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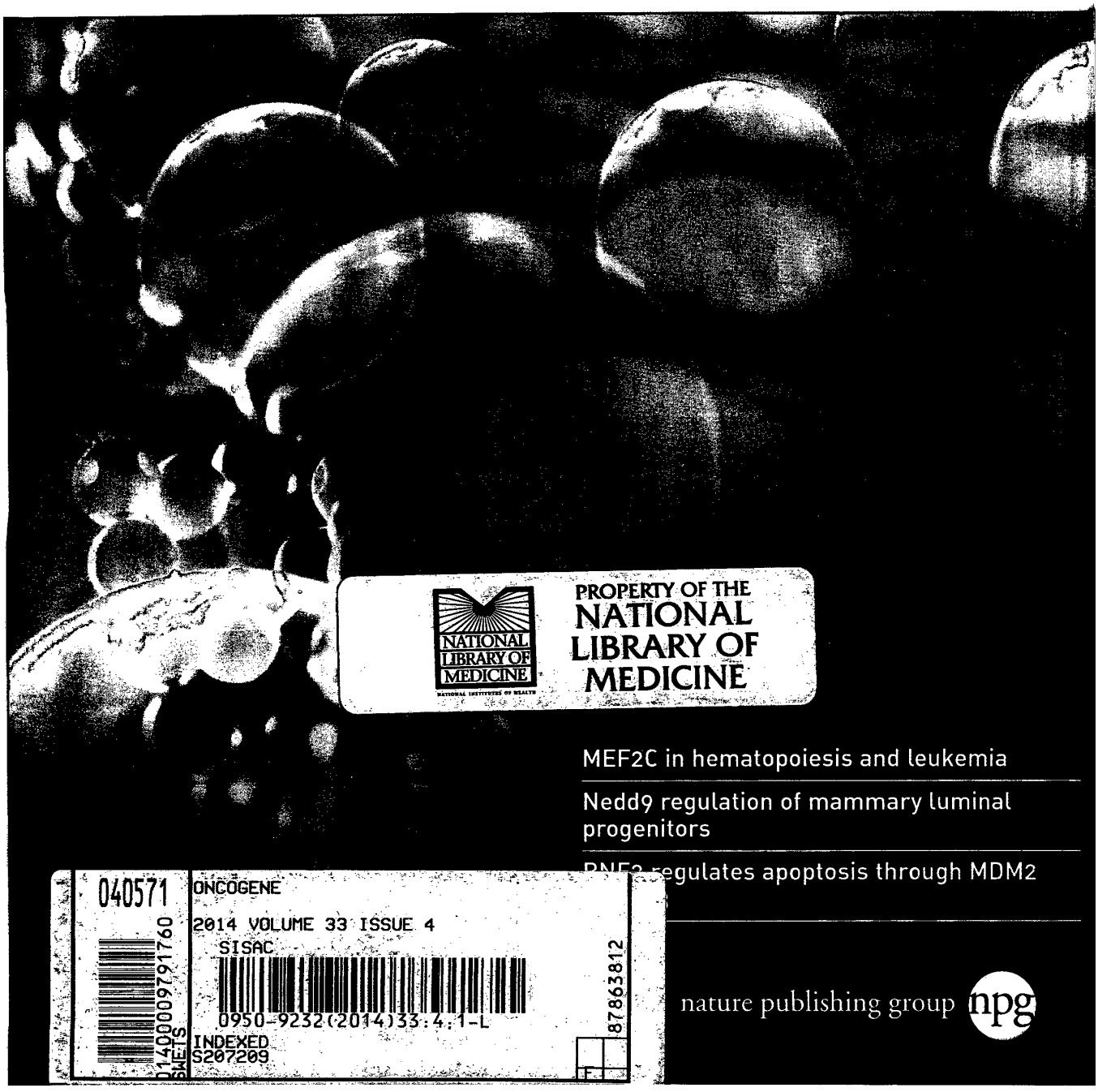

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**ORIGINAL ARTICLE**

# Design optimization and characterization of Her2/neu-targeted immunotoxins: comparative *in vitro* and *in vivo* efficacy studies

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Targeted therapeutics are potential therapeutic agents because of their selectivity and efficacy against tumors resistant to conventional therapy. The goal of this study was to determine the comparative activity of monovalent, engineered anti-Her2/neu immunotoxins fused to recombinant gelonin (rGel) to the activity of bivalent IgG-containing immunoconjugates. Utilizing Herceptin and its derived humanized single-chain antibody (single-chain fragment variable, designated 4D5), we generated bivalent chemical Herceptin/rGel conjugate, and the corresponding monovalent recombinant immunotoxins in two orientations, 4D5/rGel and rGel/4D5. All the constructs showed similar affinity to Her2/neu-overexpressing cancer cells, but significantly different antitumor activities. The rGel/4D5 orientation construct and Herceptin/rGel conjugate were superior to 4D5/rGel construct in *in vitro* and *in vivo* efficacy. The enhanced activity was attributed to improved intracellular toxin uptake into target cells and efficient downregulation of Her2/neu-related signaling pathways. The Her2/neu-targeted immunotoxins effectively targeted cells with Her2/neu expression level $>1.5\times10^5$ sites per cell. Cells resistant to Herceptin or chemotherapeutic agents were not cross-resistant to rGel-based immunotoxins. Against SK-OV-3 tumor xenografts, the rGel/4D5 construct with excellent tumor penetration showed impressive tumor inhibition. Although Herceptin/rGel conjugate demonstrated comparatively longer serum half-life, the *in vivo* efficacy of the conjugate was similar to the rGel/4D5 fusion. These comparative studies demonstrate that the monovalent, engineered rGel/4D5 construct displayed comparable *in vitro* and *in vivo* antitumor efficacy as bivalent Herceptin/rGel conjugate. Immunotoxin orientation can significantly impact the overall functionality and performance of these agents. The recombinant rGel/4D5 construct with excellent tumor penetration and rapid blood clearance may reduce the unwanted toxicity when administrating to patients, and warrants consideration for further clinical evaluation.

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## INTRODUCTION

Numerous studies have revealed that Her2/neu overexpression by tumors is a threshold event leading to a highly aggressive cellular phenotype, and therapeutic strategies directed against Her2/neu have rapidly gained recognition.[1,2] Her2/neu-targeted therapies, including Herceptin and Lapatinib, have significantly improved outcomes in Her2/neu-positive cancers; however, use of these agents can be limited by resistance and tolerability issues.[3,4] Therefore, there is a need for novel and improved therapeutic approaches targeting Her2/neu.

Immunotoxins are exquisitely powerful cytotoxic proteins.[5,6] Initial studies focused on constructs created by chemically conjugating an antibody to a protein toxin.[7,8] With advances in recombinant DNA technology, engineered antibody fragments have been employed to deliver various toxins to Her2/neu-positive tumor cells.[9,10] There have been numerous studies examining the impact of construct size, antibody affinity and the valency of constructs on overall efficacy.[11,12] Wels and colleagues[13] suggested that higher avidity and longer residence time of IgG-based immunoconjugates may outweigh the improved tumor penetration of single-chain fragment variable (scFv)-based constructs. However, immunoconjugate development has been hampered by nonspecific toxicity and vascular leak syndrome.[14] In addition, tight junctions between tumor cells and high interstitial tumor pressures could limit successful use of IgG conjugates.[12]

Recombinant engineering of immunotoxins has the potential to overcome many of these problems.[12,15] To improve Her2/neu-targeting properties, Adams *et al.*[16] suggested that $(scFv)_2$ constructs display improved tumor targeting and retention compared with scFv monomers. However, Pastan and colleagues[17] described diabody-based immunotoxins (designated e23 $(dsFv)_2$/PE) showing >10-fold greater *in vitro* cytotoxicity than their monovalent counterparts, but only about 2-fold greater activity *in vivo* than the monovalent analogs. The high affinity of a diabody may result in formation of a binding-site barrier at the periphery of tumors, which impedes immunotoxin penetration into the tumor mass.[18] Thus, the therapeutic window for Her2/neu targeting may be optimized on design changes instead of confinement to the valency argument.

Recombinant gelonin (rGel), a 29-kDa single-chain ribosome-inactivating protein, has been well established as a highly cytotoxic payload of chemical conjugates or fusion constructs for the treatment of many tumor types.[19–21] In this study, we utilized Herceptin and its humanized scFv (designated 4D5) to

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generate a conventional Herceptin/rGel conjugate and corresponding recombinant immunotoxins in two orientations: 4D5/rGel and rGel/4D5. Further characterization studies were performed, including examining the impact of valency and construct orientation on *in vitro* selectivity, specificity and efficacy of these agents, as well as comparison of their pharmacokinetics, tumor penetration and tumor-targeting efficacy *in vivo* against tumor xenografts.

## RESULTS

### Preparation of rGel-based immunotoxins

Antibody–toxin conjugates were generated with a disulfide-based succinimidyl 3-(2-pyridyldithio)propionate (SPDP) linker for facile release of toxin from the antibody carrier (Figure 1a). As shown in Figure 1b, the final product contained a mixture of immunoconjugates containing one rGel molecule (major) and two rGel molecules (minor; average molar ratio of 1.21 rGel molecules per antibody). No free Herceptin or free rGel were detected.

The monovalent immunotoxins were generated by fusing scFv 4D5 to the rGel, using the flexible GGGGS linker in two orientations (4D5/rGel and rGel/4D5, Figure 1a). Both immunotoxins were expressed in *E.coli* AD494 (DE3) pLysS. Following purification, the immunotoxins were shown to migrate at the expected molecular weight (55 kDa under nonreducing condition) with a purity $>95\%$ (Figure 1b).

### Analysis of binding affinity

The binding affinities of monovalent fusion constructs and bivalent chemical conjugates were assessed by enzyme-linked immunosorbent assay using Her2/neu extracellular domain (Figure 2a). The apparent binding affinities (*Kd*) were determined by calculating the concentration of immunotoxins that produced half-maximal specific binding. The monovalent 4D5/rGel and rGel/4D5 demonstrated apparent affinities of 0.106 and 0.142 nM, respectively, and the bivalent Herceptin/rGel conjugate had an apparent affinity of 0.201 nM. These results are in agreement with the published affinity values for native Herceptin to the Her2/neu receptor (*Kd* 0.15 nM).[22]

We next tested the cellular Her2/neu-binding activities of these immunotoxins by flow cytometry. As shown in Figure 2b, all the immunotoxins produced higher staining intensities with the Her2/neu-positive SK-OV-3 and BT-474-M1 cells, and displayed a significantly high specificity based on negative MDA-MB-468 cells. These studies confirmed that monovalent fusion constructs display virtually identical binding affinities compared with their original bivalent antibody-based conjugates.

### Cell-free protein synthesis inhibitory activity

To examine the n-glycosidic activity of rGel component of immunotoxins, these materials were tested by cell-free protein synthesis assay. Inhibition curves for 4D5/rGel, rGel/4D5 and Herceptin/rGel conjugate were compared with that of native rGel (Supplementary Figure S1). The calculated $IC_{50}$ (half-maximal inhibitory concentration) values for each immunotoxin were found to be virtually identical (55.2 pM, 48.9 pM, 38.3 pM vs 70.3 pM for rGel, respectively). The consistency of rGel-based immunotoxins clearly demonstrated that no loss of toxin enzymatic activity occurred in the different constructs.

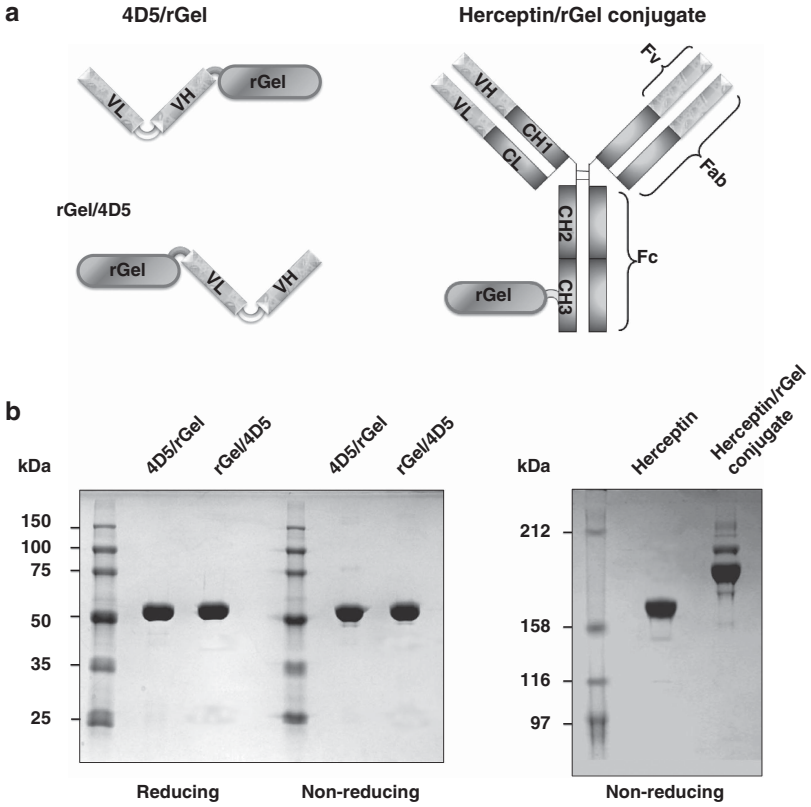


**Figure 1.** Construction and preparation of Herceptin-based immunotoxins. (**a**) Schematic diagram of immunotoxin constructs containing scFv 4D5 or full-length antibody Herceptin and rGel. (**b**) Purified immunotoxins were analyzed by SDS–polyacrylamide gel electrophoresis under nonreducing condition.

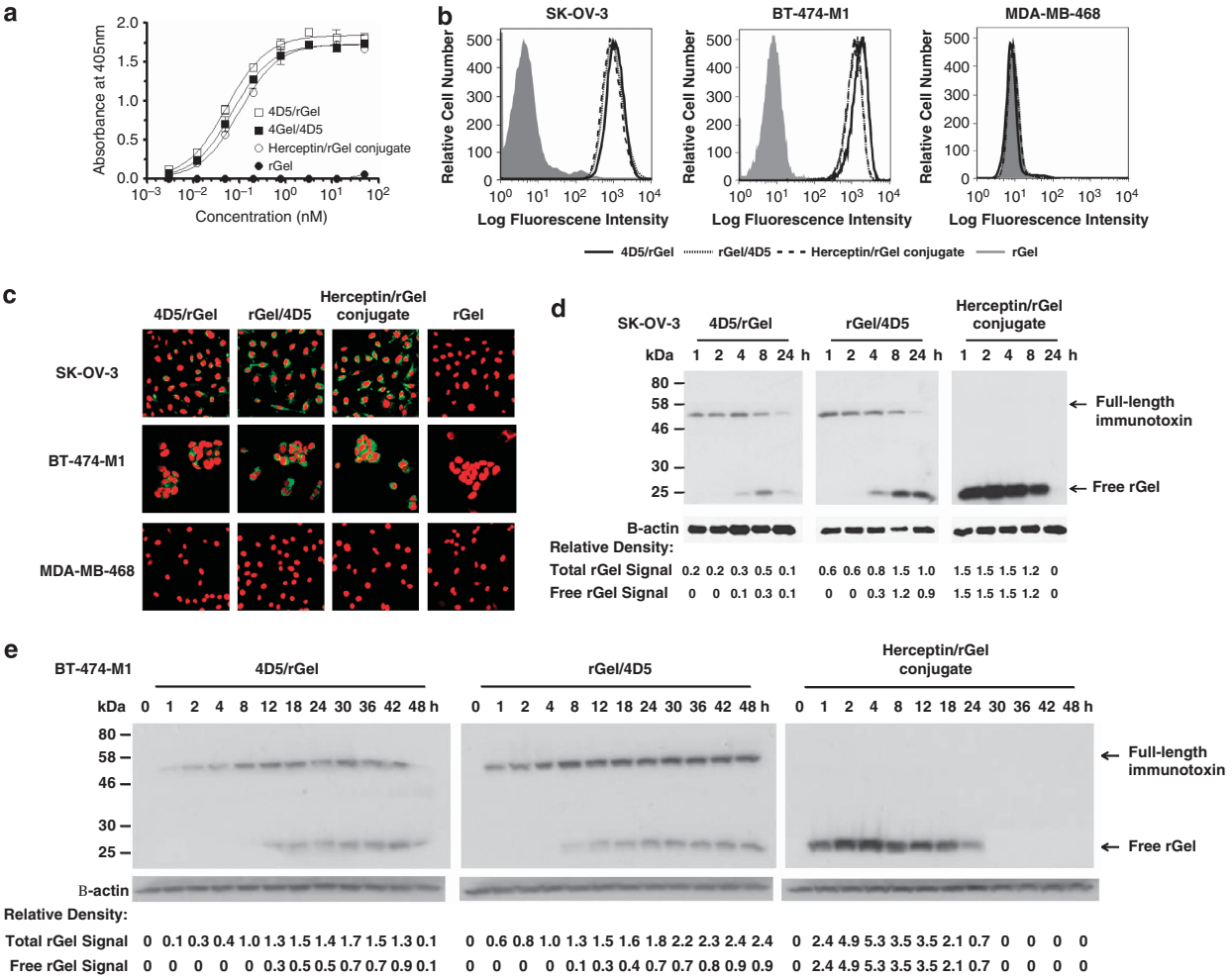


**Figure 2.** Characterization and comparison of anti-Her2/neu immunotoxins. (**a**) Binding curves of immunotoxins to Her2/neu extracellular domain by enzyme-linked immunosorbent assay. (**b**) Binding affinity analysis of 25 nM constructs on Her2/neu-positive (SK-OV-3 and BT-474-M1) and -negative (MDA-MB-468) cells by flow cytometry. (**c**) Internalization analysis on Her2/neu-positive and -negative cells after 4 h treatment of 25 nM immunotoxin. Cells were subjected to immunofluorescent staining with anti-rGel antibody (fluorescein isothiocyanate-conjugated secondary), with propidium iodine nuclear counterstaining. (**d**, **e**) Western blot analysis of intracellular behavior of 25 nM immunotoxin in SK-OV-3 and BT-474-M1 cells. Relative density of total rGel signal and free rGel signal normalized to the β-actin protein-loading control.

## Cellular uptake and toxin delivery of immunotoxins

We next examined the comparative ability of the immunotoxins to internalize into SK-OV-3, BT-474-M1 and MDA-MB-468 cells. As shown in Figure 2c, after 4 h of exposure, the rGel moiety of all the immunotoxins was observed primarily in the cytosol after treatment of SK-OV-3 or BT-474-M1 cells, but not MDA-MB-468 cells. This demonstrated that all constructs were comparable in efficient internalization after exposure to Her2/neu-positive cells. The internalization efficiency of all the immunotoxins was further examined by time-dependent western blot analysis on total rGel signal (full-length immunotoxin + free rGel; Figures 2d and e). The Herceptin/rGel conjugate showed the fastest and highest internalization efficiency into target cells. Both 4D5/rGel and rGel/4D5 internalized rapidly into SK-OV-3 cells. In contrast, rGel/4D5 internalized much faster than 4D5/rGel into BT-474-M1 cells. Compared with 4D5/rGel, rGel/4D5 displayed a long-lasting increase in cell internalization.

The intracellular release of rGel after endocytosis of various constructs was assessed on free rGel signal (Figures 2d and e). For the Herceptin/rGel conjugate, there was a rapid initial delivery of free rGel to the cytoplasm within the first hour after drug exposure. The Herceptin/rGel conjugate delivered the greatest amount of rGel to both SK-OV-3 and BT-474-M1 cells. As for monovalent constructs, both fusions displayed similar initial delivery of free rGel in target cells, but rGel/4D5 orientation construct delivered constantly high levels of rGel compared with 4D5/rGel.

## *In vitro* cytotoxicity of immunotoxins

The cytotoxic effects of the constructs were then tested against a variety of different tumor cell lines. As shown in Table 1, all the immunotoxins demonstrated specific cytotoxicity to cells expressing +3 and +4 levels of Her2/neu. Targeting indices ranged from 54 to 5120 and from 21 to 2364 for Herceptin/rGel conjugate and rGel/4D5, respectively. The 4D5/rGel construct was comparatively less potent with targeting indices only as high as 213. Treatment of Herceptin-resistant (HR) BT-474-M1 cells demonstrated no cross-resistance to rGel-based immunotoxins compared with parental BT-474-M1 cells. In addition, MCF-7 cells transfected

**Table 1.** Comparative $IC_{50}$ values of fusion constructs against various types of tumor cell lines

| *Cell line* | *Type* | *Her2//neu level* | *$IC_{50}$ (nM)* | | | | | *Targeting index*[a] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *Herceptin* | *4D5/rGel* | *rGel/4D5* | *Herceptin/rGel conjugate* | *rGel* | *4D5/rGel* | *rGel/4D5* | *Hereceptin/rGel conjugate* |
| SK-BR-3 | Breast | + + + + | 60.52 | 5.10 | 0.36 | 0.17 | 851.14 | 167 | 2364 | 5120 |
| BT-474-M1 | Breast | + + + + | 50.35 | 26.53 | 0.74 | 0.17 | 457.09 | 17 | 618 | 2689 |
| BT-474-M1 (HR) | Breast | + + + + | 7930.49 | 91.92 | 0.54 | 0.13 | 315.28 | 3 | 582 | 2425 |
| MCF-7/Her2 | Breast | + + + + | 8128.31 | 32.36 | 0.28 | 0.11 | 263.03 | 8 | 939 | 2391 |
| NCI-N87 | Gastric | + + + + | 668.21 | 2.37 | 0.16 | 0.21 | 505.48 | 213 | 3159 | 2407 |
| SK-OV-3 | Ovarian | + + + + | 1026.80 | 70.86 | 0.54 | 0.28 | 501.19 | 23 | 928 | 1790 |
| Calu-3 | Lung | + + + + | 4015.33 | 21.62 | 0.20 | 0.18 | 457.09 | 20 | 2285 | 2539 |
| MDA-MB-453 | Breast | + + + | 9120.11 | 543.70 | 1.62 | 0.79 | 435.70 | 21 | 269 | 552 |
| MDA-MB-361 | Breast | + + + | 9549.93 | 776.25 | 31.62 | 12.30 | 660.69 | 1 | 21 | 54 |
| MDA-MB-435S | Breast | + + | ND | 120.64 | 111.15 | 181.68 | 364.33 | 3 | 3 | 2 |
| BT-20 | Breast | + + | ND | 429.14 | 284.97 | 174.38 | 232.22 | 1 | 1 | 1 |
| ZR-75-1 | Breast | + + | ND | 5903.00 | 6005.10 | 6555.07 | 3473.20 | 1 | 1 | 1 |
| A-431 | Epidermoid | + | ND | 98.31 | 106.68 | 213.99 | 167.38 | 2 | 1 | 1 |
| MCF-7 | Breast | + | – | 595.39 | 322.18 | 222.89 | 197.15 | ND | ND | 1 |
| MDA-MB-231 | Breast | + | ND | 526.62 | 349.70 | 571.48 | 447.10 | 1 | 1 | 1 |
| MDA-MB-468 | Breast | – | ND | 335.66 | 364.33 | 447.10 | 485.18 | 1 | 1 | 1 |

Abbreviations: $IC_{50}$, half-maximal inhibitory concentration; ND, not determined. [a]Targeting index represents $IC_{50}$ of rGel/$IC_{50}$ of immunotoxin.

with Her2/neu (MCF-7/Her2) showed increased resistance to chemotherapeutic agents (Supplementary Figure S2), but showed increased sensitivity to these constructs. The cytotoxicity of Herceptin/rGel conjugate and rGel/4D5 outperformed that of 4D5/rGel, which resulted from the improved internalization and intracellular rGel delivery. Antigen-negative cells or those expressing + 1 and + 2 levels of Her2/neu were not specifically targeted by any of the constructs.

### Effects of immunotoxins on Her2/neu-related signaling pathways

We next examined the mechanistic effects of the constructs on Her2/neu-related signaling events in SK-OV-3 cells. As shown in Figure 3a, treatment with Herceptin/rGel conjugate and rGel/4D5 resulted in an impressive inhibition of phosphorylation of Her2/neu, epidermal growth factor receptor (EGFR), Akt and extracellular signal-regulated kinase (ERK), which are critical events in the Her2/neu signaling cascade. In contrast, 4D5/rGel showed a comparatively reduced effect on these pathways. Treatment with these immunotoxins resulted in reduction in the phosphorylation of insulin-like growth factor 1 receptor (IGF1R), a crosstalk partner of Her2/neu. These results suggest a link between downregulation of IGF1R signaling and the antiproliferative effect of anti-Her2/neu immunotoxins against SK-OV-3 cells. The rGel/4D5 construct was comparatively more cytotoxic to target cells than 4D5/rGel, and the improved cytotoxicity coincided with the increased effects on signal transduction.

### Immunotoxin effects on Her2/neu-driven multidrug resistance

Cells overexpressing Her2/neu often display an increased requirement for the phosphoinositide-3-kinase/Akt signaling pathway in anchorage-independent growth.[23] Currently, we compared the efficacy of chemotherapeutic agents against MCF-7/Her2 cells and parental MCF-7 cells. The MCF-7 cells nominally coexpress Her3 and transfection results in an increased resistance to multiple chemotherapeutic agents (Supplementary Figure S2).[24] However, we didn't observe the cross-resistance of these cells to rGel-based immunotoxins. As shown in Figure 3b, treatment with Herceptin/rGel conjugate and rGel/4D5 caused a dose-dependent inhibition of Her2/neu and Her3 phosphorylation, and inhibition of the downstream phosphoinositide-3-kinase/Akt and Ras/ERK cascade.

### Cytotoxic activity of immunotoxins against HR cells

Acquired resistance to Herceptin therapy can be mediated by concomittent upregulation of Her2/neu downstream signaling pathways and involve constitutive Akt activation, or activation from extrinsic growth factor stimulation. We developed a model of HR variant BT-474-M1 cells. We also demonstrated that addition of EGF or neuregulin-1 growth factors to parental BT-474-M1 cells can prevent the cytotoxic response to Herceptin (Supplementary Figure S3). However, the treatment of rGel-based immunotoxins displayed impressive cytotoxic effects against all these HR cells (Supplementary Figure S4).

Mammary BT-474-M1 cells in anchorage-independent three-dimensional (3D) culture have been shown to organize into structures resembling *in vivo* architecture. We examined the growth of BT-474-M1 and the HR cells in response to immunotoxins under the 3D growth on basement membrane. As shown in Figure 4a, all the immunotoxins demonstrated impressive growth inhibition in these 3D models. Treatment with Herceptin/rGel conjugate or rGel/4D5 showed potent cytotoxic effects of both Herceptin-sensitive and HR models, whereas 4D5/rGel only partially inhibited cell growth (Figure 4b).

We further investigated the signaling pathway of Her2/neu and its crosstalk receptors underlying the inhibitory potential of immunotoxins (Figure 4c). In either Herceptin-sensitive or HR cells, treatment with immunotoxins led to a potent blockade of phosphorylation of EGFR, Her2/neu and Her3. Similar results were found for inactivation of downstream Akt and ERK in these models. As indicated, Herceptin/rGel conjugate and rGel/4D5 were the most potent at inhibiting phosphorylation of these targets, whereas 4D5/rGel showed comparatively reduced effects.

### Activity of immunotoxins against cells expressing intermediate levels of Her2/neu

Previous studies have suggested that targeting of tumors with antibodies or immunotoxins to Her2/neu may result in unexpected organ toxicities due to low levels of Her2/neu expressed on normal tissues.[25,26] We tested the immunotoxins on ZR-75-1 and BT-20 cells expressing intermediate levels of Her2/neu ($\sim 1.5 \times 10^5$ sites per cell).[27,28] As shown in Supplementary Figure S5A, all the immunotoxins were shown to bind to these

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