Preclinical Development

Single-Chain Antibody-Based Immunotoxins Targeting Her2/neu: Design Optimization and Impact of Affinity on Antitumor Efficacy and Off-Target Toxicity

Yu Cao1, James D. Marks1, Qian Huang2, Stephen I. Rudnick4, Chyi Xiong2, Walter N. Hittelman3, Xiaoxia Wen2, John W. Marks3, Lawrence H. Cheung3, Kim Boland4, Chun Li2, Gregory P. Adams4, and Michael G. Rosenblum3

Abstract

Recombinant immunotoxins, consisting of single-chain variable fragments (scFv) genetically fused to polypeptide toxins, represent potentially effective candidates for cancer therapeutics. We evaluated the affinity of various anti-Her2/neu scFv fused to recombinant gelonin (rGel) and its effect on antitumor efficacy and off-target toxicity. A series of rGel-based immunotoxins were created from the human anti-Her2/neu scFv C6.5 and various affinity mutants (designated ML3-9, MH3-B1, and B1D3) with affinities ranging from 10^{-8} to 10^{-11} mol/L. Against Her2/neu-overexpressing tumor cells, immunotoxins with increasing affinity displayed improved internalization and enhanced autophagic cytotoxicity. Targeting indices were highest for the highest affinity B1D3/rGel construct. However, the addition of free Her2/neu extracellular domain (ECD) significantly reduced the cytotoxicity of B1D3/rGel because of immune complex formation. In contrast, ECD addition had little impact on the lower affinity constructs in vitro. In vivo studies against established BT474 M1 xenografts showed growth suppression by all immunotoxins. Surprisingly, therapy with the B1D3-rGel induced significant liver toxicity because of immune complex formation with shed Her2/neu antigen in circulation. The MH3-B1/rGel construct with intermediate affinity showed effective tumor growth inhibition without inducing hepatotoxicity or complex formation. These findings show that while high-affinity constructs can be potent antitumor agents, they may also be associated with mistargeting through the facile formation of complexes with soluble antigen leading to significant off-target toxicity. Constructs composed of intermediate-affinity antibodies are also potent agents that are more resistant to immune complex formation. Therefore, affinity is an exceptionally important consideration when evaluating the design and efficacy of targeted therapeutics. Mol Cancer Ther; 11(1); 143–53. ©2011 AACR.

Introduction

Immunotherapeutic approaches using antibodies have been widely explored against a variety of tumors, but an effective treatment of solid tumors remains a potential problem because therapeutic antibodies must diffuse into tumors through a disordered vasculature and against a hydrostatic pressure gradient (1, 2). Because low–molecular weight antibody fragments have been shown to have better tumor diffusion properties (3), single-chain variable fragments (scFv) were favored to deliver protein-based toxins to cancer cells (4, 5).

A variety of scFv-based immunotoxins have been engineered that are suitable for diverse therapeutic applications. An anti-CD174 scFv designated SGN-10 fused with Pseudomonas exotoxin (PE) was developed for optimal tumor penetration but clinical studies were limited by renal toxicity and gastritis (6, 7). Chaudhary and colleagues (8) and Powell and colleagues (9) generated LMB-2, anti-CD25 scFv-PE immunotoxin and described promising preclinical efficacy on malignant cells from patients with adult T-cell leukemia. However, common toxicities included transaminase evaluation. The therapeutic window for this class of constructs may be optimized by various design changes to lower the efficacious dose, improve specificity by reducing off-target effects, thereby allowing an increase in the maximal tolerated dose (10–12).

Tumor–antigen affinity and specificity of scFvs are important variables that may impact off-target tissue distribution and toxicity in vivo. These attributes have led...
to the commonly held concept that scFv must have high affinity to be therapeutically relevant. However, studies by Adams and colleagues (13) and Rudnick and Adams (14) have suggested that high-affinity scFv may be suboptimal vehicles and that lower affinity scFv appear to diffuse more uniformly throughout the tumor interior. In addition, because the presence of shed tumor antigen has the potential to misdirect the targeted constructs through immune complex formation (10, 15), higher affinity scFv could potentially be at risk compared with lower affinity constructs.

Although previous studies primarily focused on the in vivo behavior of scFv, few companion studies have been conducted to determine whether scFv-based immunotoxins display the same behavior with regard to the relationship between affinity, tumor penetration, tumor residence, and efficacy. Our present knowledge of the affinity/function relationship of scFv-based immunotoxins is insufficient to afford accurate predictions as to whether a given scFv is appropriate for toxin delivery. A comprehensive head-to-head comparison of recombinant immunotoxins with different affinities targeting the same epitope on an antigen would be useful to guide the developmental strategy for future immunotoxins.

We previously reported the construction and characterization of anti-Her2/neu immunotoxins constructed by fusing scFv C6.5 with the recombinant gelonin (rGel). These constructs showed highly efficient activity against Her2/neu-positive tumor cells (16). In the current study, we generated a series of rGel-containing fusion constructs composed of C6.5 and its mutants, ML3-9, MH3-B1, and B1D3 created by site-directed amino acid substitutions in the CDR3s; ref. 17) were supplied by Dr. James D Marks (University of California, San Francisco, San Francisco, CA; Fig. 1A). Illustrations of the immunotoxin constructs are shown in Fig. 1B. Recombinant immunotoxins containing each scFv and rGel were constructed by overlapping PCR and were designated C6.5/rGel, ML3-9/rGel, MH3-B1/rGel, and B1D3/rGel, respectively.

Materials and Methods

Plasmid construction
The gene encoding human anti-Her2/neu scFv (C6.5 and its affinity mutants, ML3-9, MH3-B1, and B1D3 created by site-directed amino acid substitutions in the CDR3s; ref. 17) were supplied by Dr. James D Marks (University of California, San Francisco, San Francisco, CA; Fig. 1A). Illustrations of the immunotoxin constructs are shown in Fig. 1B. Recombinant immunotoxins containing each scFv and rGel were constructed by overlapping PCR and were designated C6.5/rGel, ML3-9/rGel, MH3-B1/rGel, and B1D3/rGel, respectively.

Protein expression and purification
The immunotoxins were expressed in *Escherichia coli* strain AD494 (DE3) pLysS and purified by immobilized metal affinity chromatography (IMAC) essentially as previously described (16).

Binding affinity of immunotoxins
The binding affinity and specificity of the immunotoxins were tested by ELISA on Her2/neu-positive (SKBR3, BT474 M1) and -negative (MCF7) cells. Rabbit anti-rGel antibody and horseradish peroxidase–conjugated goat anti-rabbit IgG were used as a tracer in this assay as described previously (16).

Internalization and competitive inhibition analysis
Immunofluorescence-based internalization studies were conducted on Her2/neu-positive (SKBR3, BT474 M1) and -negative (MCF7) cells. Immunofluorescence staining and competitive inhibition were analyzed as described in Supplementary Methods.

Cytotoxicity of scFv/rGel and competitive cytotoxicity assays
The cytotoxicity of immunotoxins on log-phase Her2/neu-positive and -negative cell lines were tested with the crystal violet staining method, and competitive assays were conducted as described in Supplementary Methods (18).

Western blot analysis of apoptosis and autophagy
The detection of apoptosis and autophagy on BT474 M1 cells treated with immunotoxins was analyzed as described in Supplementary Methods.

In vivo efficacy studies
BALB/c nude mice bearing subcutaneous BT474 M1 tumors were established and treated (intravenously, tail vein) with immunotoxins, as described in Supplementary Methods.

Tissue distribution study
The MH3-B1/rGel and B1D3/rGel was labeled with IRDye800CW according to the manufacturer’s protocol. The tissue distribution assays and the imaging analysis are further described in Supplementary Methods.

Coimmunoprecipitation assay
Liver samples from mice after treatment with MH3-B1/rGel or B1D3/rGel were collected. Samples were examined for the presence of antigen:immunotoxin complexes as described in Supplementary Methods.

In situ immunofluorescent detection
Samples of liver tissues from mice were further prepared for immunofluorescence staining tracing Her2/neu antigen and scFv/rGel immunotoxins as described in Supplementary Methods.

Liver toxicity study
Hepatotoxicity was investigated by measuring activities of alanine transaminase (ALT), aspartate transaminase (AST), and lactate dehydrogenase (LDH) in collected serum from treated mice according to an assay kit (Roche). The histologic examination for hepatotoxicity was...
assessed by hematoxylin and eosin staining. Further details are presented in Supplementary Methods.

**Statistical analysis**

Statistical analyses were conducted with SPSS version 17.0.2 software (SPSS Inc.). Data were presented as mean ± SD, and significance was determined using a 2-sided Student t test, unless otherwise noted. A value of $P < 0.05$ was considered statistically significant.

**Results**

**Preparation of scFv/rGel fusion constructs**

The scFv/rGel constructs were created from human anti-Her2/neu scFv C6.5 and various affinity mutants (designated ML3-9, MH3-B1, and B1D3, in increasing affinity order). The affinities of the scFv ranged from $10^{-8}$ to $10^{-11}$ mol/L (Fig. 1A; refs. 17, 19). The immunotoxin genes were cloned into vector pET-32a(+) separately (Fig. 1B). Sequenced DNA clones were subsequently transformed into *E. coli* AD494 (DE3) pLysS for protein expression. As shown in Fig. 1C, after purification, all the immunotoxins migrated on SDS-PAGE at the expected molecular weight of 55 kDa under both reducing and nonreducing conditions.

**Binding and cellular internalization of the fusion constructs**

To ensure that immunotoxins retained antigen-binding ability, the fusion proteins were compared in an ELISA-
based–binding assay using Her2/neu-positive (SKBR3, BT474 M1) and -negative (MCF7) cells. All the scFv/rGel constructs showed specific and significant ELISA binding to Her2/neu-positive cells with negligible binding to negative cells (Fig. 2A). The equilibrium dissociation constants \( (K_d) \) were calculated (GraphPad Prism), and the affinities of immunotoxins for BT474 M1 cells were found to be 53.13 nmol/L (C6.5/rGel), 1.45 nmol/L (ML3-9/rGel), 0.18 nmol/L (MH3-B1/rGel), and 27 pmol/L (B1D3/rGel). The correlation between the \( K_d \) values of the scFvs and fusion constructs was found to be significant with a correlation coefficient of 0.939 \( (P < 0.01) \), indicating that introduction of the rGel component did not affect the binding affinity of the scFv.

We next examined whether the various affinity scFv/rGel fusions could specifically internalize into target cells. Immunofluorescence staining was conducted on Her2/neu-positive and -negative cells. As quantified by relative fluorescence (Fig. 2B), the internalization efficiency exhibited a moderate increase with increasing binding affinity in Her2/neu-positive cells. For BT474 M1 cells, the relative fluorescence intensities were 56.30 (C6.5/rGel), 73.69 (ML3-9/rGel), 86.29 (MH3-B1/rGel), and 90.41 (B1D3/rGel). There was a good correlation between increases in apparent affinity and internalization efficiency \( (r^2 = 0.8289; P < 0.01) \) indicating that efficient binding to the cell surface appears to be primarily responsible for rapid internalization after cell exposure.

**In vitro cytotoxicity of scFv/rGel fusion constructs**

All the scFv/rGel constructs and rGel were tested against a number of different tumor cell lines (Table 1). As expected, there appeared to be a good correlation \( (r^2 = 0.7812; P < 0.01) \) between apparent affinity and IC\(_{50}\) values. Targeting indices were found to be highest for the highest affinity B1D3/rGel construct. This study showed that for the scFv/rGel immunotoxins, binding affinity appears to mediate internalization efficiency and this appeared to directly impact the overall cytotoxic effects observed. Furthermore, against Her2/neu-negative cells, there was little or no specific cytotoxicity of the constructs compared with rGel itself.

**Effects of various fusion constructs on cytotoxic mechanisms**

The cytotoxic effects mediated by scFv/rGel immunotoxins were analyzed in BT474 M1 cells. As shown in Fig. 3A, scFv/rGel fusions did not activate caspase-dependent apoptosis in target cells, showing no cleavage of the caspase substrate PARP. We next assessed LDH release and found that exposure of BT474 M1 cells to immunotoxins did not induce necrotic cell death (data not shown).

Then, we examined whether the cytotoxic effects of these immunotoxins activate autophagic signaling. As shown in Fig. 3B, the ratio of LC3-II formation to the \( \beta \)-actin control was shown to be increased after treatment with the fusion constructs, showing that autophagic flux
was induced by rGel-based immunotoxins in BT474 M1 cells. In addition, autophagic induction by fusion constructs was further validated by the observed selective release of cellular HMGB1 (Fig. 3C; ref. 20). These data indicated that the observed cytotoxic effects of scFv/rGel fusions in BT474 M1 cells appeared to be mediated not through an apoptotic or necrotic mechanisms but by the efficient induction of autophagic cell death.

### Table 1. Comparative IC$_{50}$ values of fusion constructs against various types of tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type</th>
<th>Her2/neu level</th>
<th>IC$_{50}$ (nmol/L) with T.I.$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>C6.5/rGel</td>
</tr>
<tr>
<td>SKBR3</td>
<td>Breast</td>
<td>++++</td>
<td>6.4 (165)</td>
</tr>
<tr>
<td>BT474 M1</td>
<td>Breast</td>
<td>++++</td>
<td>18.9 (12)</td>
</tr>
<tr>
<td>NCI-N87</td>
<td>Gastric</td>
<td>++++</td>
<td>30.1 (32)</td>
</tr>
<tr>
<td>Calu3</td>
<td>Lung</td>
<td>++++</td>
<td>24.3 (22)</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Breast</td>
<td>+</td>
<td>145.8 (2)</td>
</tr>
<tr>
<td>MCF7</td>
<td>Breast</td>
<td>+</td>
<td>246.9 (1)</td>
</tr>
<tr>
<td>A375m</td>
<td>Melanoma</td>
<td>+</td>
<td>61.4 (3)</td>
</tr>
<tr>
<td>Me180</td>
<td>Cervical</td>
<td>+</td>
<td>160.8 (1)</td>
</tr>
</tbody>
</table>

Abbreviation: T.I., targeting index.

$^a$Targeting index represents IC$_{50}$ of rGel/IC$_{50}$ of immunotoxin.

Figure 3. Cell-killing mechanism analysis of the immunotoxins on BT474 M1 cells. A, Western blot analysis of PARP cleavage after 24 and 48 hours of scFv/rGel fusion treatment. B, analysis of LC3 after treatment with the scFv/rGel fusions for 24 and 48 hours. C, analysis of cell extract and medium for HMGB1 protein after immunotoxin treatment for 48 hours.
Influence of soluble Her2/neu extracellular domain on immunotoxin activity

Shedding of target antigen from the surface of tumor cells into circulation may present obstacles for antibodies to effectively target tumor cells in vivo (21). To investigate the impact of soluble antigen on scFv/rGel immunotoxins, we evaluated the internalization of the immunotoxins in the presence of free Her2/neu extracellular domain (ECD) in BT474 M1 cells (Fig. 4A and Supplementary Fig. S1). The addition of ECD reduced the internalization for all the constructs. The highest affinity B1D3/rGel construct showed a significant reduction \( (P < 0.01) \), whereas the lower affinity fusions exhibited the lesser impact of ECD on internalization.

We next applied coimmunoprecipitation to examine whether the decreased internalization observed was because of the immune complexes of the immunotoxins with ECD. As shown in Fig. 4B, the highest affinity construct (B1D3/rGel) and the lowest affinity construct (C6.5/rGel) formed the highest and lowest amount of immune complex with ECD, with intermediate-affinity molecules (ML3-9/rGel and MH3-B1/rGel) generating intermediate levels of immune complexes. Therefore, the significant reduction in cell internalization observed with B1D3/rGel fusion was the result of immune complexes formation with soluble ECD, further preventing binding of the immunotoxins via cell-associated antigen.

A competitive cytotoxicity assay was conducted on SKBR3 and BT474 M1 cells by adding 20 nmol/L ECD to various concentrations of each fusion construct (Fig. 4C). All the fusion constructs showed an increase in IC50 value in the presence of ECD. Constructs with low and medium affinity showed the least impact of ECD on cytotoxic effects whereas B1D3/rGel showed the greatest influence. Furthermore, the addition of various concentration of ECD to a fixed (20 nmol/L) dose of immunotoxins showed similar effects (Supplementary Fig. S2). B1D3/rGel was impacted to the greatest extent in the presence of ECD, whereas the constructs with low or medium affinity showed less impact on cytotoxicity.

Her2/neu antigen shed from tumor cells

Because the Her2/neu antigen can be shed from target cells and may impact the cytotoxic effects observed with

---

Figure 4. Competitive analysis of the scFv/rGel in the presence of free Her2/neu ECD. A, quantification of competitive internalization rate of the fusion proteins on BT474 M1 cells. Cells were treated with the mixture of 20 nmol/L immunotoxins and different concentration of ECD. Values are expressed as mean ± SD \( (n > 50) \). B, coimmunoprecipitation of scFv/rGel and ECD complex. The mixture supernatants were subjected to Her2/neu immunoprecipitation (IP), followed by immunoblotting (IB) for rGel. C, competitive cytotoxicity of the immunotoxins in the presence of 20 nmol/L ECD. Values are presented as IC50.

---

Mol Cancer Ther; 11(1) January 2012 Molecular Cancer Therapeutics

Downloaded from mct.aacrjournals.org on June 22, 2017. © 2012 American Association for Cancer Research.
scFv/rGel immunotoxins, we measured the endogenous Her2/neu antigen levels in cell media and serum from mice bearing tumor xenografts by quantitative ELISA. The medium of Her2/neu-positive cells (SKBR3, BT474 M1, NCI-N87, and Calu3) were collected daily for 7 days. As shown in Supplementary Fig. S3A, the Her2/neu antigen was present in the medium of all Her2/neu-positive cells, and the concentrations increased relative to cell number. Levels of antigen in culture media were ≤0.4 nmol/L from all lines tested during a 72-hour cytotoxicity assay and appeared to be well below levels that would impact immunotoxin efficacy.

Measurements of shed Her2/neu antigen in the blood of mice bearing BT474 M1 tumor showed that Her2/neu levels increase in parallel with tumor size (Supplementary Fig. S3B). Levels of Her2/neu in serum increased from 2 to 12 nmol/L for mice with 200 mm³ tumors up to 1,800 mm³, respectively. The correlation between shed Her2/neu levels and tumor volume was found to be significant with a correlation coefficient of 0.797 (P < 0.01). At sufficiently high Her2/neu levels, the efficacy of high-affinity–targeted therapeutics could be impacted.

Antitumor activity of scFv/rGel fusions in xenograft models

We next evaluated the ability of various scFv/rGel immunotoxins to inhibit the growth of established BT474 M1 tumor xenografts in BALB/c nude mice after systemic administration. BT474 M1 cells were implanted into mice and tumors were allowed to grow to 200 mm³ in volume. Mice were then treated with each fusion protein and rGel as control at a total dose of 24 mg/kg. As shown in Fig. 5A, treatment with the scFv/rGel fusions all showed great antitumor effects, with the intermediate-affinity MH3-B1/rGel showing more enhanced and long-lasting tumor inhibition effects than lower affinity C6.5/rGel and ML3-9/rGel. There was little obvious toxicity observed with the administration of the immunotoxins with the
exception of the highest affinity B1D3/rGel. As shown in Fig. 5B, mice treated with this agent showed considerable body weight loss (~27%), and all the mice in this group died after fourth injection. Further studies were initiated to examine the reason of the toxicity of B1D3/rGel compared with other constructs.

**In vivo optical imaging**
To examine the in vivo toxicity observed in mice treated with the highest affinity B1D3/rGel, we used a fluorescent molecular imaging probe (IRDye 800CW) to label MH3-B1/rGel and B1D3/rGel for in vivo biodistribution studies (22). Nude mice bearing BT474 M1 tumors were injected (intravenously) with 1.5 nmol/L IRDye800-MH3-B1/rGel (IR-MH3-B1/rGel) or IRDye800-B1D3/rGel (IR-B1D3/rGel). The mice were then imaged at different times (4, 24, 48, and 72 hours) with the IVIS Optical Imaging System (Fig. 5C and D). Both IR-MH3-B1/rGel and IR-B1D3/rGel were shown to accumulate in BT474 M1 tumors with the first 4 hours of postinjection (tumor to contralateral background ratio of 1.85 ± 0.12 and 1.85 ± 0.09 for each) and reached maximal concentrations at 48 hours (tumor to contralateral background ratio of 2.70 ± 0.31 and 2.58 ± 0.15, respectively). In addition, accumulation of IR-B1D3/rGel was observed in the liver postinjection from 48 to 72 hours, compared with IR-MH3-B1/rGel.

To avoid any measuring errors caused by limited tissue penetration of fluorophores, animals were sacrificed; tumor and major organs were collected at the 24-hour and 72-hour time points and were subjected immediately to NIRF imaging (Supplementary Fig. S4). At 24 hours postinjection, there were no significant differences between the tissue distribution of IR-MH3-B1/rGel and IR-B1D3/rGel (Fig. 5E and Supplementary Fig. S5A). However, a better biodistribution of IR-MH3-B1/rGel over IR-B1D3/rGel could be identified 72 hours after injection, with the tissue-to-muscle ratio of tumor being 1.5 times higher than the later one (e.g. 2.84 ± 0.23 vs. 1.89 ± 0.23, P < 0.002; Fig. 5F and Supplementary Fig. S5B). Correspondingly, a 1.6-fold higher liver retention of IR-B1D3/rGel over IR-MH3-B1/rGel was observed (4.65 ± 0.61 vs. 2.89 ± 0.56, respectively; P < 0.01). Both IR-MH3-B1/rGel and IR-B1D3/rGel were found to accumulate in the kidneys likely due to renal clearance of the low-molecular weight agents. In the other major tissues we collected, there were no significant differences in the distribution of these immunotoxins to tissues such as heart, lung, spleen, muscle.

**Accumulation of Her2/neu antigen and B1D3/rGel in the liver**
To identify the cause of the liver distribution with the highest affinity B1D3/rGel, we administered B1D3/rGel and MH3-B1/rGel to nude mice with or without tumors at a dose of 1.5 nmol/L. Mice were sacrificed 72 hours after injection. After homogenization, the liver samples were subjected to immunoprecipitation using anti-Her2/neu antibody and immunoblotted to assess the fusions. As shown in Fig. 6A, we found immune complexes of Her2/neu antigen and the B1D3/rGel in tumor-bearing mice but not in tumor-free mice. In contrast, we found lower amounts of immune complexes in the livers of mice treated with MH3-B1/rGel.

Immunofluorescence staining confirmed colocalization and accumulation of B1D3/rGel with Her2/neu antigen in the liver of tumor-bearing mice, but less staining of antigen was observed from the livers in the MH3-B1/rGel treatment group (Fig. 6B).

**In vivo toxicity of immunotoxins in mice**
We then examined the influence of tumor-derived shed antigen on the hepatotoxicity of the B1D3 and MH3-B1 fusion toxins. Serum samples were collected 72 hours after the administration of the fusions or rGel to mice with or without tumors, and enzymatic activities of liver enzymes were determined (Fig. 6C and Supplementary Fig. S6). We found slight increases in the serum ALT, AST, and LDH levels in tumor-bearing versus tumor-free mice treated with rGel or MH3-B1/rGel. The greatest increases in all 3 markers were found with tumor-bearing mice treated with the high-affinity B1D3/rGel construct. This increase was not observed in tumor-free mice confirming that the observed hepatotoxicity was due to immune complexes of B1D3/rGel and Her2/neu antigen localizing in the liver.

To further test the hepatotoxicity in animals, mouse livers were harvested 72 hours after immunotoxin injection and examined. Compared with animals treated with rGel or MH3-B1/rGel, the livers of mice treated with B1D3/rGel showed severe liver damage characterized by marked necrosis and vacuolar degeneration of hepatocytes and extensive hemorrhage (Fig. 6D). This suggests that the in vivo efficacy of high-affinity immunotoxins can be significantly impaired by the presence of shed antigen. Furthermore, the immune complexes formed by the immunotoxins and the shed antigen contribute to significant hepatotoxicity.

**Discussion**
Using a panel of scFv/rGel fusions specific for the same Her2/neu epitope (17) and nearly identical in their sequences and structure, we were able to assess the influence of affinity. This appears to be one of the first comprehensive examinations of the impact of affinity on the in vitro and in vivo behavior of immunotoxins. More importantly, the current study clearly described the increased potential for high-affinity immunotoxins to form immune complexes in vivo resulting in off-target toxicity in liver responsible for clearing these complexes. These data have potential relevance to a number of anti-Her2/neu approaches (23, 24), as well as other targets for which there is a level of circulating antigen present (25, 26).

Previous studies suggested that the binding affinity for antigen plays a pivotal role in the total concentration and penetration of scFv into tumors (13, 14). On the basis of scFv/rGel immunotoxins, we showed that increasing...
affinity could improve cell-binding ability, internalization efficiency, and autophagic cytotoxicity on Her2/neu-positive cells. However, fusion toxins with a 10-fold increase in affinity did not show either a corresponding improvement in internalization or a concomitant improvement in cytotoxic effects. This suggests that the internalization rate of the construct may primarily be associated with Her2/neu receptor recycling and the rate of antigen endocytosis and appears to be largely unaffected by the affinity of immunotoxin binding (27, 28). Moreover, intracellular trafficking and distribution of the toxin component to the ribosomal compartment may be critical factors that can define immunotoxin sensitivity.

In addition to the characteristics of the targeting moiety, shed antigen levels are clearly a factor in the potential therapeutic application of targeted agents. Pharmacokinetic studies of anti-Her2/neu antibodies in patients showed an inverse association between serum concentrations of antibody and the levels of shed Her2/neu antigen (29, 30). This observation may be explained, in part, by formation of soluble antibody:antibody complexes leading to a more rapid clearance by the reticuloendothelial system (RES). Clinical studies of Herceptin showed that Her2/neu plasma concentrations greater than 500 ng/mL (~10 nmol/L) were associated with shorter serum half-life and subtherapeutic trough levels of the antibody (31, 32). We showed the presence of shed Her2/neu antigen in cell culture medium and in blood from tumor-bearing mice in the nanomolar range. In vitro studies showed that the activity of the highest affinity B1D3/rGel construct was the most vulnerable to soluble Her2/neu antigen compared with the immunotoxins with lower affinity. As a result, soluble Her2/neu antigen formed immune complexes with B1D3/rGel leading to a reduction in in vitro cytotoxicity and a significant increase in in vivo toxicity. Biodistribution studies in BT474 M1 tumor-bearing mice indicated that there was a statistically significant decrease in tumor localization for B1D3/rGel compared with MH3-B1/rGel 72 hours after injection and a corresponding increase in liver accumulation. These data are consistent with the finding that B1D3/rGel high-affinity construct formed immune complexes with soluble, tumor-derived antigen leading to clearance by the hepatic RES and a reduction in ability to distribute to the tumor.

The presence of hepatotoxicity has limited the clinical dose-escalation of some immunotoxins. Anti-Her2/neu immunotoxins containing PE have shown potent antitumor activity in animal models (23, 33) but resulted in unexpected hepatotoxicity in all patients likely due to the normal presence of Her2/neu on hepatocytes (34, 35). Studies by Onda and colleagues found that overall positive charge on anti-Tac(Fv) PE38 immunotoxin contributed to nonspecific binding to liver cells and resulted in dose-limiting liver toxicity (36, 37). In this study, analyzing the behavior of different affinity immunotoxins offered a comprehensive insight into the relationships between...
antibody affinity, scFv/rGel tumor uptake, in vivo efficacy, toxicity, and hepatic accumulation of immune complexes. The importance of complex formation leading to toxicity appears to be underscored by lethal toxicity observed in tumor-bearing but not in tumor-free mice after administration of the high-affinity immunotoxin.

Shedding of cell surface antigens is an important biologic process that is used by cells to modulate responses to signals in the extracellular environment (38, 39). Our biodistribution study indicated that there was little difference in the liver and tumor uptake of MH3-B1/rGel and B1D3/rGel at 24 hours, but there was a significant difference between the two at 72 hours. These results suggested that complex formation between the Her2/neu antigen and B1D3/rGel immunotoxin may play a role in later events in the biodistribution patterns of high-affinity constructs. The observations were consistent with our findings that clearance of antigen:immunotoxin complexes are an important event in the observed off-target toxicity found in liver and that high-affinity antibodies are most susceptible to formation of these complexes.

The emergence of clinically relevant, nonspecific toxicity has limited the therapeutic potential of numerous immunotoxins (40–42) and is a significant limitation to the clinical development of this class of molecules in general. Therefore, it is of paramount importance to design immunotoxins with minimal toxicity to normal tissues. Previous concepts focused on using the highest affinity antibodies available for use in immunotoxin constructs. The current study suggests that high-affinity constructs are the most vulnerable to the presence of shed antigen. Intermediate-affinity antibodies appear to be preferable for this application because their targeting ability is high enough to show reliable specificity and targeted toxicity but low enough to avoid interference by shed antigen.

The toxicity of Her2/neu constructs to liver as a result of normal tissue expression is a common problem that has been addressed in clinical trials. Because the scFv antibodies we used for this study do not cross-react with murine Her2/neu (17, 19), antigen expression leading to toxicity was therefore not a factor contributing to hepatic toxicity. Our study clearly showed that when high-affinity immunotoxins form complexes in vivo with tumor-derived Her2/neu antigen, this, in addition, results in significantly increased hepatic toxicity.

It is important to note that murine studies of species-specific anti-human Her2/neu antibody constructs may underrepresent the eventual clinical toxicity of these agents against normal tissues expressing low levels of Her2/neu antigen. Such dose-limiting toxicities include renal and liver toxicity as well as vascular leak syndrome (43–45). The use of rGel-based constructs may be particularly important to be considered in cases of low-level expression of antigen on normal tissues because previous studies have shown that there appears to be a relatively high minimal threshold of Her2/neu antigen sites (~150,000) present on a target cell before specific toxicity is enabled. This appears to be unique compared with studies with other toxins.

The present study suggests that the efficacy of high-affinity immunotoxins appears to be most easily impaired by the presence of circulating Her2/neu antigen and the toxicity of these agents is more easily increased because of the facile formation of immune complexes leading to toxicity in RES organs (i.e., liver). The use of fusion constructs with intermediate affinity appears to be most appropriate because their antitumor efficacy and toxicity do not appear to be significantly impacted by the presence of soluble antigen. These factors should be taken into consideration when designing antitumor immunotoxins as cancer therapeutics.

Disclosure of Potential Conflicts of Interest

M.G. Rosenblum has a patent with the Clayton Foundation for Research. No potential conflicts of interest were disclosed by the other authors.

Acknowledgments

The authors thank Heidi Simmons of the FCCC Developmental Therapeutics Program for her excellent technical assistance as well as Dr. Margaret Einerson and the FCCC High Throughput Screening and Translational Research Facility.

Grant Support

This work was conducted by the Clayton Foundation for Research and supported by NCI grant R01 CA118159 (G.P. Adams). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 15, 2011; revised November 8, 2011; accepted November 8, 2011; published OnlineFirst November 16, 2011.

References

patients with metastatic melanoma induces a selective partial reduc-
11. Kreitman RJ. Immunotoxins for targeted cancer therapy. AAPS J
2006;8:E532–51.
12. Pastan I, Hassan R, Fitzgerald DJ, Kreitman RJ. Immunotoxin therapy of
RK, et al. High affinity restricts the localization and tumor penetration
14. Rudnick SI, Adams GP. Affinity and avidity in antibody-based tumor
15. Zhang Y, Pastan I. High shed antigen levels within tumors: an addi-
tional barrier to immunocojugate therapy. Clin Cancer Res 2008;14:
7981–6.
Construction and characterization of novel, recombinant immunotox-
ins targeting the Her2/neu oncogene product: in vitro and in vivo
Isolation of picomolar affinity anti-c-erbB-2 single-chain Fv by molecu-
lar evolution of the complementarity determining regions in the center
18. Rosenblum MG, Cheung LH, Liu Y, Marks JW III. Design, expression,
purification, and characterization, in vitro and in vivo, of an antitumor
single-chain Fv antibody fused to the toxin gelonin. Cancer Res 2003;
63:3995–4002.
vitro and in vivo characterization of a human anti-c-erbB-2 single-chain
Fv isolated from a filamentous phage antibody library. Immunotech-
Autophagy regulates selective HMG18 poly release in tumor cells that
Cancer J 2008;14:737–44.
visible and near-infrared wavelength-excitable fluorescent dyes for
23. King CR, Fischer PH, Rando RF, Pastan I. The performance of e23(Fv)
PEs, recombinant toxins targeting the erbB-2 protein. Semin Cancer
24. Mazor Y, Noy R, Wels WS, Benhar I. cFRP5-ZZ-PE38, a large IgG-toxin
immunocytotoxin outperforms the corresponding smaller FRP5
(Fv)-ETA immunotoxin in eradicating ErbB2-expressing tumor xeno-
25. Chan SY, Gordon AN, Coleman RE, Hall JS, Berger MS, Sherman ML,
et al. A phase 2 study of the cytotoxic immunocytotoxin CMGE-401
(hCTM01-calicheamicin) in patients with platinum-sensitive recurrent
epithelial ovarian carcinoma. Cancer Immunol Immunother 2003;52:
243–8.
results from a decrease in shed mesothelin levels in the extracellular
27. Ackerman ME, Pawlowski D, Wittprud KD. Effect of antigen turnover
rate and expression level on antibody penetration into tumor spheroids.
28. Austin CD, De Maziere AM, Piscarcie PI, van Dijk SM, Eigenbrot C,
Sliwkowski MX, et al. Endocytosis and sorting of ErbB2 and the site
of action of cancer therapeutics trastuzumab and geldanamycin. Mol
29. Pegram MD, Lipton A, Hayes DF, Weber BL, Baselga JM, Tripathy D,
et al. Phase II study of receptor-enhanced chemo-sensitivity using
recombinant humanized anti-p185HER2/new monoclonal antibody
plus cisplatin in patients with HER2/new-overexpressing metastatic
breast cancer refractory to chemotherapy treatment. J Clin Oncol
Inhibitory effects of combinations of HER-2/new antibody and che-
motherapeutic agents used for treatment of human breast cancers.
Oncogene 1999;18:2241–51.
humanized anti-p185HER2 monoclonal antibody in patients with
HER2/new-overexpressing metastatic breast cancer. J Clin Oncol
1998;16:737–44.
32. Leary AF, Hanna WM, van de Vijler MJ, Benali-Lorca F, Rousch J,
Osmuva RY, et al. Value and limitations of measuring HER-2 extra-
cellular domain in the serum of breast cancer patients. J Clin Oncol
33. Wells W, Harerth IM, Mueller M, Grorner B, Hynes NE. Selective
inhibition of tumor cell growth by a recombinant single-chain anti-
body-toxin specific for the erbB-2 receptor. Cancer Res 1992;52:
6310–7.
Hepatotoxicity in cancer patients receiving erb-38, a recombinant
immunotoxin that targets the erbB-2 receptor. Clin Cancer Res 1999;
5:2311–5.
35. Azemar M, Djahansouzi S, Jager E, Sollbach C, Schmidt M, Maurer
AB, et al. 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.
36. Onda M, Willingham M, Wang DC, Kreitman RJ, Tsutsumi Y, Nagata
S, et al. Inhibition of TNF-α-alpha produced by Kupffer cells protects
against the nonspecific liver toxicity of immunotoxin anti-Tac(Fv)-
37. Onda M, Kreitman RJ, Vasmatis G, Lee B, Pastan I. Reduction of the
nonspecific animal toxicity of anti-Tac(Fv)-PE38 by mutations in the
framework regions of the Fv which lower the isoelectric point. J
38. Arribas J, Borroto A. Protein ectodomain shedding. Chem Rev
39. Delo SP, Rovida E. Transmodulation of cell surface regulatory mole-
40. Ghetie MA, Ghetie V, Vitetta ES. Immunotoxins for the treatment of B-
41. Bang S, Nagata S, Onda M, Kreitman RJ, Pastan I, Ha22 (R490A) is a
recombinant immunotoxin with increased antitumor activity without an
42. Mathew V, Verma RS. Humanized immunotoxins: a new generation
of immunotoxins for targeted cancer therapy. Cancer Sci 2009;100:
1359–65.
43. Baluna R, Vitetta ES. Vascular leak syndrome: a side effect of immu-
44. Vallera DA, Panoskiatis-Mortari A, Blazar BR. Renal dysfunction
accounts for the dose limiting toxicity of DT390anti-CD3deltaFv, a po-
tential new recombinant anti-GVHD immunotoxin. Protein Eng 1997;10:
1071–6.
J, et al. Preclinical assessment of an anti-EpCAM immunotoxin:
locoregional delivery provides a safer alternative to systemic admin-
Molecular Cancer Therapeutics

Single-Chain Antibody-Based Immunotoxins Targeting Her2/neu: Design Optimization and Impact of Affinity on Antitumor Efficacy and Off-Target Toxicity

Yu Cao, James D. Marks, Qian Huang, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-11-0519

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2011/11/15/1535-7163.MCT-11-0519.DC1

Cited articles
This article cites 45 articles, 18 of which you can access for free at:
http://mct.aacrjournals.org/content/11/1/143.full.html#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
/content/11/1/143.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.