CHAPTER 29

# Preclinical Safety Evaluation of Immunotoxins

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## 29.1 INTRODUCTION

Chemotherapy represents the most common approach in the treatment of cancer. However, the overall clinical success of chemotherapeutics is often limited by drug resistance and nonselective targeting resulting in dose-limiting toxicities. The preference for highly potent anticancer molecules that specifically target tumor cells while demonstrating minimal toxicity toward normal

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tissue guides the rational design of the next generation of anticancer therapeutics.

Cancer immunotherapy became possible with the identification of tumor-associated antigens as well as the discovery of monoclonal antibodies (MAbs) that have the intrinsic properties of high affinity and specificity for their target antigen [38]. Antibodies exert their biological effector functions (e.g., antibody-dependant cellular toxicity—ADCC—or complement-dependant cellular toxicity—CDC once the antibody has bound to its target antigen. Despite the unique specificity of MAbs, in many cases the clinical benefit has been marginal prompting the search for more potent tumor-selective drugs that possess a different mechanism of action. The coupling of a cytotoxin to a MAb (or Mab fragment) has resulted in the generation of immunotoxins that derive their unique specificity from the antibody and impart a potent cell death signal to the targeted cells. Immunotoxins are emerging as important therapeutic agents for the treatment of a number of carcinomas and haematologic cancers [28]. In addition they are also being investigated for other diseases such as HIV [39,53], graft versus host disease [62], and autoimmune diseases [63].

This chapter will outline the development of immunotoxins and describe the preclinical development required for advancing VB4-845, an anti-EpCAM targeting scFv linked to a truncated form of *Pseudomonas* exotoxin A(252-608), into the clinic.

## 29.2 IMMUNOTOXIN DEVELOPMENT

The potency of an immunotoxin is dependent on the biochemical properties of both the antibody and toxin moieties. Of paramount importance are the characteristics of the target antigen and the antibody affinity for that antigen, rate of internalization into the cell, as well as the efficiency of the intracellular processing and the type of toxin.

### 29.2.1 Target Antigens

The development of a successful immunotoxin is clearly dependent on the choice of the antigen [7]. Prerequisites would be an antigen having a medium to high density of expression and a relatively homogeneous distribution on the tumor cell surface. As most toxins exert their mechanism of action in the cytosol by catalytically disrupting protein synthesis, an internalizing antigen is a necessity. Antigens that are shed from the cell surface, such as the carcino-embryonic antigen (CEA) and the non-Hodgkin's lymphoma (NHL) idiotype, do not represent optimal targets as free antigen would be competing with antigens displayed on cancer cells for the immunotoxin [64]. If shedding antigens are to be targeted, higher doses of drug would be required to remove the competing antigen from the circulation [36,45].

A number of solid tumor targets that are preferentially expressed on cancer cells have been identified such as the epithelial cell adhesion molecule

(EpCAM), HER2/neu (c-erbB-2), EGFR, cytokine receptors, mesothelin, as well as the carbohydrate-associated antigens such as LewisY that are highly expressed in many epithelial tumors [52]. Similarly many differentiation antigens have been identified for hemapoietic malignancies, including CD19, CD22, CD25, CD30, CD33, and CD56 [28].

### 29.2.2 Toxins

Certain plants, fungi, and bacteria produce pathogenic peptide toxins that are able to kill mammalian cells. Examples of these include ricin, gelonin, saporin, bryodin, pokeweed antiviral protein, and bouganin derived from plants, the fungal-derived toxins such as restrictocin and mitogillin, and the bacterially derived toxins diptheria toxin (DT) and *Pseudomonas* exotoxin A (ETA/PE). Although many of these toxins, as well as several other more exotic varieties, have been used for the construction of immunotoxins, the ones most commonly used have been ricin [32,33] from castor bean (*Ricinus communis*), diptheria toxin [23,61] from *Corynebacteriun diphtheria*, and *Pseudomonas* exotoxin A [46,47] from *Pseudomonas aeruginosa*. Toxins such as DT and ETA are extremely potent in killing tumor cells, and they exert their effect in the cytosol by interrupting protein synthesis, resulting in cell death [27].

## 29.3 RATIONAL DESIGN OF IMMUNOTOXINS

In general, first-generation immunotoxins consisted of chemically conjugating toxins to full-length antibodies. However, many of these toxins, such as ricin, naturally contain a cell-binding domain that targets normal tissue and proved unsuccessful in both animal models and in the clinic due to their nonspecific binding to normal tissue. In one case the attachment of ETA to the intact anti-TAC antibody that binds to CD25 on T cells and T cell malignancies resulted in severe liver toxicity in a phase 1 trial [16,46].

The design of immunotoxins has become more sophisticated, and efforts have been made to engineer safer, more efficacious molecules through a better understanding of the biochemistry of the toxins and antibodies as well as the physiological limitations surrounding effective delivery. For example, immunotoxins have been constructed with the cell-binding domain removed [25,55], thereby reducing toxicity and ensuring targeting through the antibody moiety. In addition genetically engineered fusion constructs containing antibody fragments with increased stability have been generated to enhance tumor penetration and to maximize serum half-life.

### 29.3.1 Immunogenicity and Toxicity

Despite the recent advances in immunotoxin design, there remain two major obstacles still to be resolved, namely immunogenicity and toxicity. Although the humanized or fully human antibody moiety of an immunotoxin has limited

recognition by the immune system, toxins are highly immunogenic and rapidly elicit an immune response upon administration to patients. Further many people have been immunized against diptheria and already possess neutralizing antibodies against the toxin. Up to 20% of the general population possesses anti-*Pseudomonas* antibodies as a consequence of *Pseudomonas* infections, and this number can be as high as 80% in long-term hospitals patients (VBI, unpublished data). Similarly patients who have been exposed to castor oil may have developed anti-ricin antibodies. Although considerable success has been achieved in the treatment of patients with leukemias and lymphomas [48] due in part to the immunosuppressive nature of these diseases, this is not the case for immunocompetent patients with solid tumors who may rapidly develop antibodies precluding repeat systemic administration of the immunotoxin [20,26,44].

Another serious side effect of immunotoxin treatment and a consideration in designing a preclinical program for immunotoxins is vascular leak syndrome (VLS). VLS is a nonspecific, non–antigen-related toxicity characterized by fluid leakage from the capillaries into the tissue resulting in low blood pressure and reduced blood flow to internal organs. Major symptoms are low blood pressure, edema, and low levels of albumin. VLS symptoms have been observed in many immunotoxin trials, and although these symptoms are generally manageable, reports of vascular collapse have been reported with certain ricin-based immuntoxins [14]. In order to abrogate vascular damage, several ricin A chain constructs, with mutations in the VLS-associated motif, have been engineered and are currently being evaluated [3,59].

### 29.3.2 VB4-845

VB4-845 is a recombinant fusion protein consisting of a tumor-targeting humanized single-chain antibody fragment, 4D5MOCB, specific for epithelial cell adhesion molecule (EpCAM) linked to a truncated form of *Pseudomonas* exotoxin A (ETA) that lacks the cell-binding domain, ETA(252-608). EpCAM is a cell surface marker that is highly expressed on carcinoma cells of epithelial origin, but has limited expression on normal cells [37,51,65]. Once inside the cell, ETA is a potent inhibitor of protein synthesis that induces cell death [15]. VB4-845 is a single 70 kDa protein, produced in E104 *E. coli* cells and is being developed for intratumoral injection for patients suffering from squamous cell carcinoma of the head and neck (SSCHN).

As with other immunotoxins, immunogenicity and toxicity are two of the major challenges that limit the use of VB4-845 in the clinic. Direct administration of VB4-845 into the tumor offers a number of advantages over systemic delivery. The intratumoral route provides a higher concentration of drug to the tumor than could be achieved by intravenous injection. Moreover, since the interstitial protein concentration is minimal relative to the circulation, in particular for protein in excess of 60 kDa, the concentration of antibodies preexisting or generated over the course treatment would be low relative to

the administered dose, thereby minimizing the neutralizing effects of an anti-immunotoxin response. As well, since immunotoxin uptake by the tumor should be maximized due to intratumoral injection, this route of delivery is predicted to limit the interaction of the immunotoxin with nontarget tissue and thus minimize the likelihood of dose-limiting toxicity.

### 29.3.3 Mechanism of Action

It has been wellestablished that ETA irreversibly inhibits protein synthesis in mammalian cells by adenosine di-phosphate (ADP)-ribosylation of elongation factor 2 [29,49]. To demonstrate that the activity of VB4-845 is consistent with that of ETA by the inhibition of protein synthesis, the uptake of [3H]leucine was measured in EpCAM positive and EpCAM negative tumor cell lines, following the addition of VB4-845 to the cell cultures. VB4-845 inhibited protein synthesis in EpCAM positive SW2 cells with an $IC_{50}$ of 0.01 pM but not in the EpCAM negative control cell line RL over the range of concentrations tested (0.0001–100 pM) [12]. The activity of VB4-845 was shown to be due to the inhibition of protein synthesis and is consistent with the mechanism of action of ETA (Figure 29.1). Results also indicate that EpCAM is required on the cell surface for treatment with VB4-845 to result in pharmacological activity (VBI, unpublished data).

To examine the requirements to take an immunotoxin into the clinic, the preclinical developmental strategy of VB4-845 (manuscript in preparation) is described to examine the challenges encountered to progress this antibody from bench to clinic by following the ICH S6 guidelines.

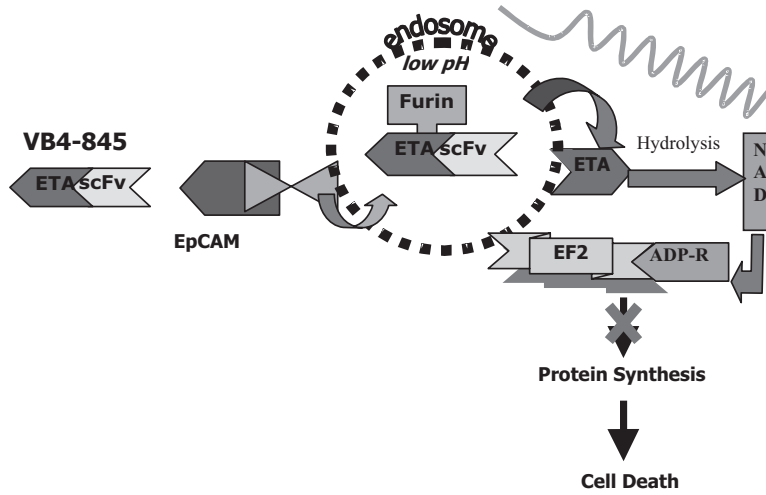


**Figure 29.1** Mechanism of action of VB4-845.

## 29.4 IMMUNOTOXIN EVALUATION USING THE ICH S6 GUIDELINES

Each immunotoxin is evaluated in a series of stages. These include assessment of specificity, cytotoxicity, and serum stability; selection of a relevant animal species and route of drug administration; and finally safety evaluation.

### 29.4.1 Specificity, Cytotoxicity, and Serum Stability

Initially immunotoxins are tested in vitro for specificity, cytotoxicity, and serum stability. The specificity of VB4-845 for EpCAM expressing tumor cells was demonstrated by flow cytometry and cell growth analyses of various epithelial-derived tumor and normal cell lines originating from different tissue types. VB4-845 showed strong cell surface reactivity to EpCAM positive tumor cell lines such as Cal 27, a squamous cell carcinoma of the tongue, but not to the colon-derived, EpCAM negative cell line, Colo-320. The specificity of VB4-845 was further highlighted by assessing cytotoxicity against a panel of cell lines exhibiting varying degrees of EpCAM expression. As predicted by the binding data, all EpCAM positive tumor cell lines were sensitive to killing by VB4-845, with $IC_{50}$ values ranging from 0.001 to 1.84 pM. Normal cell lines, on the other hand, either were not affected by VB4-845 ($IC_{50} > 500$ pM) or were anywhere between 50- to 1000-fold less sensitive to VB4-845 than their counterpart tumor cell lines. For example, VB4-845 displayed potent activity against the bladder tumor cell line, TCCSUP ($IC_{50} < 0.005$ pM) but displayed no activity against the normal bladder cell line, HMVEC-bd ($IC_{50} > 500$ pM). This differentiation was also demonstrated in EpCAM positive and negative efficacy models [12].

Because immunotoxins are specifically targeted therapies, toxicities due to binding to receptors/antigens present on normal tissues occur at a much lower frequency than toxicities experienced with other anticancer drugs such as chemotherapy. According to FDA specifications [13], all antibodies are required to be tested for immunoreactivity against a human tissue panel of 33 normal frozen human tissues using immunohistochemistry (IHC). The in vitro immunoreactivity binding pattern of VB4-845 was membrane-associated in epithelial tissues, as is consistent with the detection of EpCAM expression by other antibodies [4]. Although normal epithelial cells express EpCAM, it is generally restricted to the basolateral portion of the cell [42], and therefore minimal in vivo binding is expected by VB4-845. To further ensure minimal patient toxicity, VB4-845 will be directly administered into SCCHN tumors. It should be noted that although tumor-reactive antibodies preferentially target cancer cells, they often cross-react with certain normal tissues that have limited expression of the target antigen, giving rise to significant toxicities. This does not preclude the use of such molecules as therapeutics. However, it underscores the requirement for testing the immunotoxin in a suitable animal model to determine its degree of interaction with critical normal tissues in the body.

Serum stability is another important attribute of immunotoxins as each immunotoxin must be sufficiently stable to progress from the blood into the tissues and be capable of penetrating into a tumor in a sufficient concentration to result in tumor cell death [8,12]. Examination at 37 °C showed that VB4-845 was a stable immunotoxin [12].

Although extensive in vitro testing is performed on each immunotoxin to assess its cytotoxicity and specificity, it is animal model studies that ultimately determine the serum half-life, serum stability, as well as the efficacy of an immunotoxin. A compilation of the in vitro and in vivo data enables a decision to be made on the potential of each immunotoxin to be an effective and safe therapeutic and to determine whether human clinical trials are warranted.

### 29.4.2 Selection of Animal Model and Route of Administration

As part of evaluating a new biopharmaceutical, the drug should be administered in a relevant animal species in which it is pharmacologically active. For an immunotoxin the drug should bind to the intended receptor or epitope expressed in the animal model. However, this result may not always be possible. In the case of VB4-845, cross-reactivity using IHC was examined in several animal species commonly used for toxicology studies: mouse, rat, dog, cynomolgus and rhesus monkeys, as well as the chimpanzee. No cross-reactivity was observed in any tissues of animals normally used for toxicology studies. Some cross-reactivity was observed in chimpanzee tissue, but because the pattern was not the same as that observed in human tissues, it was not considered to be a relevant species.

The use of nonrelevant animal species is discouraged as the results of such studies may be misleading (per ICH S6). Therefore the next option, when available, is to substitute a relevant transgenic animal model for a pharmacologically relevant species. Transgenic animal models for human EpCAM have been developed, but they either have a different tissue expression pattern from that seen in humans [41,43] or the model has not been validated [40], making them unsuitable for the evaluation of the safety of anti-EpCAM immunotherapeutics.

While safety evaluation programs require studies to be conducted in two species to characterize drug toxicity, should no viable option exist for a relevant species to conduct toxicology studies, the FDA suggests that toxicity be assessed in a single species [13]. Although no animal model system was available to examine possible binding of VB4-845 to EpCAM receptors, numerous studies have examined ETA-conjugated immunotoxins. It is well documented that the intravenous administration of ETA immunotoxins to rats results in symptoms that resemble VLS as seen in human immunotoxin trials [20,57]. Thus the choice of the Sprague–Dawley rat for toxicological testing was made for the well-known effects of immunotoxins in this animal model.

The route and treatment regimen in toxicology studies are expected to be as close as possible to that in the clinic. Single-dose studies aid in selecting the

route of administration for a toxicology study if the intended human route of administration can not be mimicked in the animal. For VB4-845, the chosen route of administration in the clinic was intratumoral. While intratumoral administration was successfully demonstrated in a preclinical efficacy mouse model [12], this mode is not a viable option in toxicology studies. Therefore intradermal drug administration was used as a surrogate route of administration. Intravenous dosing, although not intended in the clinic, was used in the toxicology study with the rat as a comparison to examine the possible "worst case" effect of systemic exposure.

### 29.4.3 Safety Assessment

***Single-Dose Toxicology Studies*** Drug dosage in an animal model should be conducted so that a dose–response relationship may be examined. This may range from a no observed effect level (NOEL) and no observed adverse effect level (NOAEL) all the way to a maximum tolerated dose (MTD) of the drug. The dose response in a single-dose study will assist to determine the dose levels to be selected for a repeated-dose toxicology study. These levels will also aid in determining the first-dose level of the drug in humans as well as the therapeutic index and the margin of safety when dosing humans. When considering dosage in test animals, the volume must also be considered. Ethical maximum volumes have been determined for different species, which may mean that if there is a limitation to the drug concentration, a maximum feasible dose will be determined instead of an MTD.

Clinical signs noted in the dose-ranging study conducted in Sprague–Dawley rats administered VB4-845 locally (intradermal, ID) and systemically (intravenous, IV) were related to injection site lesions that exhibited a dose-dependent effect. There were no other findings in animals locally dosed. Animals that were systemically dosed had an increase in red blood cell parameters, total red blood cell counts, hemoglobin, and hematocrit and a decrease in albumin, total protein, and albumin–globulin ratio. While all these findings were dose dependent, these variations were within the normal physiological range.

Single-dose studies also help to determine whether dosing modifications are required based on the bioavailability or pharmacokinetics of the drug in the test species. Route of administration may also be modified if the drug has limited bioavailability by the chosen clinical route, or else treatment regimen may be changed to compensate for high clearance or low drug exposure.

***Repeated-Dose Toxicology Studies*** The repeated-dose toxicity study is expected to be a toxicology study under GLP conditions [17], which examines clinical signs, hematology, clinical biochemistry, urinalysis, and bone marrow to evaluate the effect of the drug during and after administration. Additional animal groups are required so that drug effect can be examined immediately after dosing in one group of animals and then later in another group of animals

to determine whether any observed effects are reversible or in some cases whether the drug effects are delayed. Safety pharmacology, toxicokinetics, and immunogenicity may also be examined in this study for immunotoxins.

Based on the injection site reactions in the single-dose studies, it was anticipated that there would be difficulties dosing the Sprague–Dawley rat via the ID route. Therefore a subcutaneous (SC) route of administration was used as another representative route of local administration. Injection site reactions (slight erythema, edema, superficial necrosis, ulcerations, and scab formation) attributable to VB4-845 that were dose-related and noted at or above 5.0 μg/kg in most cases had resolved by the end of the observation period. Dose-dependent but transient changes were noted in hematology, coagulation parameters, and serum chemistry and were most likely due to acute tissue injury and inflammation at the injection sites. Liver enzyme levels were elevated in rats at upper-dose levels but returned to the normal physiological range by the end of the observation period. Systemic administration of VB4-845 (77.8 μg/kg) in rats resulted in microvascular injury and pulmonary edema, with subsequent hypoxia; these findings were consistent with VLS previously observed in rats exposed to ETA-based immunotoxins [22,56].

Examination of the various parameters may identify markers that vary with dose and may be used to examine drug effects in human studies. For example, although few effects were attributable to the local administration of VB4-845 in Sprague–Dawley rats, liver enzyme levels were elevated in rats at upper-dose levels. This provides a marker to follow drug response in clinical patients. As there was no evidence of toxicity following local administration at any of the dose levels tested, no NOAEL was attained at the highest dose tested (77.8 μg/kg), and it can be assumed that higher doses may be possible. In contrast, the animals treated systemically with VB4-845 experienced lethal toxicity at the same dose (77.8 μg/kg), thus illustrating a lower NOAEL for this route of delivery.

While animal studies are used for safety assessment prior to administration to humans, preclinical testing may not always predict human effect. For example, continuous-infusion therapy with 260F9 monoclonal antibody-recombinant ricin A chain resulted in severe neurotoxic effects in humans that were not demonstrated in monkey toxicology studies [21]. In such cases where suitable animal models are not available for safety testing it is important to consider the application of an appropriate safety factor to provide a margin of safety for protection of humans receiving the initial clinical dose [11,18].

***Immunogenicity*** Immunogenicity is a significant complicating factor surrounding the administration of immunotoxins to humans [19]. Immunogenicity can arise from either the antibody or toxin portions if they are foreign proteins. The shift from murine to humanized or human antibodies has reduced the immune response due to the antibody portion [10,35]. However, immune responses are still expected because the toxins employed are either of bacterial or plant origin and are thus inherently highly immunogenic.

The immune responses to VB4-845 by both the intended local route and systemic route of administration were investigated. Not surprisingly, a dose-dependent anti-drug antibody response was observed to both the antibody and toxin portions of the construct and a similar level antibody titer was induced regardless of sex. The VB4-845 titer following local administration was significantly higher than that observed after systemic administration at the same dose level, a phenomenon that has been demonstrated to be due to the use of different routes of delivery [50].

Although relatively high serum antibody titers were produced against VB4-845, this is not expected to have a negative impact on drug administration. Local administration of drug is expected to ensure a high local drug concentration in the tumor before coming into contact with anti-VB4-845 antibodies in the circulation. This point was illustrated with scFv(FRP5)-ETA, where a patient had a complete clinical response to intratumoral treatment (second administration) even though an immune-response was generated that completely neutralized the immunotoxin [2].

***Drug Exposure Evaluation*** Immunotoxin exposure is largely dependent on the antibody portion used for targeting [10]. While whole IgGs may have half-lives up to 36 hours [5], scFv single-chain fragments can have a half-life as short as an hour or less. Although larger antibody portions are more stable and may prolong drug exposure, a larger sized molecule has more difficulty in tumor penetration [1]. A smaller sized antibody portion may not have as long a half-life, but it may permit better access to tumor cells and is able to leave the circulatory system more quickly, thereby reducing the exposure time of the endothelia to the toxin, and perhaps decrease VLS toxicity. $F_{ab}$ antibody fragments or pegylation of the molecule increases the protein size, making it more stable but still small enough for cell entry.

As no relevant species exists, biodistribution studies were conducted in xenograft mice bearing EpCAM positive and negative tumors. This study confirmed that VB4-845 was retained in EpCAM positive tumors [12]. Although there was some detection in EpCAM negative tumors, this was most likely due to increased tumor vascularization. The biodistribution study also indicated that other organs may be targeted. However, toxicology studies did not result in any toxicity findings, indicating that the accumulation of radioactivity did not reflect binding or internalization of the immunotoxin within these tissues [12].

Toxicokinetic (TK) analysis of VB4-845 indicated that there was no gender difference. Nevertheless, different profiles were generated based on the mode of administration. Local administration resulted in a maximum VB4-845 plasma concentration of 50 ng/ml 4 h after administration (Figure 29.2). The subcutaneous dose appeared to be incompletely absorbed into the circulation, resulting in low bioavailability (13%) upon sampling (Table 29.1). Systemic administration resulted in a maximum VB4-845 plasma concentration of 1000 ng/ml after 10 minutes. The disappearance profile following the systemic

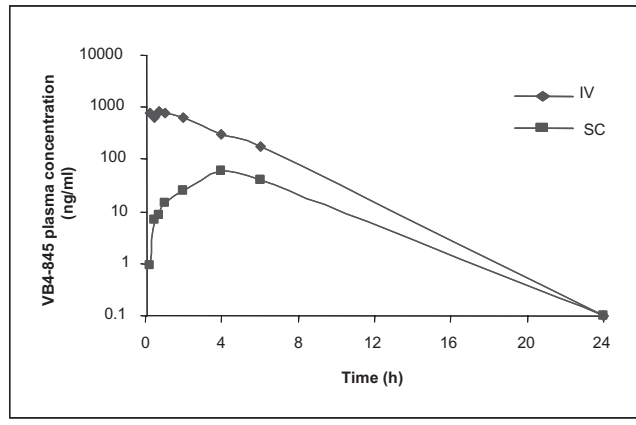


**Figure 29.2** Mean plasma concentration-time curves following either systemic (intravenous) or local (subcutaneous) administration of VB4-845 on day 1. VB4-845 was not detected at 24 hours in either mode of drug administration.

**TABLE 29.1 Toxicokinetic profile of VB4-845 administered SC or IV in Sprague–Dawley rats**

| Route | $C_{max}$ (ng/ml) | $AUC_{0\text{-inf}}$ (ng·h/ml) | k ($h^{-1}$) | Ka ($h^{-1}$) | $t_{1/2}$ (h) | CL (ml/kg/h) | $V_d$ (ml/kg) | %F |
|---|---|---|---|---|---|---|---|---|
| SC | 50 at 4 h | 421 | NC | 0.31 | NC | NC | NC | 13 |
| IV | 1000 at 10 min | 3242 | 0.3 | NC | 2.3 | 24 | 80 | NC |

Abbreviations: $AUC_{0\text{-inf}}$ = area under the curve, CL = clearance, $C_{max}$ = maximum plasma concentration, %F = bioavailability, k or Ka = elimination constant, NC = not calculated, $t_{1/2}$ = half-life, $V_d$ = volume of distribution. The pooled data set from male and female rats was used for calculations.

dose was consistent with a one-compartment distributional model and first-order elimination with a half-life of 2.3 hours.

Comparison of plasma concentrations between days 1 and 7 showed no difference with local injections. However, concentrations following systemic injections were much lower on day 7 as compared to day 1. A dramatic enlargement of the distribution space, or possibly an unspecified bioadaptation, is suggested or more likely, as the observed immune response depleted 4
the blood plasma concentration. As mentioned earlier, an immune response raised against immunotoxins can affect the TK profile of a drug. The antibodies raised against the response bind to the antibody portion of the immunotoxin, causing it to be cleared at a quicker rate. This can decrease the half-life of the drug and, in most cases, is the stopping point for re-administration of the drug. Low bioavailability after local administration will not be an issue as VB4-845 is directly targeted to the tumor but rather indicates that there will be low 5
systemic exposure to the immunotoxin.

Toxic effects of metabolites from drug degradation do not need to be monitored for immunotoxins (per IHC S6). As a recombinant protein, immunotoxins entering the human body are quickly degraded to small peptides and amino acids in the blood by proteases that specifically target foreign proteins and are cleared by the kidney.

***Safety Pharmacology*** Safety pharmacology is important to assess the drug's effect on physiological functions through in vitro and in vivo assessment of central nervous, cardiovascular, and respiratory systems. These studies are required for biotechnology-derived biopharmaceuticals and assist to establish the type of monitoring parameters that may be required in clinical studies. However, because immunotoxins are specifically targeted drugs, these studies do not need to be conducted as individual studies but may be combined with toxicology studies that evaluate safety pharmacology endpoints.

Clinical signs observed in VB4-845 toxicology studies allowed for an in vivo assessment of the central nervous system and functional ability of the dosed rats. Animals dosed locally exhibited normal behavior, whereas those dosed systemically exhibited neurological symptoms (wobbly gait) and respiratory difficulties (dyspnea). Full safety pharmacology studies have not been conducted with VB4-845 because they are not required prior to the first administration in humans for anticancer drugs examined in end-stage cancer patients (per ICH S7A). VB4-845 has progressed to pivotal clinical trials, and therefore safety pharmacology studies examining cardiovascular and respiratory response are planned.

***Immunotoxicity Studies*** Immunotoxicology studies are required for pharmaceuticals that may affect the immune system through suppression, enhancement, or sensitivity (per ICH S8). In general, immunotoxins do not have an effect on the immune system, and standard testing batteries are not recommended for biotechnology-derived pharmaceuticals. However, examination of hematology results together with detailed histopathological assessment of immune organs from single- or repeated-dose toxicology studies are routinely performed with biopharmaceuticals, and these evaluations provide initial information on whether the immune system has been affected.

***Reproductive Performance and Developmental Toxicology Studies*** The assessment of reproductive performance and developmental toxicity is dependent on the clinical indication and patient population. The majority of immunotoxins in development are currently used for the treatment of cancer, a patient population that tends to be an older, nonreproductive population. As well, potential reproductive concerns for the patients in ongoing clinical trials are usually addressed in the clinical trial protocols.

Although VB4-845 will never be administered systemically and does not bind to human placenta, studies are planned as a step for marketing registration. Because a relevant species has not been identified, and the rat is unsuit-

able for use because it cannot be dosed systemically, embryofetal development will be examined in time-mated female rabbits to determine placenta binding and potential for transplacental passage. A formal study will not be conducted unless fetal effects are observed.

***Genotoxicity Studies*** While the majority of the principles of the ICH S6 apply to immunotoxins, because they are a biotechnology-derived biopharmaceutical, certain tests such as genotoxicity and carcinogenicity studies do not directly apply.

Examination of genotoxicity of pharmaceuticals is required to assess the interaction of the drug with DNA. These studies are generally not applicable to immunotoxins. Unlike chemotherapeutics that cause cell death through DNA interaction, immunotoxins mediate cell death by preventing protein synthesis. However, immunotoxins use a linker to connect the toxin to the antibody that may need to be examined if it is an organic linker and has the ability to bind DNA (per ICH S6). The majority of immunotoxins use either a nonreducible thioether linker for intact toxins or a disulfide bond for A chains and ribosome-inactivating proteins and do not interact with DNA.

***Carcinogenicity Studies*** Immunotoxins, in general, do not have the ability to transform cells or promote the growth of transformed cells. Therefore carcinogenicity bioassays are considered inappropriate. This is in contrast to toxins fused to growth factors that may promote tumor growth under certain circumstances if, for example, a less than toxic dose were administered. Should the immunotoxin interact with growth factors or cytokines, in vitro studies may be required to examine whether growth is promoted in transformed cells.

## 29.5 PROGRESS OF IMMUNOTOXIN THERAPEUTICS

Cancers of hematologic origin are more accessible to immunotoxin therapy and thus more amenable to treatment than solid tumors. One of the most promising immunotoxins, currently in development is BL22 used for the treatment of hairy cell leukaemia. BL22 is comprised of an anti-CD22 dsFv linked to truncated PE, and it has been evaluated in a phase 1 clinical trial of patients with B cell malignancies [31] and ongoing in phase 2 clinical trials [30]. Of the 32 patients treated in the phase 1 study, 16 hairy cell leukaemia patients responded with 11 patients having a complete remission and 2 having a partial remission. Neutralizing antibodies were only observed in 4 of the 16 patients. Although VLS was observed in some patients, the dose-limiting toxicity was the cytokine release syndrome. A variety of immuntoxins have undergone clinical testing, and the testing has been comprehensively reviewed by Kreitman [28] and Schaede and Reiter [54].

Currently two immunoconjugates have been approved by the FDA for clinical use in the treatment of cancer. Mylotarg® (Gemtuzumab ozogamicin,

Wyeth-Ayerst Laboratories) is a CD33 antibody conjugated to a calicheamicin, a cytotoxic antibiotic and Ontak® (denileukin diftitox, Ligand Pharmaceuticals, Inc.) is an IL-2 cytokine conjugated to the toxin DT. Both drugs target hematological cancers. While Mylotarg® is not conjugated to a toxin, it provides a good example of the progress of an antibody immunoconjugate from preclinical development to regulatory approval for market.

The targeted antigen, CD33, of Mylotarg® is not expressed in any other species besides humans and large primates; therefore a cross-reactive species was not available for Mylotarg® [9]. Instead, repeated-dose toxicology studies were conducted in Sprague–Dawley rats and cynomolgus monkeys that indicated hepato, renal, and hematopoietic toxicities due to drug administration. Mylotarg® was more immunogenic in rats than monkeys and had a slow clearance rate in both species, three and seven days, respectively. Safety pharmacology conducted in dogs resulted in minor changes in blood pressure and cardiac output that were noted at 16-fold above human starting dose with changes in ECG and heart rate noted at 52-fold above human dose. Reproductive toxicology was not conducted; however, histopathological changes were noted in rat testes and atrophy in the mammary gland. Developmental toxicology studies conducted in rats showed dose-related development effects with decreases in fetal weights, increases in embryo/fetal mortality, and fetal digital malformations, with reabsorptions at the highest dose. The cytotoxic antibiotic used in Mylotarg®, calicheamicin, kills cells by interacting with DNA and causing breakage. Therefore genotoxicity studies were conducted using an in vivo mouse micronucleus assay and confirmed that it was clastogenic. Mylotarg® was determined to have an acceptable toxicity profile in clinical trials and few patients developed antibodies to the drug. Infusion reactions were observed that sometimes occur with monoclonal antibody infusions. Preclinical studies predicted the liver toxicity observed in patients, which for the most part was transient and reversible [9]. The preclinical studies together with the response rate in clinical trials were satisfactory for approval of Mylotarg® for CD33 positive acute myeloid leukemia patients in first relapse who are 60 years of age or older. Since then, additional studies indicate further use of Mylotarg® with combination therapy [58] as well as other population groups [6].

## 29.6 SUMMARY

Immunotoxins continue to be actively investigated as viable alternatives to conventional therapies for a variety of diseases. An array of different recombinant, antibody formats are now available for use in immunotoxins. While these design changes have improved the overall in vitro and preclinical in vivo efficacy of immunotoxins, increased potency does not address either of the two major concerns for drugs of this type: immunogenicity and toxicity. As such, immunotoxins in their current form may have limited application other than to those disease conditions either where the patients are immunocompro-

mised, as in the case for leukemia, or where the drug can be delivered directly, as was demonstrated for VB4-845 in the treatment of SCCHN and transitional cell carcinoma of the bladder. In order to expand the utility of immunotoxins to achieve a comparable safety profile, design considerations will be required to minimize immunogenicity and toxicity. To this end, pegylation [34,60] or T and/or B cell epitope depletion [24] from the toxin portion of the immunotoxin may be an alternative means to minimize an immune response while the discovery of newer toxins with better safety profiles may minimize nonspecific toxicities. In addition increasing the safety profile of immunotoxins through the discovery of new and more selective tumor targets will only serve to broaden their clinical use in the treatment against cancer.

## REFERENCES


1. Adams GP, Schier R. Generating improved single-chain Fv molecules for tumor targeting. *J Immunol Meth* 1999;231:249–60.
2. Azemar M, Djahansouzi S. Regression of cutaneous tumor lesions in patients intratumorally injected with a recombinant single-chain antibody-toxin targeted to ErbB2/HER2. *Breast Cancer Res Treat* 2003;82:155–64.
3. Baluna R, Coleman E. The effect of a monoclonal antibody coupled to ricin a chain-derived peptides on endothelial cells in vitro: insights into toxin-mediated vascular damage. *Exp Cell Res* 2000;258:417–24.
4. Balzar M, Winter MJ, de Boer CJ, Litvinov SV. The biology of the 17-1A antigen (Ep-CAM). *J Mol Med* 1999;77:699–712.
5. Behr T, Becker W, Hannappel E, Goldenberg DM, Wolf F. Targeting of liver metastases of colorectal cancer with IgG, F(ab′)2, and Fab′ anti-carcinoembryonic antigen antibodies labeled with 99mTc: the role of metabolism and kinetics. *Cancer Res* 1995;55:5777s–85s.
6. Brethon B, Auvrignon A, Galambrun C, Yakouben K, Leblanc T, Bertrand Y, Leverger G, Baruchel A. Efficacy and tolerability of gemtuzumab ozogamicin (anti-CD33 monoclonal antibody, CMA-676, Mylotarg) in children with relapsed/refractory myeloid leukemia. *BMC Cancer* 2006;6:172.
7. Brinkmann U. Recombinant immunotoxins: protein engineering for cancer therapy. *Mol Med Today* 1996;439–46.
8. Brinkmann U. Recombinant antibody fragments and immunotoxin fusions for cancer therapy. *In vivo* 2000;14:21–8.
9. Bross PF, Beitz J, Chen G. Approval summary: gemtuzumab ozogamicin in relapsed acute myeloid leukemia. *Clin Cancer Res* 2001;7:1490–6.
10. Colcher D, Goel A, Pavlinkova G, Beresford GW, Booth B, Batra SK. Effects of genetic engineering on the pharmacokinetics of antibodies. *Quart J of Nucl Med* 1999;43:132–9.
11. DeGeorge JJ, Ahn CH, Andrews PA, Brower ME, Giorgio DW, Goheer MA, Lee-Ham DY, McGuinn WD, Schmidt W, Sun CJ, Tripathi SC. Regulatory considerations for preclinical development of anticancer drugs. *Cancer Chemother Pharmacol* 1998;41:173–85.

12. Di Paolo C, Willuda J, Kubetzko S, Lauffer I, Tschudi D, Waibel R, Pluckthun A, Stahel RA, Zangemeister-Wittke U. A recombinant immunotoxin derived from a humanized epithelial cell adhesion molecule-specific single-chain antibody fragment has potent and selective antitumor activity. *Clin Cancer Res* 2003;9:2837–48.
13. FDA. Points to consider in the manufacture and testing of monoclonal antibody products for human use. *J-Immunother* 1997;20:214–43.
14. Fidias P, Grossbard ML, Lynch T. A phase II study of the immunotoxin N901-blocked ricin in small-cell lung cancer. *Clin Lung Cancer* 2002;3:219–22.
15. FitzGerald D, Pastan I. Pseudomonas exotoxin: recombinant conjugates as therapeutic agents. *Biochem Soc Transact* 1992;20:731–4.
16. FitzGerald DJ, Waldmann TA, Willingham MC, Pastan I. Pseudomonas exotoxin-anti-TAC. cell-specific immunotoxin active against cells expressing the human T cell growth factor receptor. *J Clin Invest* 1984;74:966–71.
17. Food and Drug Administration. Good Laboratory Practice for Nonclinical Laboratory Studes. 21 Code Federal Regulation 1996;58.
18. Food and Drug Administration. Estimating the Safe Starting Dose in Clinical Trial for Therapeutics in Adult Healthy Volunteers, 2002.
19. Frankel AE. Reducing the immune response to immunotoxin. *Clin Cancer Res* 2004;10:13–15.
20. Frankel AE, Kreitman RJ, Sausville EA. Targeted toxins. *Clin Cancer Res* 2000; 6:334.
21. Gould BJ, Borowitz MJ, Groves ES, Carter PW, Anthony D, Weiner LM, Frankel AE. Phase I study of an anti-breast cancer immunotoxin by continuous infusion: report of a targeted toxic effect not predicted by animal studies. *J Natl Cancer Inst* 1989;81:775–81.
22. Haggerty HG, Warner WA. BR96 sFv-PE40 Immunotoxin: Nonclinical safety assessment. *Toxicol Pathol* 1999;27:87–94.
23. Herschman HR, Simpson DL, Cawley DB. Toxic ligand conjugates as tools in the study of receptor-ligand interactions. *J Cell Biochem* 1982;20:163–76.
24. Jones TD, Phillips WJ, Smith BJ, Bamford CA, Nayee PD, Baglin TP, Gaston JS, Baker MP. Identification and removal of a promiscuous CD4+ T cell epitope from the C1 domain of factor VIII. *J Thromb Haemost* 2005;3:991–1000.
25. Kondo K, FitzGerald DJ, Chaudhary V. Activity of immunotoxins constructed with modified pseudomonas exotoxin a lacking the cell recognition domain. *J Biol Chem* 1988;263:9470–5.
26. Kreitman RJ. Immunotoxins in cancer therapy. *Curr Opin Immunol* 1999; 11:570–8.
27. Kreitman RJ. Recombinant toxins for the treatment of cancer. *Curr Opin Mol Ther* 2003;5:44–51.
28. Kreitman RJ. Immunotoxins for targeted cancer therapy. *AAPS J* 2006; 8:E532–51.
29. Kreitman RJ, Pastan I. Accumulation of a recombinant immunotoxin in a tumor in vivo: fewer than 1000 molecules per cell are sufficient for complete responses. *Cancer Res* 1998;58:968–75.
30. Kreitman RJ, Pastan I. BL22 and lymphoid malignancies. *Best Pract Res Clin Haematol* 2006;19:685–99.

31. Kreitman RJ, Wilson WH, Bergeron K, Raggio M, Stetler-Stevenson M, FitzGerald DJ, Pastan I. Efficacy of the anti-CD22 recombinant immunotoxin BL22 in chemotherapy-resistant hairy-cell leukemia. *N Engl J Med* 2001;345:241–7.
32. Krolick KA, Uhr JW, Slavin S, Vitetta ES. In vivo therapy of a murine B cell tumor (BCL1) using antibody-ricin A chain immunotoxins. *J Exp Med* 1982;155: 1797–809.
33. Krolick KA, Villemez C, Isakson P, Uhr JW, Vitetta ES. Selective killing of normal or neoplastic B cells by antibodies coupled to the A chain of ricin. *Proc Natl Acad Sci USA* 1980;77:5419–23.
34. Kubetzko S, Balic E, Waibel R, Zangemeister-Wittke U, Pluckthun A. PEGylation and multimerization of the anti-p185HER-2 single chain Fv fragment 4D5: effects on tumor targeting. *J Biol Chem* 2006;281:35186–201.
35. Kuus-Reichel K, Grauer L. Will immunogenicity limit the use, efficacy and future development of therapeutic monoclonal antibodies. *Clin Diagnost Lab Immunol* 1994;365–72.
36. Levy R, Miller RA. Therapy of lymphoma directed at idiotypes. *J Natl Cancer Inst Monogr* 1990;61–8.
37. Litvinov SV, Velders MP, Bakker HA, Fleuren GJ, Warnaar SO. Ep-CAM: a human epithelial antigen is a homophilic cell-cell adhesion molecule. *J Cell Biol* 1994; 125:437–46.
38. Maynard J, Georgiou G. Antibody Engineering. *Annu Rev Biomed Eng* 2000; 2:339–76.
39. McHugh L, Hu S, Lee BK, Santora K, Kennedy PE, Berger EA, Pastan I, Hamer DH. Increased affinity and stability of an anti-HIV-1 envelope immunotoxin by structure-based mutagenesis. *J Biol Chem* 2002;277:34383–90.
40. McLaughlin PM, Harmsen MC, Dokter WH, Kroesen BJ, van der MH, Brinker MG, Hollema H, Ruiters MH, Buys CH, de Leij LF. The epithelial glycoprotein 2 (EGP-2) promoter-driven epithelial-specific expression of EGP-2 in transgenic mice: a new model to study carcinoma-directed immunotherapy. *Cancer Res* 2001; 61:4105–11.
41. McLaughlin PM, Kroesen BJ, Dokter WH, van der MH, de Groot M, Brinker MG, Kok K, Ruiters MH, Buys CH, De Leij LF. An EGP-2/Ep-CAM-expressing transgenic rat model to evaluate antibody-mediated immunotherapy. *Cancer Immunol Immunother* 1999;48:303–11.
42. Momburg F, Moldenhauer G. Immunohistochemical study of the expression of a Mr 34,000 human epithelium-specific surface glycoprotein in normal and malignant tissues. *Cancer Res* 1987;47:2883–91.
43. Mosolits S, Campbell F. Targeting human Ep-Cam in transgenic mice by anti-idiotype and antigen based vaccines. *Int J Cancer* 2004;112:669–77.
44. Pai LH, Wittes R, Setser A, Willingham MC, Pastan I. Treatment of advanced solid tumors with immunotoxin LMB-1: An antibody linked to Pseudomonas exotoxin. *Nat Med* 1996;2:350–3.
45. Parker BA, Vassos AB, Halpern SE, Miller RA, Hupf H, Amox DG, Simoni JL, Starr RJ, Green MR, Royston I. Radioimmunotherapy of human B-cell lymphoma with 90Y-conjugated antiidiotype monoclonal antibody. *Cancer Res* 1990;50: 1022s–8s.

46. Pastan I. Immunotoxins containing pseudomonas exotoxin A: a short history. *Cancer Immunol Immunother* 2003;52:338–41.

47. Pastan I, FitzGerald D. Pseudomonas exotoxin: chimeric toxins. *J Biol Chem* 1989; 264:15157–60.

7 48. Pastan I, Hassan R, FitzGerald DJ, Kreitman RJ. Immunotoxin Treatment of Cancer. *Annu Rev Med* 2006.

49. Perentesis JP, Miller SP, Bodley JW. Protein toxin inhibitors of protein synthesis. *Biofactors* 1992;3:173–84.

50. Porter S. Human immune response to recombinant human proteins. *J Pharm Sci* 2001;90:1–11.

51. Proca DM, Niemann TH, Porcell AI, DeYoung BR. MOC31 immunoreactivity in primary and metastatic carcinoma of the liver: report of findings and review of other utilized markers. *Appl Immunohistochem Mol Morphol* 2000;8:120–5.

52. Reiter Y. Recombinant immunotoxins in targeted cancer cell therapy. *Adv Cancer Res* 2001;81:93–124.

53. Saavedra-Lozano J, Cao Y, Callison J, Sarode R, Sodora D, Edgar J, Hatfield J, Picker L, Peterson D, Ramilo O, Vitetta ES. An anti-CD45RO immunotoxin kills HIV-latently infected cells from individuals on HAART with little effect on CD8 memory. *Proc Natl Acad Sci USA* 2004;101:2494–9.

54. Schaedel O, Reiter Y. Antibodies and their fragments as anti-cancer agents. *Curr Pharm Des* 2006;12:363–78.

55. Siegall CB, Chaudhary V. Functional analysis of donaims II, Ib and III of pseudomonas exotoxin. *J Biol Chem* 1989;264:14256–61.

56. Siegall CB, Liggett D. Characterization of vascular leak syndrome induced by the toxin component of pseudomonas exotoxin-based immunotoxin and its protential inhibition with nonsteroidal anti-inflammatory drugs. *Clin Cancer Res* 1997;3: 339–45.

57. Siegall CB, Liggitt D. Prevention of immunotoxin-mediated vascular leak syndrome in rats with retention of antitiumor activity. *Proc Natl Acad Sci USA* 1994; 91:9514–18.

58. Sievers EL, Larson RA, Stadtmauer EA, Estey E, Lowenberg B, Dombret H, Karanes C, Theobald M, Bennett JM, Sherman ML, Berger MS, Eten CB, Loken MR, van Dongen JJ, Bernstein ID, Appelbaum FR. Efficacy and safety of gemtuzumab ozogamicin in patients with CD33-positive acute myeloid leukemia in first relapse. *J Clin Oncol* 2001;19:3244–54.

59. Smallshaw JE, Ghetie V, Rizo J, Fulton JRJ, Trahan L, Ghetie MA, Vitetta E. Genetic engineering of an immunotoxin to eliminate pulmonary vascular leak in mice. *Nat Biotechnol* 2003;21:387–91.

60. Tsutsumi Y, Onda M, Nagata S, Lee B, Kreitman RJ, Pastan I. Site-specific chemical modification with polyethylene glycol of recombinant immunotoxin anti-Tac(Fv)-PE38 (LMB-2) improves antitumor activity and reduces animal toxicity and immunogenicity. *Proc Natl Acad Sci USA* 2000;97:8548–53.

61. Uchida T, Pappenheimer AM, Jr, Harper AA. Diphtheria toxin and related proteins. II. Kinetic studies on intoxication of HeLa cells by diphtheria toxin and related proteins. *J Biol Chem* 1973;248:3845–50.

62. van Oosterhout YV, Van EL, Schattenberg AV, Tax WJ, Ruiter DJ, Spits H, Nagengast FM, Masereeuw R, Evers S, de WT, Preijers FW. A combination of anti-CD3 and anti-CD7 ricin A-immunotoxins for the in vivo treatment of acute graft versus host disease. *Blood* 2000;95:3693–701.
63. van Vuuren AJ, Van Roon JA, Walraven V, Stuij I, Harmsen MC, McLaughlin PM, Van de Winkel JG, Thepen T. CD64-directed immunotoxin inhibits arthritis in a novel CD64 transgenic rat model. *J Immunol* 2006;176:5833–8.
64. White CA, Weaver RL, Grillo-Lopez AJ. Antibody-targeted immunotherapy for treatment of malignancy. *Annu Rev Med* 2001;52:125–45.
65. Willuda J, Honegger A, Waibel R. High thermal stability is essential for tumor targeting of antibody fragments: engineering of a humanized anti-epithelial glycoprotein-2 (epithelial cell adhesion molecule) single-chain Fv fragment. *Cancer Res* 1999;59:5758–67.

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