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REVIEW ARTICLE

CYTOTOXIC THERAPY WITH DIPHTHERIA TOXIN FUSION PROTEINS

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SUMMARY

Diphtheria toxin (DT) fusion proteins can selectively target malignant cells and induce cell death by a different mechanism than conventional chemotherapeutic drugs. Cytotoxic therapy with DT fusion proteins is a potential treatment option for relapsed/refractory cancers that are resistant to chemotherapeutic drugs. Due to the heterogeneity of chemical conjugates, the majority of DT fusion proteins used in clinical trials are recombinant DT fusion proteins. These consist of the catalytic and translocation domains of DT fused to tumor-selective ligands (single-chain antibody fragments [sFv], cytokines and growth factors). In this review, recent progress in DT fusion protein design and synthesis, clinical updates of DT fusion protein trials, and the challenges and perspectives for DT fusion proteins are discussed. Although DT fusion proteins have a unique mechanism of action toward tumor cells, their clinical use is limited by nonspecific cell toxicity, particularly endothelial damage. Immunogenicity also limits repeated dosing. New approaches to overcome these limitations are needed.

INTRODUCTION

Chemotherapy resistance in the treatment of many cancers is a major clinical problem. Most chemotherapeutic drugs are small molecules that target DNA, mitotic mechanisms or transcription factors. These small molecules can be exported by various pump mechanisms, which can be enhanced by selection processes, leading to cancer chemotherapy resistance. Alternative approaches to cancer treatment have therefore been sought. One such approach is a diphtheria toxin (DT) fusion protein consisting of the catalytic and translocation domains of DT genetically fused to tumor-selective ligands (single-chain antibody fragments [sFv], cytokines and growth factors). DT fusion proteins can selectively target malignant cells and induce cell death by a different mechanism than conventional chemotherapeutic drugs. Therefore, cytotoxic therapy with DT fusion proteins is a potential treatment option for relapsed/refractory cancers that are resistant to chemotherapeutic drugs.

DT is a 535-amino-acid protein comprised of two subunits consisting of an A chain (catalytic domain) and a B chain connected by a furin-cleavable linker that is an arginine-rich loop formed by one disulfide bond (1). The cleavage of the furin-cleavable linker is a prerequisite for DT intoxication of cells (2, 3). The B chain contains a translocation domain and a receptor binding domain (1). In DT fusion proteins, the receptor binding domain is replaced with tumor-selective ligands. Binding induces receptor-mediated endocytosis, followed by localization to the endosomes. Because different receptors have different preferences for intracellular routing, some receptors achieve limited penetration into the late acidic endosome. These DT–ligand combinations exhibit suboptimal target toxicity (4). In the acidic environment of the endosomes, DT partially unfolds. This exposes hydrophobic regions on the translocation domain and triggers membrane insertion to form a channel for translocation of the catalytic domain (5-7). Unfolding of the catalytic domain is also required for cytosolic entry through the translocation channel (8, 9). The chaperone activity of cytosolic heat shock protein HSP90 and the membrane-inserted translocation domain translocates the unfolded catalytic domain to the cytosol (10, 11). For the release of the catalytic domain into the cytosol, cytosolic thioredoxin reductase TR1 reduces the disulfide bond between the catalytic domain and translocation (11). The translocated catalytic domain catalytically inactivates cellular protein synthesis by ADP-ribosylating the diphthamide residue in domain IV of elongation factor 2 (EF-2) (12). One single molecule of the catalytic domain in the cytosol is sufficient to block protein synthesis and lead to death of the targeted tumor cell (13).

Many DT fusion proteins have been designed and tested preclinically and clinically. $DAB_{389}IL2$ was first approved for the treatment of cutaneous T-cell lymphoma (CTCL) in 1999 by the FDA. Four DT

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fusion proteins –DAB_{389}IL2, A-dmDT390-bisFv(UCHT1), DT_{388}IL3 and DT2219ARL– are being tested in clinical trials. Many other DT fusion proteins are also being developed at the preclinical stage. In this review, recent progress in DT fusion protein design and synthesis, clinical updates of DT fusion protein trials, and challenges and perspectives for DT fusion proteins are discussed.

RECENT PROGRESS IN DESIGN AND SYNTHESIS OF DT FUSION PROTEINS

Potency improvement is an obvious way to increase efficacy and reduce nonspecific toxicity. The first generation of DT-based therapeutic agents were chemical conjugates of mutant DTs and tumor-selective ligands. Only one chemical conjugate –transferrin-CRM107– reached clinical trials due to the inherent heterogeneity of chemical conjugates. The second-generation DT fusion proteins –DAB_{389}IL2, DT_{388}IL3, DAB_{389}EGF and DT_{388}GMCSF– were developed using protein engineering technology. They have only one tumor-selective ligand and a relatively simple structure, such that these molecules were efficiently produced in *Escherichia coli*.

The third-generation DT fusion proteins –A-dmDT390-bisFv(UCHT1) and DT2219ARL– have a more complicated structure to increase binding affinity for target antigens. These molecules were designed by the addition of another identical ligand or a different ligand. The third-generation DT fusion proteins are being investigated in clinical trials. For molecules with two identical ligands (normally sFv), there are two formats –bivalent and fold-back formats. In the bivalent format (DT-sFv-$[G_4S]_3$-sFv), the additional sFv (V_L-$[G_4S]_3$-V_H) is fused to the *C*-terminus of the monovalent DT fusion protein through the $(G_4S)_3$ linker. The bivalency provides much higher binding to targeted cells (20- to 30-fold increase) (14). In the fold-back format (DT-V_L-G_4S-V_H-$[G_4S]_3$-V_L-G_4S-V_H), the G_4S linkers between V_L and V_H are shorter than the $(G_4S)_3$ linker between the first and second sFv. The shorter linkers prevent adjacent V_L and V_H domains from forming an sFv. The $(G_4S)_3$ linker is long enough so that the second V_L and V_H domains can fold back to pair with the first V_H and V_L, respectively, forming two sFv antigen-binding sites. This provides a more rigid structure compared to the bivalent proteins, resulting in an increase in potency of 5- to 7-fold (15).

However, the production of these DT fusion proteins by in vitro refolding from *E. coli* was very inefficient due to the multidomain structure (16). Good refolding could be achieved in a toxin-resistant CHO cell line, but production capacity was limited to 5 µg/mL (17). We demonstrated that *Pichia pastoris* is an ideal host strain to express the bivalent DT fusion proteins via the secretory route, because secretory expression minimizes exposure of DT to the cytosol, where the catalytic domain inactivates EF-2 by ADP-ribosylation (16). Furthermore, we have developed a DT-resistant strain by a point mutation (G710R) in EF-2 (18). The DT-resistant strain (120 mg/L) produced three times higher concentrations of the bivalent DT fusion proteins than the wild-type strain (37 mg/L) after optimization of fermentation conditions (19, 20). Also, borate anion exchange chromatography proved to be an efficient step to separate the bivalent DT fusion protein from host glycoproteins (21).

The bispecific DT2219ARL is a good example of a molecule with two different ligands. This molecule contains an *N*-terminal methionine, the first 389 amino acid residues of DT, the EASPEEA linker and two different sFv moieties recognizing different antigens (CD22 and CD19). The V_L and V_H domains in each sFv moiety are connected by a flexible aggregation-reducing linker (ARL) derived from human muscle aldolase (the first 20 amino acid residues). Two sFv molecules are linked by the $(G_4S)_3$ linker. This ARL increased potency and product yield in *E. coli* (22). This bispecific molecule was 1,000-fold more potent than DT22 alone or an equal mixture of DT22 and DT19. Interestingly, the enhanced potency of DT2219ARL was attributed to possibly improved internalization or optimized intracellular routing to compartments for translocation, but not to increased binding affinity. However, no internalization or intracellular compartment studies were reported with DT2219ARL.

Three other bispecific DT fusion proteins (DTEGF13, DTEpCAM23 and DTAT13) have been reported (23-25). DTEGF13 has human epidermal growth factor (EGF) and human IL-13 as tumor-selective ligands (23), DTEpCAM23 contains anti-EpCAM sFv and anti-HER2 sFv (24), and DTAT13 consists of human IL-13 and the 135-amino-acid residue *N*-terminal fragment of human urokinase (25). The bispecific fusion proteins DTEGF13 and DTEpCAM23 were more potent than monospecific equivalents, but in vitro cytotoxicity was not enhanced for DTAT13.

UPDATES ON CLINICAL DEVELOPMENT OF DT FUSION PROTEIN THERAPY

Among DT fusion proteins for cancer treatment, eight have reached clinical testing. Several protein molecules showed antitumor activity and one of them has been approved by the U.S. FDA for the treatment of persistent or recurrent CTCL. Table I summarizes clinical results for DT fusion proteins in patients with various malignant diseases.

DAB_{389}IL2 (Ontak®, denileukin diftitox) was the first FDA-approved DT fusion protein for the treatment of persistent or recurrent CTCL. DAB_{389}IL2 is comprised of the catalytic and translocation domains of DT (DAB_{389}) and *C*-terminal human interleukin-2 (IL-2). DAB_{389}IL2 was produced in *E. coli*, formulated in 20 mM citrate/0.05 mM EDTA/1% polysorbate 20 (pH 6.9-7.2) at 0.15 mg/mL, sterile-filtered, vialed in 2 mL, frozen and stored at –20 °C. DAB_{389}IL2 was selectively cytotoxic for human and murine IL-2 receptor-positive cells (26). Patients with CTCL, non-Hodgkin's lymphoma (NHL) or Hodgkin's disease showed a maximum tolerated dose (MTD) of 27 µg/kg/day for 5 days in a phase I trial (27). In a phase III trial, DAB_{389}IL2 was administered at 9 or 18 µg/kg/day by i.v. infusion over 30-60 min for 5 consecutive days every 3 weeks for eight cycles. Of the 71 patients with CTCL, 30% experienced an objective response (10% complete remission [CR] and 20% partial remission [PR]) (28). In the other placebo-controlled phase III trial, 10% CR and 34% PR were observed in CTCL patients (29). Adverse events included fever, chills, nausea, vomiting, myalgias, arthralgias, hypotension, hypoalbuminemia, edema, dyspnea, chest pain and back pain. The serum half-life was 70-80 min.

DAB_{386}IL2 produces transient T-cell depletion in melanoma patients, causing regression of melanoma metastases in 4 of 16 patients. This suggested that transient T-cell depletion may disrupt the homeostatic control of cognate immunity and allow for the expansion of effector T cells with specificity against neoplastic cells during homeostatic T-cell proliferation (30). In a phase II trial in

Table I. *Clinical results for DT fusion proteins.*

Drug	Phase	Indications	Dose levels (doses/course)	Response rate (CR)	Toxicities	Ref.
$DAB_{389}IL2$	I	CTCL NHL HD	3-31 μg/kg (5)	13(5)/35 3(1)/17 0(0)/21	Acute infusion events, VLS	27
$DAB_{389}IL2$	III	CTCL	9 or 18 μg/kg (5)	21(7)/71	Flu-like symptoms, acute infusion events, VLS	28
$DAB_{389}IL2$	III	CTCL	Placebo 9 or 18 μg/kg (5)	7(1)/44 44(10)/100	Flu-like symptoms, acute infusion events, VLS	29
$DAB_{389}IL2$ + steroid premedication	II	CTCL	9 or 18 μg/kg (5)	9(1)/15	Acute infusion events, VLS	76
$DAB_{389}IL2$	I	GVHD	9 μg/kg (2) 9 μg/kg (6) 9 μg/kg (7)	5(1)/7 9(6)/13 17(8)/24	Transaminasemia	88
$DAB_{389}IL2$	II	GVHD	4.5 or 9 μg/kg (9)	9(9)/22	VLS, opportunistic infection	89
$DAB_{389}IL2$	II	CLL	9 or 18 μg/kg (5) 18 μg/kg (5) 18 μg/kg (5)	2(0)/12 2(0)/7 6(1)/22	Transaminasemia, VLS Transaminasemia, VLS, anorexia Transaminasemia, VLS, opportunistic infection	90 91 92
$DAB_{389}IL2$	II	Melanoma	12 μg/kg (4)	4(0)/16	Mild dermatitis and arthritis	30
$DAB_{389}IL2$	II	Psoriasis	Placebo 5, 10 or 15 μg/kg (3)	2(0)/12 7(0)/29	Flu-like symptoms, rash, VLS	93
$DAB_{389}IL2$ + bexarotene	I	CTCL	18 μg/kg (3)	8(4)/14	Lymphopenia, leukopenia	94
$DAB_{389}IL2$	II	T-cell NHL	18 μg/kg (5)	13(6)/27	VLS, transaminasemia, skin reaction	31
$DAB_{389}IL2$	II	B-cell NHL	18 μg/kg (5)	11(3)/45	Transaminasemia, leukopenia, thrombocytopenia, VLS	95
$DAB_{389}IL2$+ rituximab	II	B-cell NHL	18 μg/kg (5)	12(6)/38	Transaminasemia, fatigue, VLS	96
$DAB_{389}IL2$	II	Indolent NHL	18 μg/kg (5)	3(0)/29	Fatigue, transminasemia, neutropenia, VLS	97
$DAB_{389}IL2$	II	NSCLC	18 μg/kg (5)	0(0)/41	VLS, constitutional symptoms	98
A-dmDT390-bisFv(UCHT1)	I	CTCL, PTCL	2.5 μg/kg (8) 5.0 μg/kg (8)	3(2)/6 2(0)/2	Flu-like symptoms, VLS, opportunistic infection	36
$DT_{388}IL3$	I	AML, MDS	4-12.5 μg/kg (6)	3(1)/45	Flu-like symptoms, VLS	45
$DT_{388}IL3$	I	AML, MDS	7.07 μg/kg (5)	Ongoing		
DT2219ARL	I	B-cell malignancy		Ongoing		
$DAB_{486}IL2$	I	NHL, HD, CLL, CTCL HD, CTCL	0.7-200 μg/kg (5) 75-200 μg/kg (5) 75 or 100 μg/kg (5)	3(1)/18 1(1)/15 3(1)/5	Transaminasemia, rash Transaminasemia, hypoalbuminemia Transaminasemia	99 100 101
DAB_{389}-EGF	1	Metastatic solid tumors	0.3-15 μg/kg (5) 6-9 μg/kg (3)	1(0)/72	Liver and renal toxicity	59
$DT_{388}GMCSF$	I	AML	1-5 μg/kg (5)	3(1)/31	Liver and renal toxicity, VLS	73
Transferrin-CRM107	I	Glioma	5-180 mL of 0.1-1.0 μg/mL (1)	9(2)/15	Cerebral edema, peritumoral brain injury	55
Transferrin-CRM107	II	Glioma	40 mL of 0.67 μg/mL (1)	12(5)/44	Cerebral edema, seizures	57

CTCL, cutaneous T-cell lymphoma; NHL, non-Hodgkin's lymphoma; HD, Hodgkin's disease; GVHD, graft versus host disease; CLL, chronic lymphocytic leukemia; NSCLC, non-small cell lung cancer; PTCL, peripheral T-cell lymphoma; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; CR, complete remission; VLS, vascular leak syndrome.

T-cell NHL (peripheral T-cell lymphoma, anaplastic large cell lymphoma, angioimmunoblastic T-cell lymphoma, Sézary syndrome and NK/T-cell lymphoma), 6 CRs and 7 PRs were observed in 27 patients (overall response rate of 48.1%). Interestingly, an objective response was achieved in 8 of 13 patients (61.5%) with $CD25^+$ tumors (4 CRs and 4 PRs) (31). $DAB_{389}IL2$ is currently being evaluated in clinical trials either alone or in combination with other therapies for the treatment of various cancers, including B-cell NHL, T-cell NHL, melanoma, advanced breast cancer, pancreatic cancer, ovarian cancer and acute myeloid leukemia (AML).

A-dmDT390-bisFv(UCHT1) is a CD3-targeted DT fusion protein for the treatment of T-cell malignancies and autoimmune diseases, and for the induction of immune tolerance for transplantation (14). A-dmDT390-bisFv(UCHT1) contains DT preceded by an alanine residue (A), truncated at amino acid residue 390, doubly mutated (dm) to remove glycosylation sites within the toxin, which reduced bioactivity when expressed in *P. pastoris* and eukaryotic cells (17), and two tandem sFv molecules derived from the UCHT1 parental antibody. A-dmDT390-bisFv(UCHT1) was produced in *P. pastoris* (32), formulated in 5 mM Tris/1 mM EDTA/150 mM NaCl/5% glycerol (pH 8) at 0.8 mg/mL, sterile-filtered, vialed in 2.2 mL, frozen and stored at −80 °C. This type of CD3-targeted DT fusion protein kills human malignant T cells in animal models (33). It can also kill 99.4% of the resting cells in a transgenic animal model where the T cells express human CD3 (14). However, it was not toxic to CD3-negative normal cell lines (34). The MTD of A-dmDT390-bisFv(UCHT1) in squirrel monkeys and Sprague-Dawley rats was 200 µg/kg in total (eight doses twice daily for 4 consecutive days). Dose-limiting toxicity (DLT) was reversible elevation of liver transaminases. Treatment of human peripheral blood mononuclear cells with A-dmDT390-bisFv(UCHT1) did not induce T-cell proliferation or cytokine release (35).

A-dmDT390-bisFv(UCHT1) is being tested in a phase I clinical trial (36). After eight infusions over a 4-day period blood T cells were depleted by 3 logs. CD4 and CD8 T-cell numbers returned to pretreatment levels by day 20, but the naïve subsets were markedly depressed for several months. One course of A-dmDT390-bisFv(UCHT1) treatment produced durable remissions lasting for over a year. Of eight eligible patients with CTCL, two had CR (25%) and three had PR (37.5%), with an overall response rate of 62.5%. One patient with peripheral T-cell lymphoma developed underlying disease (congestive heart failure [CHF]) and vascular leak syndrome (VLS) during treatment. CHF was therefore included in patient ineligibility criteria. Adverse events included fever, chills, nausea, hypoalbuminemia, transaminasemia, lymphopenia, reactivation of Epstein–Barr virus and cytomegalovirus, and hypophosphatemia. Since the pharmacodynamic activity of A-dmDT390-bisFv(UCHT1) was to deplete tumor and normal T cells, lymphopenia and secondary opportunistic infections were not considered as DLTs. The serum half-life was 48 min. Dose escalation is proceeding.

DT_{388}IL3 consists of the first 388 amino acid residues of DT with a His-Met linker fused to human IL-3. DT_{388}IL3 was designed for the treatment of AML and myelodysplastic syndrome (MDS). DT_{388}IL3 was produced in *E. coli*, formulated in 0.25 M NaCl/5 mM Tris (pH 8) at 1.8-1.9 mg/mL, sterile-filtered, vialed in 1 mL, frozen and stored at −80 °C (37). DT_{388}IL3 produced potent and selective killing of AML cell lines and patient leukemic progenitors expressing the IL-3 receptor (38-41) and demonstrated in vivo efficacy in mice bearing human leukemia xenografts (42, 43). The safety of DT_{388}IL3 was confirmed in cynomolgus monkeys having cross-reactive IL-3 receptor (44).

In a phase I trial, 45 patients with AML and MDS received 1-6 doses of 4-12.5 µg/kg/day as 15-min i.v. infusions every other day (Monday, Wednesday and Friday) over a 2-week period. One AML patient had a CR (8 months) and two patients had a PR (3 and 4 months). Adverse events included transaminasemia, fever, chills, hypoalbuminemia, hypocalcemia and hypotension. The MTD was > 12.5 µg/kg/dose (45). Because patient tolerance to the 2-week course was poor due to long inpatient stay, the dose schedule was modified to five daily doses over 5 days. The starting dose was 7.07 µg/kg/day. Dose escalation is proceeding.

To improve the efficacy of DT_{388}IL3, a point mutation (Lys to Trp) at the 116-amino-acid residue of the IL-3 moiety in DT_{388}IL3 was introduced, yielding a second-generation DT_{388}IL3, namely DT_{388}IL3[K116W]. DT_{388}IL3[K116W] showed significantly better potency in AML cell lines (46), AML patient blasts (47) and AML progenitors (48) compared to DT_{388}IL3. Clinical-grade DT_{388}IL3[K116W] was prepared in *E. coli*, formulated in 0.25 M NaCl/5 mM Tris (pH 8) at 1.0 mg/mL, sterile-filtered, vialed in 1 mL, frozen and stored at −80 °C. Clinical-grade DT_{388}IL3[K116W] was 12- to 15-fold more potent than clinical-grade DT_{388}IL3 (37, 49). Safety evaluation of DT_{388}IL3[K116W] is needed for IND approval by the FDA in order to test the product in refractory AML and MDS patients.

DT2219ARL is a bispecific DT fusion protein consisting of two sFv ligands recognizing CD19 and CD22, two ARL linkers (the first 20 amino acid residues of human muscle aldolase), an EASPEEA linker, a G_4S linker and the first 389 amino acid residues of DT, for the treatment of B-cell malignancies. DT2219ARL was produced in *E. coli*, formulated in 150 mM NaCl/10 mM sodium phosphate/0.5% polysorbate 80 (pH 7.4) at 1 mg/mL, sterile-filtered, vialed in 1 mL, frozen and stored at −80 °C. DT2219ARL was associated with selective tumor cell-killing activity in Daudi cells and in a mouse xenograft model of Raji Burkitt's lymphoma (22). The MTD of i.v. infusion of DT2219ARL every other day for four doses in New Zealand White rabbits was 200 µg/kg/dose (22). A phase I clinical trial is ongoing.

Transferrin-CRM107 (TransMID) is a chemical conjugate of transferrin and a mutant DT (CRM107) lacking receptor-binding function (50) for the treatment of malignant brain tumors. CRM107 was produced in *Corynebacterium diphtheria* and the conjugate was made with a thioether linkage. Transferrin-CRM107 was highly toxic to malignant tumor cells such as glioblastoma or medulloblastoma, but was less effective against slow-growing and benign tumor cells (51). Transferrin-CRM107 demonstrated in vivo efficacy in a nude mouse model of s.c. human glioma (U-251) (52) and in a nude rat model of intracerebral human glioblastoma biopsy specimens, but not in a nude rat model of intracerebral U-87 MG glioma (53). The MTD of intracerebral and intrathecal infusion of transferrin-CRM107 in guinea pigs and rhesus monkeys was 2 pM (54).

In a phase I trial, 18 patients with recurrent malignant glioma were treated intratumorally with high-flow interstitial microinfusion of transferrin-CRM107 by convection-enhanced delivery (CED), which permits direct drug delivery to the brain tumor interstitium. Of 15 eligible patients, 9 had objective responses (2 CR and 7 PR). The MTD was 40 mL of 0.67 µg/mL and adverse events included cerebral edema and peritumoral focal brain injury, but no symptomatic systemic toxicity was seen (55). Brain injury was related to low levels of transferrin receptor on capillary endothelial cells (56). In a phase II trial, 44 patients received 40 mL of 0.67 µg/mL over 4-5 days by CED. Of 34 patients evaluated for efficacy, 5 had CR and 7 PR (overall response rate: 35.3%). Adverse events included symptomatic cerebral edema (14%) and seizures (6.8%) (57).

$DAB_{389}EGF$ is comprised of the catalytic and translocation domains of DT (DAB_{389}) fused with a His–Ala linker to human epidermal growth factor (EGF). $DAB_{389}EGF$ was initially developed for the treatment of EGFR-positive metastatic malignancies (58). It was produced in *E. coli*, formulated in phosphate-buffered saline (pH 7.2) with 1% mannitol and 0.05 mM EDTA at 0.25 mg/mL, sterile-filtered, vialed in 2 mL, lyophilized and stored at –20 °C (59). Various tumor cells with large numbers of EGF receptors were sensitive to $DAB_{389}EGF$. Sensitivity to $DAB_{389}EGF$ was related to EGF receptor numbers per cell and tumor origin (60). Antitumor efficacy for $DAB_{389}EGF$ was observed in nude mice and rats bearing human lung adenocarcinoma A549 cells. Its MTD in rats and cynomolgus monkeys was 30 and 20 µg/kg/day for 14 days, respectively. DLT was renal and hepatic injury in both species of animals. Serum half-life was < 1 min. [^{35}S]-Methionine-labeled $DAB_{389}EGF$ was rapidly distributed to the liver (61% of injected dose) and kidneys (6% of injected dose) in rats (59).

In three phase I clinical trials, three different dosing schedules were employed to treat EGFR-positive metastatic malignancies: 1) 23 patients received five daily doses at 0.3-15 µg/kg/day (58); 2) 29 patients received five doses on days 1, 8, 9, 15 and 16 at 0.3-15 µg/kg/day; and 3) 20 patients received 6-9 µg/kg/day on days 1, 3 and 5 every 2 or 3 weeks. Liver and renal damage was observed. Adverse effects included asthenia, anorexia, pain, fever, chills, nausea and vomiting. One patient with non-small cell lung carcinoma experienced a partial remission lasting 6 months (59).

Due to the liver and renal toxicity produced by systemic infusion of $DAB_{389}EGF$, interstitial and intravesical drug delivery therapies are being investigated for refractory glioblastoma multiforme and bladder cancer treatment, respectively. Local delivery into the brain or bladder would increase drug exposure to tumors, but minimize exposure of $DAB_{389}EGF$ to liver and kidneys. The incidence of EGFR positivity in bladder cancer (61) and glioblastoma multiforme (62) is 48% and 46%, respectively. Human EGFR-positive glioma cell lines (63) and bladder cancer cell lines were sensitive to $DAB_{389}EGF$ and in vivo efficacy was observed in athymic nude mice bearing human glioblastoma multiforme xenografts (64). These diseases are reasonable targets for local delivery of $DAB_{389}EGF$.

$DT_{388}GMCSF$ is a fusion protein consisting of a truncated DT (DT_{388}) linked to human granulocyte–macrophage colony-stimulating factor (GM-CSF). $DT_{388}GMCSF$ was designed for the treatment of AML because GM-CSF receptors are overexpressed on the majority of myeloid leukemias but are poorly expressed on early normal hematopoietic stem cells. It was produced in *E. coli*, formulated in phosphate-buffered saline (pH 7.4) at 1.5 mg/mL and stored at –80 °C (65). The fusion protein was cytotoxic to chemotherapy-resistant AML cell lines and refractory AML patient progenitors, but was noncytotoxic to normal human myeloid progenitors (66-69). Its antileukemic efficacy was demonstrated in SCID mice bearing human AML xenografts (70). $DT_{388}GMCSF$ was infused i.v. daily for 5 days. The MTD in C57BL/6 mice and cynomolgus monkeys was 84 (71) and 7.5 µg/kg/day (72), respectively. DLT was renal tubular necrosis in mice and neutropenia, anemia and hypoalbuminemia in monkeys.

In a phase I trial, 31 relapsed AML patients were treated at 1 of 6 dose levels (1, 2, 3, 4, 4.5 or 5 µg/kg/day for 5 days). The MTD was 4 µg/kg/day and DLT was liver injury by targeting Kupffer cells. Renal damage was observed. Adverse events included hypoalbuminemia, transaminasemia, fever, hypocalcemia and hypotension. Drug serum half-lives were approximately 30 min in humans and monkeys and 24 min in mice. Of the 31 AML patients, 1 had a complete remission and 2 had partial remissions. These three patients were treated at or above the MTD. Overall response rate was 9.7% (73).

To reduce the liver toxicity of $DT_{388}GMCSF$, its furin cleavage region was changed to a tumor-selective urokinase plasminogen activator (uPA) cleavage sequence, yielding DTU2GMCSF. DTU2GMCSF retained cytotoxicity to AML cell lines with both uPA receptors (uPARs) and GM-CSF receptors, but was less toxic to normal cells expressing only uPAR or GM-CSF receptors (74). A murine equivalent of DTU2GMCSF was not hepatotoxic in rats and was 227- to 682-fold less toxic to Kupffer cells compared to a murine equivalent of $DT_{388}GMCSF$ (75). This dual specificity of DTU2GMCSF may improve safety and efficacy in refractory AML and MDS patients.

CHALLENGES AND PERSPECTIVES FOR DT FUSION PROTEIN THERAPY

As discussed above, three major adverse events (VLS, infusion-related hypersensitivity and liver toxicity) were commonly observed in all DT fusion protein trials. Acute infusion-related hypersensitivity is characterized by fever, chills, nausea, vomiting, myalgias, arthralgias, hypotension, dyspnea, chest pain and back pain, symptoms which occur within 24 h of drug infusion. Acute infusion-related hypersensitivity occurred in up to 80% of patients receiving monoclonal antibody- or other protein-based therapies (76).

Acute hypersensitivity events are common for most protein-based therapies. Liver toxicity may be related to nonspecific uptake of DT fusion proteins by Kupffer cells. Alternatively, targeted toxicity can occur when the liver has a targeted receptor/antigen on the cell surface, as seen in the $DT_{388}GMCSF$ trial. Generally, liver toxicity is mild and reversible. The major DLT of DT fusion protein therapies is VLS, which is characterized by hypotension, peripheral edema and hypoalbuminemia; VLS in severe form causes pulmonary and cardiovascular failure. Symptoms are variable among patients and the causes remain unclear. The pathogenesis of endothelial cell damage is complex and includes activation or damage to endothelial cells and leukocytes, release of cytokines and inflammatory mediators, and change in cell–cell and cell–matrix adhesion. Vascular leak syndrome is observed with IL-2, monoclonal antibodies, some chemotherapeutic drugs such as docetaxel, and many immunotoxins, including DT fusion proteins. A higher incidence of VLS was found in immunotoxin trials compared to other protein-based therapy trials. This may be related to the presence of two potential VLS motifs (VDS sequences located at 6-8 and 289-291 amino acid residues) in the DT moiety of DT fusion proteins; VLS motifs, (x)D(y) where x = L, I, G or V and y = V, L or S, are commonly found in toxins, ribosome-inactivating proteins and IL-2 (77). Vascular leak syndrome restricts the doses of DT fusion proteins and necessitates the cessation of therapy in severe cases. Due to VLS, CHF and cirrhosis are contraindications to treatment with DT fusion proteins; 65% of CHF patients have clinically unrecognized fluid overload (78). In the clinical setting, fluid infusion for adequate hydration and DT fusion

protein treatment could induce fluid overload and VLS, aggravating CHF. It is also difficult to distinguish between VLS and CHF because symptoms overlap. Cirrhosis patients should also be excluded because DT fusion proteins can cause irreversible liver damage and subsequent severe VLS (79).

One way to reduce the incidence of VLS is to use prophylactic steroids. Premedication with steroids improved the tolerability of DAB_{386}IL2 without compromising the clinical response (76). In this trial, the incidence of VLS was significantly reduced from 27% to 13% compared to the phase III studies. In addition to steroids, theophylline, terbutaline, thalidomide and immunoglobulins are used to treat systemic capillary leak syndrome (80-83). Prophylactic use of theophylline, terbutaline and thalidomide may enhance the tolerability of DT fusion proteins. Since interactions of the DT moiety with endothelial cells and/or the cell matrix appear to be a major cause of VLS, integrin-binding motif peptides of fibronectin and vitronectin as antagonists may also reduce the VLS incidence. Importantly, clinical experience with VLS can also minimize the risk of VLS complications.

The immunogenicity of DT fusion proteins limits repeated use in patients. Most individuals are also immunized with diphtheria toxoid as children. However, 67.6% of children and adolescents have anti-DT antibody titers below 2 µg/mL, which does not block the in vivo efficacy of DT_{388}GMCSF (84). Instead, the treatment of DT fusion proteins boosts anti-DT antibody production in most patients. Interestingly, in 7 of 8 patients treated with A-dmDT390-bisFv(UCHT1), anti-drug antibody titers declined below an acceptable level for retreatment within a year (unpublished data). In this trial a patient with an anti-drug antibody titer of 61.1 µg/mL had a durable PR lasting 14+ months. These results are encouraging because patients can be retreated yearly with DT fusion proteins.

There are two ways to overcome the blocking effect of preexisting anti-DT antibodies in human sera. One is to modify the DT structure for deimmunization, and the other is to neutralize the antibodies with nontoxic DT mutants. A deimmunized DT with similar potency was generated by extensive mutagenesis on T-cell epitopes of DT (85). If the deimmunized DT fusion proteins are less immunogenic in animals, time to patient retreatment with deimmunized DT fusion proteins may be shorter as compared to normal DT fusion proteins. The other method is to neutralize existing anti-DT antibodies by infusion of nontoxic DT mutants such as CRM197. This hypothesis was tested in rhesus monkeys using an FN18-CRM9 chemical conjugate (a monkey equivalent of the anti-CD3 DT fusion protein) (86). Infusion of a 100-fold excess of CRM197 (20 mg/kg) prior to FN18-CRM9 (0.2 mg/kg) was performed in two rhesus monkeys with preexisting anti-DT antibodies. In one monkey, this treatment caused a similar T-cell-depleting effect for FN18-CRM9 conjugate as observed in rhesus monkeys, with no anti-DT antibodies. However, the other monkey died due to multiple kidney infarcts. This could be related to either the formation of immune complex precipitates or weak toxicity of CRM197, or both. Although CRM197 is considered a nontoxic DT, further work demonstrated that CRM197 has about 10^{-6} the toxicity of wild-type DT because of weak ADP-ribosylating activity. In addition, a large dose of CRM197 (50 mg/kg) caused death in DT-sensitive animals (87). Therefore, it would be worth testing the safety and neutralizing efficacy of different nontoxic DT mutants in animals.

Cytotoxic therapy with DT fusion proteins is a potential treatment option for chemoresistant relapsed/refractory cancers because DT fusion proteins can selectively kill target malignant cells by a different mechanism than conventional chemotherapy. Four DT fusion proteins are being tested in clinical trials. However, their clinical use is limited by immunogenicity and unwanted toxicity, such as VLS. To overcome these problems, studies on the development of a standard clinical protocol for premedication and antagonists for VLS motif binding, improvement of DT fusion protein potencies, and a new strategy to avoid immunogenicity are needed. The success of these studies would enhance the safety and efficacy of DT fusion proteins in cancer patients.

DISCLOSURES

Jung Hee Woo is a co-inventor of A-dmDT390-bisFv(UCHT1). David Neville is an employee of Angimmune.

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