as well as to a lesser extent on normal activated T cells and T-regulatory cells [12]. It was well tolerated in patients with CTCL and patients with NHL in a Phase I trial [13]. Objective response rates of 49–63% were reported in early and advanced disease in a Phase II study with CTCL patients [11]. In the pivotal Phase III trial, 30% of the 71 CTCL patients with refractory stage IB to IV chronic T-cell lymphoma (as per Rai and Binet classification) demonstrated an objective response (10% CR and 20% PR). Ninety-eight percent of the patients developed human antitoxin antibody (HATA) response by the second treatment, which imposed dose limitations and prevented retreatment [14,15]. Significant adverse events included flu-like symptoms, acute hypersensitivity reactions, nausea, vomiting, transaminase elevations, hypoalbuminemia, rashes, hypotension and vascular leak syndrome (VLS) [15].

The potency of denileukin diftitox is limited because of the lack of high affinity IL2Rs in a large percentage of cases, usually owing to lack of CD122. DAB_{389}IL2 is being currently evaluated in combination with other treatments for various cancers including B-cell NHL, T-cell NHL, melanoma, pancreatic cancer, ovarian cancer and acute myeloid leukemia (AML).

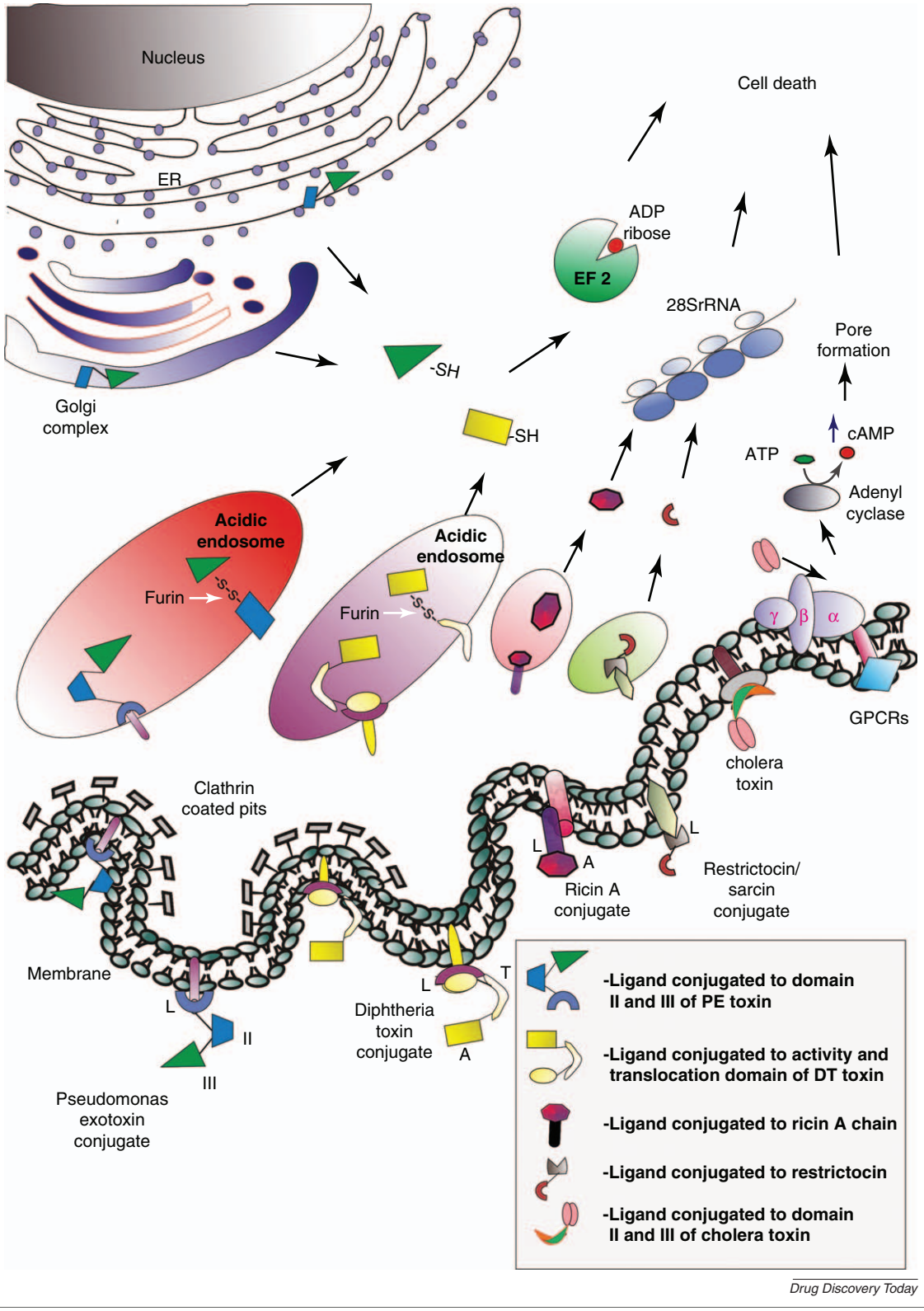

FIGURE 1

Mechanism of action of immunotoxins. Immunotoxins enter the cell via receptor-mediated endocytosis. Upon binding to a cell-specific receptor, the toxin receptor complex is internalized through clathrin-coated pits into an endosome. For PE and DT, low pH induces unfolding of the protein, proteolytic cleavage and the release of the activity domain in the cytosol, where it inhibits protein synthesis by ADP ribosylation of the diphthamide residue of elongation factor 2 (EF2). Release of ricin in the cytosol leads to *N*-glycosylation of residues in 28S rRNA and prevents association of EF1 and EF2 with 60S ribosome, while restrictocin cleaves the 28S rRNA and leads to protein synthesis inhibition. Cholera toxin acts by ADP ribosylation of the Gs-α subunit of G proteins leading to an increased cAMP level and pore formation in the membrane resulting in cell death.

TABLE 1

Classification of toxins.

Toxins	Source	Mechanism	Structure	Modifications	Refs
ADP ribosylating toxins					
Diphtheria toxin	*Corynebacterium diphtheria*	ADP ribosylation of EF2	Activity (A chain), translocation (T) and binding (B) domains	a) DT_{486} b) DT_{388} or DT_{389} (deletion of cell-binding domain) c) CRM107 (point mutation in cell-binding domain of DT)	[61,62,63,69]
Pseudomonas exotoxin	*Pseudomonas aeroginosa*	ADP ribosylation of EF2	Binding (Ia) translocation (II and Ib) and activity domains (III)	a) PE40 and PE40KDEL b) PE38 and PE38KDEL c) PE38QQR d) PE35	[61,64,65,69]
Pore-forming toxins					
Cholera toxin	*Vibrio cholera*	ADP ribosylation of Gs-α subunit of G protein	Activity (A chain) and cell-binding (pentameric B chain)	CET40 (domains II and III)	[66,67,69]
Ribosome inactivating toxins					
Holotoxins – ricin	*Ricinus communis*	*N*-glycosylation of 28S rRNA	Activity and binding domain	a) Ricin b) Ricin chain A (RTA) c) bR (blocked ricin) d) dgA (deglycosylated ricin A-chain)	[68,69]
Hemitoxins – saporin (SAP), pokeweed antiviral protein (PAP)	*Saponaria officinalis, Phytolacca americana*	*N*-glycosylation of 28S rRNA	Single-chain proteins without binding domain		[2,69]
Ribonucleases					
Fungal toxins – α-sarcin, restrictocin	*Aspergillus* sp.	Cleavage of 28S rRNA	Single-chain proteins without binding domain		[2,69]
HPR, ECP, EDN	Human	Degradation of RNA	Single-chain proteins		[9,70]

Abbreviations: DT: diphtheria toxin; DT_{388} or DT_{389}: truncated forms of DT that lack receptor-binding activity: CRM107 or cross-reacting material – mutant of DT lacking receptor binding; PE: Pseudomonas exotoxin A; PE40 and PE38: truncated forms of PE that lack receptor-binding domain Ia; CET40: cholera exotoxin A; RTA: ricin toxin A; HPR: human pancreatic ribonuclease A; ECP: eosinophilic cationic protein; EDN: eosinophil-derived neurotoxin.

Anti-CD25 immunotoxins

CD25 (low affinity IL2 receptor) greatly outnumbers CD122 and CD132 (high affinity receptors) on most malignancies [16]. To target IL2R+ disorders expressing CD25, regardless of other subunits, anti-Tac antibody, which binds with a higher affinity to CD25 alone, was used instead of IL2. An immunotoxin with a single-chain Fv fragment of the anti-CD25 monoclonal antibody (MAb) anti-Tac fused to truncated PE, PE40, was also constructed [17]. It showed promising results in preclinical trials on CD25+ cells and malignant cells from ATL patients [18] and in mice bearing CD25+ human xenografts [19]. This was followed by the development of a slightly smaller derivative of this immunotoxin, anti-Tac(Fv)-PE38 (LMB-2) which used PE38 as the truncated toxin.

In a Phase I study, LMB-2 was administered to 35 patients with chemotherapy-resistant leukemia, lymphoma and HD resulting in 1/35 (3%) CR and 7/35 (20%) PR. Dose-limiting toxicities (DLTs), such as transaminase elevations and neutralizing antibodies, developed in patients after one cycle and prevented retreatment [20]. Phase II trials have been initiated for CTCL, chronic lymphocytic leukemia (CLL), and HCL. Phase II trials involving the administration of the immunosuppressants fludarabine plus cyclophosphamide (FC), before LMB-2 for adult T-cell leukemia (ATL), are also currently underway (http://clinicaltrials.gov/ct2/show/NCT00924170). LMB-2 is also being tested in solid cancers such as melanomas because it can penetrate solid tumor masses [21].

BL22 (CAT-3888)

BL22 consists of a disulfide stabilized anti-CD22 MAb RFB4(dsFv) fused to PE38, which targets CD22 molecules present on the surface of certain B-cell malignancies, for example lymphoma and leukemia [22]. Although CD22 is also present on normal B cells, the usual B-cell repertoire can be replenished even after BL22 treatment, because the molecule is absent on B-cell stem cells. Preclinical testing of BL22 in several lymphoma cell lines [22] and leukemic patient samples [23] delivered promising results. Mice bearing human CD22+ CA46 Burkitt's lymphoma xenografts demonstrated complete regressions [24]. A Phase I trial of BL22 was carried out in 46 patients with CD22+ malignancies including HCL, CLL and NHL patients. The overall response rate in HCL patients resistant to purine analog therapy was very high with 81% responders including 61% achieving CR and 19% achieving PR [25]. The high response rate in HCL patients was attributed to a high level of CD22 expression even after purine analog treatment. BL22 was not immunogenic in B-cell CLL or NHL patients and, thus, allowed use of multiple cycles of BL22. An added advantage of CR with BL22 is the achievement of hematologic remission. The

TABLE 2

Immunotoxins targeting hematological tumors.

Immunotoxin	Targeting moiety	Toxic moiety	Target	Type of tumor	Route of administration	Clinical phase	Refs
DAB389IL2	IL2	DAB389	IL2R	CTCL, NHL, CLL, NSLC, melanoma, ovarian and breast cancers	Intravenous	I, II, III, IV FDA approved for CTCL	[10–15]
LMB-2	Anti-CD25/Tac(scFv)	PE38	α LewisY receptor	Hematopoietic malignancies	Intravenous	I	[20,21]
BL22, RFB4(dsFv)-PE38	Anti-CD22 dsFv	PE38	CD22	HCL, CLL, ALL	Intravenous	I	[25]
BL22	Anti-CD22 dsFv	PE38	CD22	Cladribine-resistant HCL	Intravenous	II	[26]
HA22	Anti-CD22 dsFv	PE38	CD22	HCL, ALL, NHL, CLL	Intravenous	I	[29]
DT388 GMCSF	GMCSF	DAB388	GMCSFR	AML	Intravenous	I	[32]
RFB4-dgA	Anti-CD22 Fab′	Deglycosylated RTA	CD22	B-NHL	Intravenous	I	[33,34]
HD37-dgA	Anti-CD19 MAb	Deglycosylated RTA	CD19	NHL	Intravenous	I	[33,34]
Combotox (RFB4-dgA + HD37-dgA)	Anti-CD22 Fab′ + Anti-CD19 MAb	Deglycosylated RTA	CD22, CD19	NHL, ALL	Intravenous	I	[34,35]
RFT5-dgA (IMTOX-25)	Anti-CD25 MAb	Deglycosylated RTA	CD25	HD, CTCL, melanoma	Intravenous	I,.II	[33,36]
Ki-4.dgA	Anti-CD30 MAb	Deglycoylated RTA	CD30	HD, NHL	Intravenous	I	[33,37]
HuM195/rGel	Anti-CD33 MAb	Gelonin	CD33	AML, CML	Intravenous	I	[39]

Abbreviations: CTCL, cutaneous T-cell lymphoma; NHL, non-Hodgkin lymphoma; CLL, chronic lymphocytic leukemia; NSCLC, non-small-cell lung carcinoma; HCL, hairy cell leukemia; ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; B-NHL, B-cell NHL; HD, Hodgkins disease; dgA, deglycosylated ricin A-chain; RTA, ricin A-chain; GMCSF, granulocyte macrophage colony stimulating factor; rGel, recombinant gelonin; MAb, monoclonal antibody; scFv, single chain variable fragment; dsFv, disulfide-stabilized Fv antibody fragment.

TABLE 3

Immunotoxins targeting solid tumors.

Immunotoxin	Targeting moiety	Toxic moiety	Target	Type of tumor	Route of administration	Clinical Phase	References
Erb38	Anti-Her2/neu dsFv	PE38	erbB2/HER2	Breast carcinoma	Intratumoral	I	[50]
scFv(FRP5)-ETA	Anti-Her2/neu scFv(FRP5)	ETA	erbB2/HER2	Melanoma, breast, colon	Intravenous	I	[51]
SS1P [SS1(dsFv)-PE38]	Antimesothelin dsFv	PE38	Mesothelin	Mesothelioma, ovarian, pancreatic cancers	Intravenous	I	[43–45]
LMB-1	Anti-Lewis Y MAb	PE38	Lewis Y	Adenocarcinoma	Intravenous	I	[47]
LMB-7	Anti-Lewis Y scFv(B3)	PE38	Lewis Y	Adenocarcinoma	Intravenous	I	[10]
LMB-9	Anti-Lewis Y dsFv(B3)	PE38	Lewis Y	Adenocarcinoma	Intravenous	I	[10]
SGN-10	Anti-Lewis Y dsFv(BR96)	PE40	Lewis Y	Adenocarcinoma	Intravenous	I	[49]
OvB3-PE	Murine MAb	PE	Ovarian cancer	Ovarian carcinoma	Intraperitoneal	I	[60]
TP40	TGFα	Modified PE40	EGFR	Bladder cancer	Intratumoral	I	[59]

Abbreviations: EGFR: epidermal growth factor receptor; TGF: transforming growth factor.

most common toxicities observed were transaminase elevations, hypoalbuminemia, fatigue and reversible hemolytic uremic syndrome (HUS) [25]. The Phase II trial results with cladribine-resistant HCL patients confirmed the high response rate to BL22, with 25% CR and 25% PR upon a single cycle, and 47% CR upon retreatment [26].

A recent Phase I trial of BL22 for pediatric B-lineage acute lymphoblastic leukemia (ALL) and NHL patients revealed an acceptable toxicity profile and transient clinical activity in most subjects. As compared with adults, BL22 was tolerated at a greater dose in children and, besides, the most common DLTs in adults, HUS, were not found [27]. The trial established a dose and schedule for the subsequent testing of HA22 (discussed below). Future trials are planned in combination with standard chemotherapy agents (http://www.clinicaltrials.gov).

BL22 variants

Low CD22 expression is a limiting factor in treating CLL with the recombinant immunotoxin BL22. The amino acid residues T-H-W at positions 100, 100a and 100b in the heavy-chain

complementary determining region 3 of the Fv of BL22 were substituted with the residues S-S-Y, to produce the molecule HA22 [28]. This agent has been reported to have a tenfold higher binding affinity for CD22 leading to an approximately 10–100-fold improvement in potency against CD22+ cell lines and samples from ALL, CLL and HCL patients [28]. Phase I trials of HA22 in HCL patients achieved an overall response rate of 79% including 43% CR and without any DLTs [29]. BL22 has produced many complete remissions in drug-resistant HCL, where many treatment cycles can be given, because neutralizing antibodies do not form. By marked contrast, only minor responses have been observed in trials with immunotoxins targeting solid tumors, because only a single treatment cycle can be given before antibodies develop. To allow more treatment cycles and increase efficacy a 'less immunogenic' immunotoxin has been produced by identifying and eliminating most of the B-cell epitopes on PE38. This was accomplished by mutation of specific large hydrophilic amino acids (Arg, Gln, Glu and Lys) to Ala, Ser or Gly. This new immunotoxin (HA22-8X) was reported to be significantly less immunogenic in three strains of mice, yet retains full cytotoxic and antitumor activities [7]. Subsequent innovations led to the development of the 'protease-resistant' immunotoxin HA22-LR, by removal of protease-cleavable clusters in PE38. This prevented its proteolytic processing by lysosomal proteases upon internalization. HA22-LR was found to be more potent against CLL cells than HA22, and was tolerated at least tenfold more than lethal doses of HA22 by xenograft mice [30]. These approaches have enhanced the therapeutic efficacy and, thus, could be applied to other PE-based immunotoxins.

DT388GMCSF

Acute myleloid leukemia blast cells overexpress the GMCSF receptor (GMCSFR) [31]. To target these cells, DT388GMCSF (or DTGM), a fusion protein composed of DT388 and human granulocyte macrophage colony stimulating factor (GMCSF), was produced. Upon intravenous (IV) administration of DTGM to 31 relapsed AML patients one CR and two PRs were achieved [32]. Overall response rate was 9.7%. Liver toxicity was seen in two patients and is attributed to the release of an inflammatory cytokine from liver Kupffer cells [32].

Combotox (RFB4-dgA plus HD37-dgA)

CD22 and CD19 are surface antigens highly expressed on malignant B cells. To target B-cell lymphoma cells, two separate immunotoxins – RFB4-dgA [deglycosylated ricin A-chain (dgA) conjugated to RFB4 (anti-CD22 MAb)] and HD37-dgA (dgA conjugated to anti-CD19 MAb) – were constructed. Separate Phase I trials using continuous infusion (CI) of RFB4-dgA and HD37-dgA showed evidence of antitumor activity [33]. Combotox is a 1:1 mixture of these two immunotoxins, used to improve antitumor specificity in patients with minimal residual disease. In a Phase I trial using CI of Combotox involving 22 patients with refractory B-cell lymphoma a 9% partial remission rate and drug tolerance at all doses were observed, and one patient died owing to aggregation in HD37-dgA formulation [34]. Recently, a Phase I dose-escalation study was done using Combotox in 17 children with refractory or relapsed B-lineage-ALL. The MTD was found to be 5 mg/m^2, and three CRs and seven PRs were recorded [35].

RFT5-dgA

RFT5-dgA is composed of RFT5 (anti-CD25 MAb), chemically linked to dgA [33]. A Phase I/II study of IV-administered RFT5-dgA on 18 refractory HD patients resulted in two PRs, one MR and five SDs. DLTs observed were VLS and anti-mouse and/or anti-dgA immune response in 60% of patients [36].

Ki-4.dgA

Ki-4.dgA is an anti-CD30 immunotoxin composed of MAb Ki-4 conjugated to dgA via a sterically hindered disulfide linker. A Phase I study on 15 patients with NHL and HD induced one PR, one MR and two SDs [33,37]. VLS was the DLT observed.

HuM195/rGel

CD33 is expressed on myeloid leukemia blasts as well as myeloid progenitor cells but not on the hematopoietic progenitor stem cells. To target leukemic cells, HuM195/rGel was constructed by chemical conjugation of the recombinant plant toxin gelonin (rGel), a single-chain RIP to HuM195, an anti-CD33 humanized antibody. Preclinical studies with CD33-expressing leukemic cell lines and subcutaneous xenografts in nude mice show promising results [38]. In addition, HuM195/rGel was also effective *in vitro* against blood samples obtained from nine AML patients. A dose-escalation Phase I study suggested that the HuM195/rGel immunotoxin appears to be safe and well-tolerated with an antileukemic response in certain patients [39].

Targeting of solid tumors

Decreased access to the immunotoxin and relatively intact immunity in solid tumors limit the number of treatment cycles that can be administered. The strategies to overcome these obstacles would refine the immunotoxin design. Among the targeted toxins against solid tumors the most promising results have been observed with brain tumors in which locoregional or convectional infusion therapy is possible.

Targeting mesothelin: SS1P

Mesothelin is a differentiation surface antigen highly expressed in various tumors such as malignant mesothelioma, ovarian cancer and pancreatic cancers, as well as nominally on normal tissues, rendering it an attractive candidate for ligand-targeted therapies [40]. To target such cancers, PE38 was fused with SS1 (an anti-mesothelin dsFv) to construct SS1P [SS1(dsFv)-PE38], a recombinant immunotoxin with high affinity for mesothelin [41]. Encouraging results were obtained from SS1P treatment of athymic nude mice bearing A431/K5 (human epidermoid carcinoma cell line) xenografts and tumor cells obtained directly from patients with mesothelioma and ovarian cancer [42]. Hence, two Phase I trials, differing in mode of administration – bolus *versus* continuous, were conducted at the US National Cancer Institute (NCI) [43,44]. Interestingly, neither of the methods was superior to the other but significant antitumor responses were observed in both. The DLT in both trials was pleuritis, and neutralizing antibodies against SS1P prevented retreatment cycles. VLS observed was reversible and not dose limiting. Further clinical development of SS1P in Phase II trials is proceeding by bolus dosing in combination with chemotherapy incorporating two drugs (pemetrexed plus cisplatinum) before SS1P treatment. The

TABLE 4

Immunotoxins against gliomas.

Immunotoxin	Targeting moiety	Toxic moiety	Target	Type of tumor	Route of administration	Clinical Phase	References
Tf-CRM107	Tf	CRM107	TfRs	GBM, AA	Intratumoral (CED)	I/II	[53,54]
IL13-PE38QQR	IL13	PE38QQR	IL13R	GBM, AA	Intratumoral (CED)	I/II/III	[52,53,55]
IL4(38-37)-PE38KDEL	Circularly permuted IL4	PE38KDEL	IL4R	GBM, AA	Intratumoral (CED)	I/II	[53,55,56]
TP38	TGFα	PE38	EGFR	GBM	Intratumoral (CED)	I	[53,57]

Abbreviations: GBM: glioblastoma multiforme; AA: anaplastic astrocytoma; TGF: transforming growth factor; CED: convection-enhanced delivery; Tf: transferrin; TfRs: transferrin receptors.

combination of SS1P and taxol has shown striking synergy in recent studies in nude mice bearing mesothelin-expressing tumor xenografts [45].

Anti-Lewis Y immunotoxins

Lewis Y (Le^Y) antigen is an oncofetal carbohydrate antigen overexpressed on many epithelial carcinomas. Many generations of immunotoxins using MAb B1 and MAb B3 have been produced for targeting the Le^Y antigen [46]. The first generation immunotoxin was a chemical conjugate of truncated PE toxin PE38 and anti-Lewis Y MAb B3 – called LMB-1. In a Phase I study in 38 patients with Le^Y-expressing carcinomas, LMB-1 produced one CR in a breast cancer patient and one PR in a colon cancer patient. DLT was seen in the form of endothelial damage, due to nonspecific binding to the Le^Y antigen on endothelial cells [47].

A series of recombinant immunotoxins developed later, employing Lewis Y as the targeting moiety [viz. B3(Fv)-PE38 (LMB-7), B3(dsFv)-PE38 (LMB-9), B1(dsFv)-PE33 and BR96 sFv-PE40 (SGN-10)], resulted in DLTs such as renal toxicity and gastritis, and no significant responses in Le^Y expressing epithelial tumors [2,48,49]. Low affinity of the immunotoxin for this carbohydrate antigen coupled with renal toxicity has limited the clinical benefits of the anti-Le^Y immunotoxins.

Anti-c-erb2/Her2/neu-erb38

Owing to high surface expression of gp185 on breast tumors, and its relatively restricted expression in normal adult tissues, this protein has been targeted in a subset of breast and ovarian carcinomas. erb38 is an immunotoxin with a dsFv fragment of the erbB2-specific monoclonal antibody e23 linked to PE38. Upon IV administration, erb38 resulted in hepatotoxicity in all six patients, owing to erbB2 expression on hepatocytes [50]. A Phase I trial was conducted with another Her2/neu-targeting agent, scFv(FRP5)-ETA [a single-chain immunotoxin with anti-Her2 scFv(FRP5) genetically linked to truncated PE A (ETA)], in 18 patients with Her2/neu-expressing cancers [51]. Dose-limiting liver toxicity was observed with no objective responses, although direct intratumoral injections of this immunotoxin into cutaneous lesions of metastatic breast cancer, colorectal cancers and malignant melanoma did cause tumor shrinkage in six of the ten patients evaluated [51].

Targeting gliomas

The blood–brain barrier has been a major impediment to using targeted toxins for the treatment of malignant glioma. With the advent of convection-enhanced delivery (CED), a novel locoregional drug delivery method for intracranial tumors, the direct administration of toxins to brain tumors or to surrounding brain tissue infiltrated by tumor cells, has become a reality [52]. The intratumor administration of a drug facilitates the complete eradication of established large tumors. Four targeted toxins advanced to Phase II clinical trials, at least, and are being used for the treatment of adult or pediatric patients with recurrent or progressive malignant glioma (Table 4). These are IL4-PE, IL13-*P. aeruginosa* exotoxin (IL13-PE38) and transferrin-*C. diphtheriae* toxin (Tf-CRM107) and tumor growth factor (TGF)α-*P. aeruginosa* exotoxin (TP-38) [53].

CRM107 (TransMID)

Transferrin receptors (TfRs) are overexpressed on tumor cells of gliobastoma multiforme (GBMs), whereas they are relatively scarce on normal brain cells. Human transferrin (Tf) was linked to CRM107 via. a thioester bond to construct Tf-CRM107. In a Phase I trial Tf-CRM107 was delivered into the tumor region by high flow interstitial microinfusion. It resulted in two CRs and tumor volume reduction in nine of the 15 evaluable patients without any symptomatic systemic toxicity [53,54]. In a Phase II study with 34 patients Tf-CRM107 resulted in a 35% response rate (five CRs and seven PRs). Severe neurotoxicities, for example progressive cerebral edema and seizures, were seen in some patients. A Phase III trial compared Tf-CRM107 with the current gold standard treatment and determined that it was ineffective and further development was terminated [53].

IL13-PE38

Enhanced expression of the IL13 receptor in GBM and limited presence in normal brain allows IL13 receptor to be used as a promising GBM target. In a Phase I trial cintredekin besudotox [recombinant cytotoxin composed of human IL13 and a truncated PE38 (PE38QQR)] was delivered via CED in GBM patients [55]. This route of IL13 cytotoxin administration appears to be well tolerated and has shown a good risk:benefit profile for use in gliomas [52].

IL4-PE38

IL4 is a pleiotropic cytokine produced by mast cells. Whereas low levels of IL4R is seen on B cells, astrocytes and microglial cells, glioma cell lines and astrocytoma tumor specimens overexpress IL4R, thus potentiating it as a target in glioma. Recombinant fusion protein IL4(38-37)-PE38KDEL (also called cpIL4-PE or IL4-PE) consists of circularly permuted IL4 and a mutated form of PE [55]. IL4-PE was reported to be highly and specifically cytotoxic to glioma cell lines *in vitro*, and caused partial or com-

plete regression of established human GBM tumors in nude mice [53,55]. Phase I/II clinical trials conducted to determine safety, tolerability and efficacy of IL4-PE, when injected directly into recurrent GBM by CED, suggested that cpIL4-PE can be safely administered without systemic toxicities and with side effects limited to CNS toxicity [56].

TP38

Epidermal growth factor receptor (EGFR) has been found to be overexpressed in a large proportion of GBM cells. Immunotoxin TP38 is a 43.5 kDa recombinant fusion protein of PE-38 and TGFα (which targets the EGFR). The Phase I clinical trial of TP38 in 20 patients with recurrent primary or metastatic malignant brain tumor delivered by CED was well tolerated with some durable radiographic responses [52,57]. Hemipharesis and constitutional symptoms (fatigue) were the dose-limiting neurologic toxicities observed.

Future prospects and concluding remarks

Integration of the immunotoxins to the current clinical modalities presents a promising approach to fighting cancers. The cumulative knowledge gathered about the structure and action of the toxins and major developments in rDNA technology and protein engineering have enabled the exploitation of lethal toxins for the production of therapeutic agents. Still, their clinical application faces many challenges – nonspecific toxicities, complexities involved in production and immunogenicity. The most common toxicities seen are hepatotoxicity and vascular leak syndrome, due to nonspecific binding of the toxic or targeting component to hepatic and endothelial cells, respectively. Serial modifications in the immunotoxin constructs used to date have reduced nonspecific toxicities, increased stability, enhanced tissue penetration and improved targeted cellular killing.

Another major challenge with application is immunogenicity. Owing to the development of the neutralizing antibodies in most patients, the immunotoxin treatment cycles have been limited to one or sometimes two cycles in most cases (except B-cell malignancies). Antibodies are raised against the nonhuman components: the toxic moiety and/or the antibody of mouse origin. The problem of human anti-mouse antibody (HAMA) has been overcome by the use of chimeric and humanized antibodies. Potential strategies to reduce the immunogenicity also include the coadministration of immunosuppressants and the engineering of the parent molecule to remove major epitopes. The prospect of engineering a bacterial toxin to render it nonimmunogenic is challenging but the success achieved with the less immunogenic variant of PE developed by Onda *et al.* is heartening [7]. A recent development based upon this strategy is a bispecific ligand-directed toxin (BLT) EGF4KDEL-7mut construct, in which human EGF and IL4 are linked to low immunogenicity PE38. It confers the dual benefit of increased targeting capability and reduced immunogenicity [58].

Synergistic studies with other chemotherapeutic drugs might offer a significant improvement in the clinical potential of immunotoxins toward cancer treatment. A newer variation to this is provided by humanized immunotoxins that exploit human proteins as the toxic and the targeting moiety and, thus, solve the problem of immunogenicity.

Among the several immunotoxins developed, Ontak™ and BL22 have shown remarkable success against hematologic tumors. The development of the CED mode for the intratumoral administration of the drugs into malignant gliomas has enabled four drugs to reach Phase II clinical trials. However, the development of immunotoxins for the treatment of solid tumors poses challenges such as the generation of an immune response against the toxin moiety, poor tumor penetration and reduced half-life. Strategies that promise to overcome these limitations are: (i) reduction of immunogenicity by removing B- and T-cell epitopes; (ii) use of humanized immunotoxins; and (iii) development of bispecific constructs as next-generation molecules. A further step toward improvement is to enhance the catalytic activity of the toxin while, at the same time, mitigating unwanted toxicity. Identification of new antigenic targets is another avenue that needs to be explored.

Conflict of interest

The authors hereby state that they have no conflicts of interest to declare.

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