Single-chain antibody based immunotoxins targeting Her2/neu. Design optimization and impact of affinity on antitumor efficacy and off-target toxicity

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Abstract

Recombinant immunotoxins, consisting of single-chain antibodies (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 $10^{-11}$ M. 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 due to 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 demonstrated growth suppression by all immunotoxins. Surprisingly, therapy with the B1D3-rGel induced significant liver toxicity due to 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 demonstrate 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 which are more resistant to immune complex formation. Therefore, affinity is an exceptionally-important consideration when evaluating the design and efficacy of targeted therapeutics.
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 since 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 which 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). Pastan et al generated LMB-2, anti-CD25 scFv-PE immunotoxin and described promising preclinical efficacy on malignant cells from ATL patients. However, common toxicities included transaminase evaluation (8, 9). 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 which 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 in order to be therapeutically relevant. However, studies by Adams et al suggested that high affinity scFv may be suboptimal vehicles and that lower-affinity scFv appear to diffuse more uniformly throughout the tumor interior (13, 14). In addition, since the presence of shed tumor antigen has the potential to mis-direct the targeted constructs through immune complex formation (10, 15), higher affinity scFv could potentially be at risk compared to 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 demonstrated 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 with varying affinities to Her2/neu, and examined the impact of affinity on in vitro cytotoxicity, pharmacodynamics, and antitumor efficacy. Additionally, we investigated the effect of antibody affinity on behavior in the presence of soluble antigen, formation of immune complexes and the coincident development of off-target toxicity.
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 (17)) were supplied by Dr. James D Marks (UCSF, San Francisco, CA) (Fig. 1A). Illustrations of the immunotoxin constructs were 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 *E.coli* strain AD494 (DE3) pLysS and purified by 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 HRP-conjugated goat anti-rabbit IgG was used as a tracer in this assay as described previously (16).

**Internalization and competitive inhibition analysis.** Immunofluorescence-based internalization studies were performed on Her2/neu positive (SKBR3, BT474 M1) and negative (MCF7) cells. Immunofluorescence staining and competitive inhibition were analyzed as described in Supplemental 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 using the crystal violet staining method, and competitive assays were carried out as described in Supplemental 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 Supplemental Methods.
**In vivo efficacy studies.** Balb/c nude mice bearing subcutaneous BT474 M1 tumors were established and treated (i.v., tail vein) with immunotoxins, as described in Supplemental Methods.

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

**Co-immunoprecipitation 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 Supplemental 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 Supplemental 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 were presented in Supplemental Methods.

**Statistical analysis.** Statistical analyses were performed using SPSS version 17.0.2 software (SPSS Inc.). Data were presented as mean ± SD, and significance was determined using a 2-sided Student’s 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 $10^{-11}$ M (Fig. 1A) (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 non-reducing 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 demonstrated 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 nM (C6.5/rGel), 1.45nM (ML3-9/rGel), 0.18nM (MH3-B1/rGel) and 27 pM (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 performed 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.
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 of 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 demonstrated 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 to 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 β-actin control was shown to be increased after treatment with the fusion constructs, demonstrating 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) (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.

**Influence of soluble Her2/neu ECD 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 ECD in BT474 M1 cells (Fig. 4A and Supplemental 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 co-immunoprecipitation to examine whether the decreased internalization observed was due to 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 performed on SKBR3 and BT474 M1 cells by adding 20nM ECD to various concentrations of each fusion construct (Fig. 4C). All the fusion constructs showed an increase in IC₅₀ in the presence of ECD. Constructs with low and medium
affinity showed the least impact of ECD on cytotoxic effects while B1D3/rGel showed the greatest influence. Furthermore, the addition of various concentration of ECD to a fixed (20nM) dose of immunotoxins demonstrated similar effects (Supplemental Fig. S2). B1D3/rGel was impacted to the greatest extent in the presence of ECD while the constructs with low or medium affinity showed less impact on cytotoxicity.

**Her2/neu antigen shed from tumor cells.**

Since the Her2/neu antigen can be shed from target cells and may impact the cytotoxic effects observed with 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 Supplemental 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 ≤0.4nM from all lines tested during a 72 h cytotoxicity assay and appeared to be well below levels which 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 (Supplemental Fig. S3B). Levels of Her2/neu in serum increased from 2nM to 12nM for mice with 200 mm$^3$ tumors up to 1800 mm$^3$ 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 200mm$^3$ in volume. Mice were then treated with each fusion protein and rGel as control at a total dose of 24mg/kg. As shown in Fig. 5A, treatment with the scFv/rGel fusions all demonstrated great antitumor effects, with the intermediate-affinity MH3-B1/rGel showing more enhanced and long-lasting tumor inhibition effects compared to 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 to other constructs.

**In vivo optical imaging.**

To examine the in vivo toxicity observed in mice treated with the highest-affinity B1D3/rGel, we utilized 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 (i.v.) with 1.5nM 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 72h) using 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 4h postinjection (tumor to contralateral background ratio of 1.85±0.12 and 1.85±0.09 for each), and reached maximal concentrations at 48h (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 post injection from 48-72h, 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 h and 72 h time points and
were subjected immediately to NIRF imaging (Supplemental Fig. S4). At 24h postinjection, there were no significant differences between the tissue distribution of IR-MH3-B1/rGel and IR-B1D3/rGel (Fig. 5E and Supplemental Fig. S5A). However, a better biodistribution of IR-MH3-B1/rGel over IR-B1D3/rGel could be identified 72h post injection, with the TMR 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 Supplemental 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, etc.

**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 1.5nM. Mice were sacrificed 72h 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 h 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 Supplemental Fig. S6). We found slight increases in the serum ALT, AST and LDH levels in tumor-bearing vs tumor-free mice treated with rGel or MH3-B1/rGel. The greatest increases in all three 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 verify the hepatotoxicity in animals, mouse livers were harvested 72h after immunotoxin injection and examined. Compared to 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. Further, 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. This data has 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). Based on scFv/rGel immunotoxins, we demonstrated 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 a corresponding improvement in either 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 which 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 demonstrated 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 antigen: 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 500ng/mL (~10nM) were associated with shorter serum half-life and sub-therapeutic trough levels of the antibody (31, 32). We demonstrated 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 demonstrated that the activity of the highest-affinity B1D3/rGel construct was the most vulnerable to soluble Her2/neu antigen compared to 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 to MH3-B1/rGel 72h after injection, and a corresponding increase in liver accumulation. This data is 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 et al 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 tumor-free mice after administration of the high affinity immunotoxin.
Shedding of cell surface antigens is an important biological 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 24h, but there was a significant difference between the two at 72h. 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 employing 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 since 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 which has been addressed in clinical trials. Since the scFv antibodies we employed 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 demonstrated that when high affinity immunotoxins form complexes in vivo with tumor-derived Her2/neu antigen, this additionally results in significantly increased hepatic toxicity.
It is important to note that murine studies of species-specific anti-human Her2/neu antibody constructs may under-represent 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 consider in cases of low level expression of antigen on normal tissues since previous studies have demonstrated 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 to 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 due to 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 does 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.

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References


(23) King CR, Fischer PH, Rando RF, Pastan I. The performance of e23(Fv)PEs, recombinant toxins targeting the erbB-2 protein. Semin Cancer Biol 1996;7:79-86.


<table>
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<th>Cell line</th>
<th>Type</th>
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<th>ML3-9/rGel</th>
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<td>A375m</td>
<td>Melanoma</td>
<td>*</td>
<td>61.4 (3)</td>
<td>126.9 (2)</td>
<td>153.9 (1)</td>
<td>173.9 (1)</td>
<td>207.3</td>
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<tr>
<td>Me180</td>
<td>Cervical</td>
<td>*</td>
<td>160.8 (1)</td>
<td>185.4 (1)</td>
<td>194.6 (1)</td>
<td>213.1 (1)</td>
<td>222.5</td>
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</table>

*Targeting index represents IC50 of rGel/IC50 of immunotoxin.
Figure 1. Construction and preparation of scFv/rGel immunotoxins. A, Amino acid mutations and affinity parameters of the C6.5 and its mutants, ML3-9, MH3-B1 and B1D3. The listed amino acids for each scFv indicate mutations to the sequence and the substituting amino acids. Dashes indicate no changes from the original sequence. B, Schematic diagram of immunotoxin constructs containing scFv (C6.5, ML3-9, MH3-B1 or B1D3) and rGel. C, SDS-PAGE analysis of purified immunotoxins.

Figure 2. Characterization and comparison of scFv/rGel immunotoxins. A. Evaluation binding activity of the scFv/rGel to Her2/neu positive (SKBR3, BT474 M1) and negative (MCF7) cells by whole-cell ELISA. B, Quantification of Internalization rate of the immunotoxins on Her2/neu positive and negative cells. Cells were subjected to immunofluorescent staining with anti-rGel antibody (FITC-conjugated secondary). The bar graphs were calculated from relative fluorescence estimation, and the values are expressed as mean ± S.D (n>50).

Figure 3. Cell-killing mechanism analysis of the immunotoxins on BT474 M1 cells. A, Western blot analysis of PARP cleavage after 24 and 48h of scFv/rGel fusions treatment. B, Analysis of LC3 after treated with the scFv/rGel fusions for 24 and 48h. C, Analysis of cell extract and medium for HMGB1 protein after immunotoxins treatment for 48h.

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 20nM immunotoxins and different concentration of...
ECD. Values are expressed as mean ± S.D (n>50). B, Co-immunoprecipitation of scFv/rGel and ECD complex. The mixture supernatants were subjected to Her2/neu immunoprecipitation, followed by western blot for rGel. C, Competitive cytotoxicity of the immunotoxins in the presence of 20nM ECD. Values are presented as IC\textsubscript{50}.

Figure 5. In vivo study of the immunotoxins against BT474 M1 tumor xenografts in nude mice. A, Treatment of BT474 M1 tumors with immunotoxins at the dose of 24mg/kg. Mean tumor volume was calculated by W×L×H as measured by digital calipers. B, Average body weight of the mice during the immunotoxin treatment. C and D, Whole-body imaging results of the mice i.v. injected with either IR-MH3-B1/rGel or IR-B1D3/rGel at 4, 24, 48 and 72h. Arrows pointed at tumor (T) and liver (L). E and F, The Comparison of Tissue-to-Muscle Ratio (TMR) of IR-MH3-B1/rGel and IR-B1D3/rGel at 24h and 72h after injection into xenograft mice (n=5).

Figure 6. Analysis of the accumulation of Her2/neu antigen and scFv/rGel (MH3-B1/rGel or B1D3/rGel) complex driven liver toxicity after 72h injection. A, Co-immunoprecipitation of Her2/neu antigen and scFv/rGel in the liver from the mice with or without BT474 M1 tumors. Liver homogenates were subjected to Her2/neu immunoprecipitation, followed by western blot for rGel. B, Immunofluorescence colocalization of scFv/rGel (green) and Her2/neu antigen (red) in the liver of the BT474 M1 burden mice. C, Enzymatic activities of ALT in the serum of mice with or without tumors, treated with rGel, MH3-B1/rGel or B1D3/rGel. Values are expressed as mean ± S.D (n=5). *, ** and ***, statistically different from own control.
mice at $P < 0.05$, $P < 0.01$ and $P < 0.002$, respectively. D, Histologic findings of the livers from tumor burden mice after immunotoxin application. All the sections were stained with hematoxylin and eosin (primary magnification: 100×). Inset showed multiple necrosis of hepatocytes (primary magnification: 400×).
Fig. 1

A

C6.5  
<table>
<thead>
<tr>
<th>$V_H$</th>
<th>$V_L$</th>
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<tr>
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<td>A--DS</td>
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ML3-9  
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<th>$V_H$</th>
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MH3-B1  
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<th>$V_H$</th>
<th>$V_L$</th>
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<tr>
<td>TDRD--YFQH</td>
<td>S--YT</td>
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B1D3  
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<th>$V_H$</th>
<th>$V_L$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDRD--WLDN</td>
<td>S--YT</td>
</tr>
</tbody>
</table>

$K_d$  
- C6.5: 16 nM
- ML3-9: 1 nM
- MH3-B1: 0.12 nM
- B1D3: 0.013 nM

B

C6.5/ML3-9/MH3-B1/B1D3-rGel cDNA

scFv $G_{\delta}$ rGel

Bacterial Expression Vector

BspHI

NcoI

XhoI

pET 32a (Novagen)

C

[Image of gel electrophoresis showing bands for reducing and non-reducing conditions for different constructs]
Fig. 2

A

SKBR3

BT474 M1

MCF7 (-)

Absorbance at 405nm

Conc. nM

0.01 1 10 100

C6.5/rGel

ML3-9/rGel

MH3-B1/rGel

B1D3/rGel

rGel

0.01 1 10 100

B

SKBR3

BT474 M1

MCF7 (-)

Relative Fluorescence

C6.5/rGel ML3-9/rGel MH3-B1/rGel B1D3/rGel rGel

C6.5/rGel ML3-9/rGel MH3-B1/rGel B1D3/rGel rGel

C6.5/rGel ML3-9/rGel MH3-B1/rGel B1D3/rGel rGel

0 20 40 60 80 100

0 20 40 60 80 100

0 20 40 60 80 100
Fig. 4

A

- Graphs showing relative fluorescence for different concentrations of Her2/ neu ECD for C6.5/rGel, ML3-9/rGel, MH3-B1/rGel, and B1D3/rGel.

B

- Western blots for IP: Her2/neu ECD and IB: rGel.

C

- Graphs showing IC50 values for SKBR3 and BT474 M1 treated with different immunotoxins.
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.

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