Minireview

Targeted Toxins

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Abstract

Targeted toxins, consisting of tumor-selective ligands coupled to polypeptide toxins, represent a new class of cancer therapeutics that kills malignant cells by inactivating cytosolic protein synthesis and inducing apoptosis. A number of these molecules have been produced under good manufacturing practice conditions and given systemically to patients with a variety of neoplasms. The promising results to date and the remaining pharmacological hurdles are discussed.

Introduction

Targeted toxins consist of a targeting polypeptide covalently linked to a peptide toxin. The peptide protein may be an antibody or antibody fragment, such as a single-chain antibody. These reagents are called immunotoxins. When the targeting moiety is a cytokine, growth factor, or peptide hormone, the molecule is referred to as a fusion protein toxin. The targeting protein or ligand directs the molecule to a cell surface receptor or determinant; the toxin moiety then enters the cell and induces apoptosis by, in most cases, inactivating protein synthesis. Extremely potent catalytic toxins that can kill cells with as few as 1 molecule/cell are found in plants, bacteria, and fungi. The toxins most commonly modified for the construction of targeted molecules that have been clinically evaluated include DT2 and PE from bacteria and ricin and Gel and PAP isolated from plants.

The two initial challenges in synthesis of a clinically effective targeted toxin are: (a) to identify a ligand that will selectively target to every malignant cell in the body; and (b) to modify the toxin so that it will no longer bind normal tissues. The ligand and modified toxin are then covalently linked together.

Recently, a number of ligands have been found that bind with high affinity to antigens or receptors on neoplastic tissues. These are listed in Table 1. None of the targets are truly tumor specific. In fact, most are differentiation antigens or growth factor receptors. Nevertheless, initial clinical data suggest that in certain cases, there may be more target on the surface of cancer cells, or that the loss of particular normal tissues in patients bearing the target does not produce serious side effects. One would expect the rapid overgrowth of tumor cells lacking the target, but these “resistant” relapsing tumors have not been commonly observed to date in clinical trials of these agents (31). An additional note is that the ligand after binding the target must internalize by receptor-mediated endocytosis to permit the toxin to gain entry to the cytosol. Many targets have had to be abandoned prior to in vivo or clinical testing because they lacked this crucial property. Nevertheless, a number of targeted toxins have been made that bind to and internalize tumor cells in tissue culture in a selective fashion. Once produced in sufficient quantities, many of these have been tested clinically. The encouraging results with targeting the IL-2 receptor, the B-cell differentiation antigen CD22, and local targeting of transferrin receptors in brain tumors suggest that sufficient biological delivery can be obtained for clinical benefit.

Toxins must be modified, both to remove normal-tissue binding sites and for linkage to the tumor-selective ligand. DT is a M r 58,000 protein with an NH2-terminal ADP-ribosylation catalytic domain that inactivates cellular protein synthesis, a hydrophobic middle domain responsible for translocation of the ADP-ribosylation domain to the cytosol, and a COOH-terminal cell-binding domain (32). DT has been genetically modified for targeted toxin synthesis by either a point mutation in the binding domain, altering a critical Ser-525 to a phenylalanine (CRM107; Ref. 33), or deletion of the 147-amino acid residue cell-binding domain (DT388 or DAB395; Ref. 34). PE is a M r 68,000 polypeptide with an NH2-terminal cell-binding domain, followed by the amphipathic helix-containing translocation domain and a COOH-terminal ADP-ribosylation domain (35). PE has been altered to eliminate normal tissue binding by deleting amino acid residues 1–252 and 365–384, yielding PE38 (36). Ricin toxin is a M r 64,000 heterodimer consisting of an A subunit with rRNA N-glycosidase enzyme activity that blocks cell protein synthesis linked by a disulfide bond to a B lectin subunit, which binds to normal mammalian cell surface oligosaccharides (37). Ricin is prepared for targeted toxin synthesis by reduction and removal of the cell-binding B subunit so that only the A subunit (RTA2) is used (38) or by chemically blocking the lectin sites of the B subunit with reactive glycopeptides to create a bR molecule (39). Both Gel and PAP are also rRNA N-glycosidases capable of inactivating protein synthesis (40). However, they lack cell-binding moieties and hence can be used directly in targeted toxin constructions. Currently, there is insufficient information to choose one toxin over another in the assembly of a targeted toxin for a particular malignancy.

The ligand and modified toxins are linked together, either
chemically or genetically, and the conjugates or fusion protein toxins are purified, usually by chromatographic separations. Once purified, selective cytotoxicity to malignant cells has been demonstrated with a number of these molecules, both in tissue culture and animal models. Subsequently, many of these were produced and purified under good manufacturing practice conditions for clinical evaluation.

A number of clinical trials have been conducted with immunotoxins and fusion protein toxins over the last 16 years. These studies defined a number of pharmacological and toxicological barriers that needed to be overcome. Table 2 lists these clinical studies as well as current clinical trials of targeted toxins with a description of the compound, the diseases for which it is being tested, the response rate, and toxicities, where known.

Excitingly, the first of the targeted toxins has received Food and Drug Administration approval for human use, and many additional agents are showing significant anticancer activity in Phases I and II clinical studies, primarily in patients with chemotherapy-refractory cancers. The original rationale for the production and testing of these reagents was that they had a different mechanism of action than DNA- or cell division-damaging therapeutics and thus might be effective either alone or in combination in patients with chemotherapy-resistant malignancies. This rationale appears to be at least partly vindicated.

Efficacy

Dramatic anticancer efficacy has been seen in five targeted toxin clinical trials. ONTAK, composed of the catalytic and transmembrane domains of DT fused to human IL-2, has received Food and Drug Administration approval for sale. The

| Table 1 Ligands used in clinically tested targeted toxins |
|----------------|----------------|----------------|------------------|------------------|
| Ligand        | Receptor/antigen | Toxin | Linker | Name | Ref. |
| B3 Mab        | Lewisα           | PE    | C     | LMB-1 | (1) |
| B3 sFv        | Lewisα           | PE    | G     | LMB-7b | (2) |
| B3 dsFv       | Lewisα           | PE    | G     | B96(sFv)PE40 | (3) |
| RFB4 Mab      | CD22             | Ricin | C     | IgG-RFB4-dgA | (4) |
| RFB4 Fab      | CD22             | Ricin | C     | Fab'-RFB4-dgA | (5) |
| RFB4 dsFv     | CD22             | PE    | G     | BL22  | (6) |
| HD37 Mab      | CD19             | Ricin | C     | IgG-HD37-dgA | (7) |
| B43 Mab       | CD19             | PAP   | C     | B43-PAP | (8) |
| Anti-B4 Mab   | CD19             | Ricin | C     | Anti-B4-bR | (9) |
| IL2           | IL2R             | DT    | G     | ONTAKc | (10) |
| Anti-Tac Mab  | CD25             | PE    | C     | Anti-Tac-PE | (11) |
| RFT5 Mab      | CD25             | Ricin | C     | RFT5-dgA | (12) |
| Anti-Tac sFv  | CD25             | PE    | G     | LMB-2  | (13) |
| Tf            | TIR              | DT    | C     | HuM195-gel | (14) |
| 454A12 Mab    | TIR              | Ricin | C     | 454A12-rTf | (15) |
| IL4           | IL4R             | PE    | G     | IL4(38-37)PE38KDEL | (16) |
| HuM195 Mab    | CD33             | Gel   | C     | HuM195-gel | (17) |
| GM-CSF        | GM-CSFR          | DT    | G     | DTGM  | (18) |
| N901 Mab      | CD55             | Ricin | C     | N901-bR | (19) |
| TGFR          | EGFR             | PE    | G     | TP40  | (20) |
| EGF           | EGFR             | DT    | G     | DAB389-EGF | (21) |
| 991T/36 Mab   | M, 72,000 antigen| Ricin | C     | Xomazyme-791 | (22) |
| 3409 Mab      | M, 55,000 antigen| Ricin | C     | 260F9-rA | (23) |
| Ber-H2 Mab    | CD30             | SO6   | C     | Ber-H2-SO6 | (24) |
| T101 Mab      | CD5              | Ricin | C     | T101-RTA | (25) |
| H65 Mab       | CD5              | Ricin | C     | H65-RTA | (26) |
| XMME-M01 Mab  | Proteoglycan     | Ricin | C     | XMME-M01-RTA | (27) |
| OVB3 Mab      | Ovarian antigen  | PE    | C     | OVB3-PE | (28) |
| 3A1 Mab       | CD7              | Ricin | C     | 3A1-dgRTA | (29) |
| TXU Mab       | CD7              | PAP   | C     | TXU-PAP | (30) |
| e33 dsFv      | erbB-2           | PE    | G     | ErbB-38 | (31) |

α G, genetic fusion; C, chemical conjugate; SO6, saporin; EGF, epidermal growth factor; TGFR, tumor growth factor α; sFv, single-chain Fv antibody; dsFv, disulfide-stabilized sFv; TIR, Tf receptor; EGFR, epidermal growth factor receptor; GM-CSFR, GM-CSF receptor; Lewisα, a carbohydrate antigen; CD5, CD7, CD19, CD22, CD25, CD30, and CD56, “cluster-designation” cell surface antigens; erbB-2, Her-2/new gene product; Fab9, Fab antibody fragment.


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other four are still being studied, but their clinical activity warrants description.

ONTAK (DAB₃₈₉ IL2), administered 9 or 18 mg/kg/day daily for 5 consecutive days every 3 weeks for up to eight cycles, yielded a 30% objective response rate in 71 patients with refractory stage IB–IV CTCLs.³ The median response lasted 6.9 months, with a range of 2.7–23.7 months. Patients must have shown $20\%$ CD25-positive lymphocytes within a biopsy specimen by immunohistochemistry. Among patients with advanced disease (stage $IIB$), responses were more common at the higher dose (38% versus 10%, $P = 0.07$). Decreased tumor burden correlated with amelioration of symptoms, measured by a quality of life questionnaire. In addition, 52% of patients classified as having stable disease showed a 50% decrease in tumor burden at some point during the study, but for 6 weeks.

Photographs before and after treatment of one of the complete responders are shown in Fig. 1.

LMB-2 [anti-Tac(Fv)-PE38] was administered to four 2-CdA- and IFN-refractory HCL patients (12). One patient received 63 mg/kg every other day for three doses for a total of two cycles. He had a complete remission, with no evidence of progression after 13 months. A second patient received 30 mg/kg every other day for three doses for a single cycle. The patient had a 95% reduction in leukemia burden by day 30 but could not be retreated because of infections. A third patient received 40 mg/kg for three doses over 6 days and showed a 95% reduction in leukemic cells but developed high titer neutralizing antibodies. A second cycle was given to this patient, but in the presence of antibody, no detectable LMB-2 was


observed in the circulation, and there was no further response. The fourth patient received a single infusion of 63 μg/kg of LMB-2, and no further agent was given because of diarrhea and reversible cardiomyopathy. A 99% reduction in leukemia cell burden occurred by day 22. Fig. 2 is a graph of the normal and leukemic blood cell counts over time after treatment with LMB-2. LMB-2 has also induced partial responses in patients with CLL, CTCL, HD, and ATL.

HN66000 (Tf-CRM107; CRM107 is a Tf-binding site mutant DT) was infused interstitially via catheters implanted into the tumor beds of patients with high-grade gliomas (13). Fifteen evaluable patients were treated with 0.1–1 μg/ml conjugate infused over 2–16 days with a total volume of 5–120 ml. A 60% partial and complete response rate has been observed, with median survival of responding patients exceeding 1 year. Gadolinium-enhanced T1-weighted MRI scans of one of the complete responders are shown in Fig. 3.

BL22 [anti-CD22(Fv)-PE38] has produced a complete remission in one HCL patient at 10 μg/kg every other day for three doses given in two cycles (6). Both eradication of circulating CLL cells and shrinkage of peripheral adenopathy have been observed.

In some of the other targeted toxin trials, responses have been observed in chemotherapy-refractory patients but at rates of <30%. RFB4-dgA produced a 25% response rate when given by continuous infusion or intermittent bolus schedule in patients with heavily pretreated B-cell non-Hodgkin’s lymphoma (4, 42). These included 1 complete remission and 9 partial remissions of 32 patients. RFB4-dgA also produced an unmaintained complete remission of >3 years in a posttransplant lymphoma (43). HD37-dgA (anti-CD19-dgA) produced a lower response rate (~10%; Ref. 7), and the combined anti-CD22 (RFB4-dgA) plus anti-CD19 (HD37-dgA) toxins, referred to as Combotox, when administered at 10, 20, or 30 mg/m² over 192 h by continuous infusion yielded a 9% partial response rate with remissions lasting 1 and 5 months. N901-br (anti-CD56-br) given at 5–40 μg/kg/day for 7 days by continuous infusion produced 1 partial remission in 21 refractory small cell lung cancer patients (18). LMB-1 (anti-Lewisα-PE38) was administered by single i.v. bolus infusion at 10–100 μg/kg to 38 patients with advanced solid tumors (1). There was one complete response and one partial response lasting 2 and 7 months, respectively. RFT5-dgA gave 2 of 10 partial responses in HD (11). Ongoing studies with LMB-7, RFT5-dgA, N901-br, DTGM, IL4(38–37)PE38KDEL, TXU-PAP, BR96(sFv)-PE40, and B43-PAP are too early to assess response rates.

**Toxicities**

In some instances, the targeted toxin receptor/antigen is present on normal tissues, and side effects have occurred. LMB-7 binds Lewisα antigen on normal stomach mucosa. Initial dose-limiting toxicities at 7–24 μg/kg every other day for three doses were nausea, vomiting, and diarrhea. Endoscopy con-

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4 E. Sausville and R. Messman, unpublished observations.
5 L. H. Pai-Scherf, D. Pearson, R. Wittes, M. C. Willingham, and I. Pastan. A Phase I study of LMB-7, [B3(Fv)PE38], a recombinant
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A, cycle 2 on day 32. In

cells/mm$^3$. In publication.

toxicity was observed at 2–3
cytes, macrophages, and neutrophils. Again, early dose-limiting

DTGM binds the GM-CSF receptor present on mature mono-
greatly reduced the side effects and permitted dose escalation.

Prophylaxis with omeprazole, antiemetics, and loperamide

confirmed gastritis with apoptotic cells in the body and fundus.

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greatly reduced the side effects and permitted dose escalation.

DTGM binds the GM-CSF receptor present on mature mono-
cytes, macrophages, and neutrophils. Again, early dose-limiting
toxicity was observed at 2–3 µg/kg given daily for 5 days (17).

Evidence of cytokine release or systemic inflammatory response
syndrome was seen with transient fever, chills, nausea, vomiting,
transaminasemia, hypotension, hypoalbuminemia, mild renal
and pulmonary insufficiency, edema, and weight gain. One
patient was tested and showed a rise in IL-6 and IL-1 receptor
antagonist, correlating with symptoms. Once corticosteroid pro-
phylaxis was initiated, these side effects were prevented, and
dose escalation continued. HN66000 (TF-CRM107) binds Tf
receptors present on normal brain capillaries. Peritumoral focal
brain injury occurred in some infused patients 2–4 weeks after
treatment (13). There were stereotypic MRI changes consisting
of serpentine strips of increased signal in the peritumoral cortex
evident on unenhanced T1-weighted MRI. Biopsy confirmed
thrombosed cortical venules and/or capillaries. By lowering the
concentration of HN66000 in the infusate and lowering the
volume, toxicity to the normal adjacent cortex was ameliorated.
Erb-38 bound receptors present on normal hepatocytes and
causedit hepatotoxicity at low doses (29). OVB3-PE and
260F9-rA reacted with antigen present in the central nervous
system and Schwann cells, respectively, and produced dose-
limiting neurotoxicities (21, 26).

Toxicities that are independent of ligand have been observed
with most targeted toxin clinical studies. These consist of
either hepatocyte injury, causing abnormal liver function tests,
or vascular endothelial damage with resultant VLS. Both the
liver lesion and the vascular lesion may be attributable to
nonspecific uptake of targeted toxins by normal human tissues.
The normal tissue mediating these injuries may be the tissue
showing the observed toxicity, liver or vascular endothelium, or
may be macrophages that secondarily release cytokines, produc-
ing the liver and blood vessel damage (Fig. 4). There are data
supporting both hypotheses. Hepatocytes have been exposed to
targeted toxins in vitro. The agent binds to the hepatocyte cell
surface dependent on the pl of the ligand in the conjugate (48).
Separately, animals have been treated with toxins, and macro-
phage release of cytokines was demonstrated (49). These cyto-
kines, including tumor necrosis factor α, may also directly
injure hepatocytes. Clinical proof of which mechanism is more
responsible for the transaminasemia in patients treated with
fusion toxins is lacking, but it is likely that several mechanisms
may operate simultaneously. Nevertheless, the use of targeted
toxins with lower nonspecific binding and efforts to block
macrophage cytokine release appear warranted. VLS is charac-
terized by weight gain, increased vascular permeability, hy-
poalbuminemia, myalgias, mild renal and pulmonary insuffi-
ciency, and hypotension, and in some cases, aphasia and
pulmonary edema. The syndrome occurs transiently, but at
times severely, after targeted toxin treatment. VLS usually oc-
curs 4–6 days after initiating therapy and lasts 4–10 days. The
cause of the endothelial lesion is unknown. Again, both uptake
by the vascular endothelium and macrophages has been postu-
lated as triggering events. There are a number of studies in vitro
showing endothelial cell culture apoptosis after toxin conjugate
exposure (50). Proteins containing a three amino acid motif
(x)D(y), where x can be L, I, G, or V and y is V, L, or S appear
to bind and damage human endothelial cells in vitro (41). This
motif has been found in IL-2, PE, and RTA chain and may be
partly responsible for VLS. A slight increase in loss of endo-
thelial surface relative to replacement could lead to a significant
“leak.” Endothelial cells may be uniquely sensitive because of
exposure to high concentrations of the targeted toxin in the
bloodstream. A correlation between AUC (blood concentration
in vivo may yield less VLS at comparable doses.

Alternatively, inflammatory cytokines released by toxin-
ingested macrophages could also produce profound systemic
alterations in vascular integrity, as seen with the systemic in-
flammatory response syndrome. Although no animal model
reproduces human VLS, a syndrome of hydrothorax, hypoalbu-
mimia, hemoconcentration, and neutrophilia developed in rats

Fig. 2 Response of HCL patient to LMB-2. The patient received
LMB-2 at 63 µg/kg i.v. QOD × 3 for two cycles: cycle 1 on day 1 and
cycle 2 on day 32. In A, the number of HCL is shown: ▼, HCL
cells/mm$^3$. In B, normal blood cells are represented by ▲, absolute
neutrophil count/mm$^3$ × 10$^{-1}$; ○, platelet count/mm$^3$ × 10$^{-3}$; ●,
hemoglobin in gm/dl × 10. Figure and legend provided by Dr. Robert
Kreitman, NIH (Bethesda, MD), and reproduced with permission (12).

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single-chain immunotoxin for advanced solid tumors, submitted for
publication.
after i.v. injections of anti-Lewis\textsuperscript{a} Fv-PE40 (51). Rats treated with PE40 alone or IL6-PE\textsuperscript{4E} also developed a VLS-like syndrome (52). The syndrome in rats was prevented by prophylaxis with steroids or nonsteroidal anti-inflammatory drugs. This may be because of the blocking of macrophage cytokine release by these agents. To date, however, there is no clinical data, such as elevated circulating inflammatory cytokines, supporting cytokine-induced vascular trauma. Regardless, it still appears prudent to select smaller targeted toxins. The role of steroid prophylaxis is less certain, because anecdotal reports suggest either “protection” (17) or lack of “protection” in humans (53) as a role.

Other side effects that have been reported with targeted toxins include mild constitutional symptoms when the biological agent is infused rapidly (<30–60 min).\textsuperscript{3} These symptoms include fever, chills, myalgias, headaches, chest discomfort, and transient hypotension. The infusion may be stopped. The patient may then be symptomatically treated and usually the agent infusion restarted at a slower rate without complications. Corticosteroid prophylaxis appears to prevent these constitutional symptoms. Rarely, patients may have an anaphylactoid response to targeted toxin. Because the agent is protein in nature, rare hypersensitivity is not unexpected. However, the rare occurrence of this complication mandates the administration of the biological agent in a setting where detection and treatment of anaphylaxis are routine. Therapy may include 0.3 ml of epi

ephrine, 100 mg of Solumedrol, and 25 mg of Benadryl, all given i.v. along with supplemental boluses of i.v. normal saline and nasal prong or face mask oxygen, with continuous cardiac monitoring until resolution. Patients should not be retreated with the same targeted toxin, if anaphylaxis is attributable to the agent, because the reaction is likely to recur with further exposure.

**Pharmacology**

Recent improvements in disease selection and targeted toxin design has led to an improvement in tumor localization and reduced immunogenicity. However, these remain important pharmacological barriers.

The circulating half-life varied with the size of the molecule. Larger molecules had longer half-lives, with 24 h for Combotox and N901-bR and 9 h for LMB-1 (1, 18, 31). All of these molecules consisted of whole IgGs coupled to peptide...
toxins. Smaller molecules, including the single-chain immunotoxins LMB-2 and LMB-7 and the cytokine fusion protein toxin ONTAK (DAB389IL2), had shorter half-lives of 5, 2, and 1 h, respectively (12). Some of the clearance of the smaller molecules (\(M_r \sim 60,000\)) may be renal glomerular filtration. However, most of the clearance of these foreign proteins is likely by the liver or reticuloendothelial system.

No clinical protocols have been reported that comprehensively correlated percentage of extravascular tumor cell saturation with dose of targeted toxin. The assumption has been that toxicities, including VLS, hepatotoxicity, or neurotoxicities, prevented sufficient doses to saturate extravascular tumor sites. In vitro studies with multicellular tumor spheroids and mathematical models using data from other proteins suggest that smaller-sized fusion toxins and permeability enhancers, such as cisplatin or hyaluronidase, may improve tumor uptake (54, 55). Clinical responses with targeted toxins in lymphomas and leukemias may be attributable, in part, to a significant fraction of circulating malignant stem cells in these diseases.

Targeted toxins generated humoral immune responses in all patients, except those with CLL. Clearly, the development of neutralizing antibodies is detrimental to targeted toxin antitumor efficacy. In many trials, retreatments have been limited to a few cycles because of the development of neutralizing antibodies. Even when the antibodies generated are nonneutralizing, they may form immune complexes and accelerate clearance from the circulation. This antibody response also reduces clinical benefit. Rituximab is a human Mab to the B-cell differentiation antigen CD20. It is being tried in combination with LMB-1 to reduce immune responses to that immunotoxin. Other methods include coadministration of 15-deoxyspergualin or CTLA4Ig and to have to date been tried only in animal models (56, 57). Finally, the use of human RNases as the toxophore may be an additional method for reducing conjugate immunogenicity (58).

**Conclusions**

The potential for targeted toxins as postulated by Paul Ehrlich one century ago (59) has not been fully realized to date. However, over the last 20 years with the advent of genetic engineering and advances in receptor physiology, we have progressed to the point that several targeted toxins have demonstrated clinical utility. Over the next decade, additional ligand-receptor systems should be defined that extend the applications of targeted toxins to additional disease states. Control of the nonspecific toxicities and immune responses with various prophylactic maneuvers should further improve the therapeutic index of these molecules. Finally, combination therapy trials with cytotoxic chemotherapeutic agents are likely to yield even higher response rates with more durable responses, based on preclinical results and clinical studies with other biological/cytotoxic agent combinations. The next decade should see exciting advances in the development of these reagents.

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