HER2-Affitoxin: A Potent Therapeutic Agent for the Treatment of HER2-Overexpressing Tumors

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Abstract

Purpose: Cancers overexpressing the HER2/neu gene are usually more aggressive and are associated with poor prognosis. Although trastuzumab has significantly improved the outcome, many tumors do not respond or acquire resistance to current therapies. To provide an alternative HER2-targeted therapy, we have developed and characterized a novel recombinant protein combining an HER2-specific Affibody and modified Pseudomonas aeruginosa exotoxin A (PE 38), which, after binding to HER2, is internalized and delivered to the cytosol of the tumor cell, where it blocks protein synthesis by ADP ribosylation of eEF-2.

Experimental Design: The effect of the Affitoxin on cell viability was assessed using CellTiter-Glo (Promega). To assess HER2-specific efficacy, athymic nude mice bearing BT-474 breast cancer, SK-OV-3 ovarian cancer, and NCI-N87 gastric carcinoma xenografts were treated with the Affitoxin (HER2- or Tag-specific), which was injected every third day. Affitoxin immunogenicity in female BALB/c mice was investigated using standard antibody production and splenocyte proliferation assays.

Results: In vitro experiments proved that HER2-Affitoxin is a potent agent that eliminates HER2-overexpressing cells at low picomolar concentrations. Therapeutic efficacy studies showed complete eradication of relatively large BT-474 tumors and significant effects on SK-OV-3 and NCI-N87 tumors. HER2-Affitoxin cleared quickly from circulation (T1/2 < 10 minutes) and was well tolerated by mice at doses of 0.5 mg/kg and below. Immunogenicity studies indicated that HER2-Affitoxin induced antibody development after the third injected dose.

Conclusions: Our findings showed that HER2-Affitoxin is an effective anticancer agent and a potential candidate for clinical studies. Clin Cancer Res; 17(15); 1–11. ©2011 AACR.

Introduction

HER2 is a tyrosine kinase receptor belonging to the epidermal growth factor receptor (EGFR) family (1, 2).

When overexpressed in tumor cells, HER2 constitutively triggers activation of the cell signaling network, leading to upregulated transcription of genes that drive cellular proliferation, migration, differentiation, and angiogenesis, as well as apoptosis suppression/cell survival (3).

Affibody molecules are a new class of relatively small (~7 kDa) affinity proteins, that are structurally based on a 58-kDa protein A, and are obtained by combinatorial protein engineering (4, 5). HER2-specific Affibody molecules strongly bind to their target (Kd = 22 pmol/L) without changing the receptor activation status (6). It was previously reported that Affibody molecules are capable of being labeled with radionuclides including 99mTc, 111In, 68Ga, 90Y, 125I, and 18F (7–13), optical beacons (14–16), or reporter enzymes (15). The aforementioned probes have been successfully applied to characterize HER2 expression in vitro as well as in xenografts.

Previously, we have successfully cloned, expressed, purified, and characterized Affitoxin, an HER2-specific tumoricidal agent (HER2-Affitoxin) consisting of an Affibody molecule as a targeting modality and a modified Pseudomonas exotoxin A (PE38) as an effector (17). On the basis of the well-described Pseudomonas aeruginosa cytotoxic pathway, it is assumed that after binding to HER2, at least a...
fraction of the HER2-Affitoxin pool is internalized and redistributed to the cytosol, where it acts as an ADP ribosylating agent for eEF-2. This in turn blocks the enzymatic activity of eEF-2 and hinders protein synthesis on the ribosomal level (18, 19).

We have shown that HER2-Affitoxin was expressed as soluble protein and can be purified to homogeneity after 2 chromatographic steps. It binds to HER2 with high affinity ($K_d \sim 1 \text{ nmol/L}$), and the binding epitope is distinct from that being recognized by trastuzumab. HER2-Affitoxin binding is well correlated with the expression of HER2. Our previous in vitro toxicity studies confirmed that cells expressing high levels of HER2 are more sensitive to HER2-Affitoxin than cells with low receptor numbers, and the excess of Affibody molecules competing with HER2-Affitoxin for receptor binding prevents induction of cell death (17).

In this study, in vivo characterization of HER2-Affitoxin was carried out including pharmacokinetics and biodistribution analyses of the drug. This was followed by efficacy evaluation in a mouse model bearing HER2-positive breast, ovarian, or gastric cancer tumors. Finally, assuming that repeated drug administration might become inevitable during the therapy design, the ability of HER2-Affitoxin to induce immune response was evaluated.

**Material and Methods**

**Cloning and purification of Affitoxins**

HER2-Affitoxin was expressed and purified as described recently (17). Briefly, an isopropyl $\beta$-D-1-thiogalactopyranoside (IPTG)-induced culture of One Shot BL21 Star (DE3) cells (Invitrogen) transformed with pAFtXKDEL31 plasmid was lysed by ultrasonication. Soluble fractions of protein were subjected to purification by the AKTA Purifier 10 Chromatographic System (Amersham Bioscience) using an Ni-affinity HisTrap and anion exchange HiTrap Q column (Amersham Bioscience), followed by buffer exchange to PBS using sample ultrafiltration on an Amicon Ultra centrifugal filter device with a 30-kDa cutoff membrane (Millipore). The last step of purification involved endotoxin removal, which was carried out according to the provided protocol (ToxinEraser Endotoxin Removal Kit, GenScript). The endotoxin level was measured using the ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript). Protein concentration was measured using the BCA Protein Assay Kit (Pierce) according to the provided protocol.

Non–HER2-specific Affitoxin containing an Affibody against bacterial Taq polymerase ($Z_{\text{TaqS1,1}}$-Affibody), called Taq-Affitoxin, was cloned according to the following procedure. Plasmid pAfTxKDEL31 was used as a template for PCR to amplify the PE38 toxin moiety using primers 60-3 and T7term70 (Supplementary Table S1). The $Z_{\text{TaqS1,1}}$ moiety was PCR amplified using the pAfTxKDEL31 template and primers 5BSal-His, 60-1c, and 60-2c (Supplementary Table S1). The resulting PCR products were gel purified and combined by megaprimer PCR using primers 5BSal-His and T7term70. The megaprimer PCR product containing DNA coding for the $Z_{\text{TaqS1,1}}$-PE38 fusion, and the pET23d vector (EMD Chemicals) were separately digested with Ncol and HindIII restriction endonucleases (New England Biolabs), gel purified, and ligated and then transformed into XL10-Gold competent cells (Agilent Technologies). The resulting pNT3 plasmid, which contains the predicted nucleotide sequence, was transformed into One Shot BL21 Star (DE3) cells (Invitrogen), and the protein was processed according to the protocol used for purifying HER2-Affitoxin, as described above.

**HER2-Affitoxin modification**

For near-infrared (NIR) optical imaging, HER2-Affitoxin was labeled with DyLight 750 (Pierce) using maleimide chemistry to attach the dye to the C-terminal cysteine. First, HER2-Affitoxin was reduced by incubation in Tris(2-carboxyethyl)phosphine (TCEP) for 30 minutes at 4°C and then mixed with 4-fold excess of DyLight 750 maleimide derivative. The labeled HER2-Affitoxin was repurified on a HisTrap column to remove nonbound dye. After purification, the buffer was changed to PBS and, finally, the conjugate was sterilized by 0.22-μm filtration. The same protocol was applied for labeling HER2-Affibody molecules containing C-terminal cysteine.

**Cell culture**

Human breast cancers BT-474 and MDA-MB-468, gastric carcinoma NCI-N87, and ovarian carcinoma SK-OV-3 cell lines were obtained from the American Type Culture Collection. An SK-OV-3-luc-D3 cell line was purchased from Caliper Life Sciences. The cells were grown in RPMI (BT-474, NCI-N87) or DMEM/F12 (SK-OV-3 and MDA-MB-468) culture media supplemented with 10% FBS and
LD50 value was calculated by probit analysis in StatPlus. The postinjection period were taken into consideration. The indicated doses of HER2-Affitoxin by intravenous injection into the tail vein. Any reported death cases or morbid conditions that occurred within the 2-week period were excluded. The cell viability was assessed using CellTiter-Glo (Promega). The IC50 values were calculated using GraphPad Prism software (GraphPad Software, Inc.).

The minimal time necessary to affect the HER2-positive population was determined as follows: preplated NCI-N87 cells were treated with HER2-Affitoxin or Taq-Affitoxin for various exposure times, washed 3 times with medium, and incubated for additional time up to 72 hours. The cells' viability (assessed as above) was expressed as the percentage of viable cells in a reference to the nontreated control group.

Animals
Female athymic nude mice (nu/nu genotype, BALB/c background) and BALB/c mice, 5 to 8 weeks old, were purchased from the Animal Production Program (NCI-Frederick). This study was approved by the Animal Safety and Use Committee of NIH and carried out in accordance with the Department of Health and Human Services' Guide for the Care and Use of Laboratory Animals.

Non–HER2-specific toxicity
BALB/c mice (n = 3–10 per group) were administered the indicated doses of HER2-Affitoxin by intravenous injection into the tail vein. Any reported death cases or morbid conditions that occurred within the 2-week postinjection period were taken into consideration. The LD50 value was calculated by probit analysis in StatPlus 2009 software (AnalytSoft).

The liver toxicity of HER2-Affitoxin was investigated in athymic mice receiving 6 injections of the drug (administered intravenously, 0.25 mg/kg each, every third day). Blood samples were obtained by submandibular bleeding and collected in heparinized tubes. Plasma samples were analyzed using a liver panel test carried out by the NCI Pathology/Histotechnology Laboratory (SAIC-Frederick, Inc., NCI-Frederick).

Pharmacokinetics of HER2-Affitoxin
Three athymic mice were intravenously administered 0.5 mg/kg of HER2-Affitoxin. A total of 20 to 30 μL of blood was withdrawn at 1, 5, 15, 30, 60, and 120 minutes postinjection. At 5 hours postinjection, the mice were anesthetized and terminal bled. All samples were incubated for 15 minutes in EDTA-coated tubes and centrifuged at 2,000 × g for 3 minutes. Plasma was stored at −30°C for further analysis.

The concentration of HER2-Affitoxin in plasma was measured by cell-based ELISA. NCI-N87 cells (7.5 × 10⁴ cells per well) seeded on a 96-well plate were allowed to attach for 24 hours and then fixed in 4% buffered parafomaldehyde for 20 minutes at room temperature. After 3 washing steps (PBS + 2% FBS, 5 minutes each), the cells were exposed to 20 μL of HER2-Affitoxin (concentration range: 2 pg/mL–1 μg/mL) or plasma samples diluted 1:50. Incubation was carried out at room temperature with intensive shaking for 1 hour. HER2-Affitoxin detection was conducted using anti–Pseudomonas exotoxin A polyclonal antibody (Sigma-Aldrich) and anti-rabbit IgG conjugated with horseradish peroxidase (Millipore). Chemiluminescent signal was recorded using a FLUOStar Optima plate reader (BMG Labtech) 10 minutes after incubation with LumiGLO Peroxidase Chemiluminescent Substrate (KPL). The half-life and initial concentration of the drug were estimated using GraphPad Prism Software.

In addition, the residual activity of HER2-Affitoxin present in the plasma as a function of time was tested by 72-hour in vitro exposure of NCI-N87 cells to the plasma samples obtained from mice at different time points post-injection. Following this treatment, the viability of the cells was assessed by CellTiter-Glo (Promega).

Biodistribution studies
Biodistribution of DyLight 750–labeled Affitoxin was studied using a subcutaneous BT-474 tumor model, the previously described NIR fluorescence small-animal imager (20) and IVIS Lumina (Caliper Life Sciences).

Immunohistochemistry
Immunohistochemical service was provided by the Pathology/Histotechnology Laboratory, SAIC-Frederick, Inc. Briefly, tissue specimens from xenografts were fixed in 10% neutral-buffered formalin. Five-μm paraffin-embedded sections were immobilized on positively charged slides and Affibody staining was conducted on Leica Microsystems Bond Autostainer (Leica), followed by a citrate buffer antigen-retrieval step. After a blocking step in 2% normal rabbit serum (Vector Laboratories), slides were exposed to goat anti-Affibody antibody (Abcam) for 30 minutes at a dilution of 1:100. Signal detection was conducted using a Bond Intense R Detection Kit (Leica) after a 1-hour incubation with biotinylated rabbit anti-goat IgG (Vector Laboratories), at a dilution of 1:100. HER2 detection was conducted after immobilized slides were deparaffinized and rehydrated using a DAKO HercepTest Kit (Dako). Both Affibody- and HER2-stained slides were contrasted according to Gill’s hematoxylin staining protocol.

In vivo efficacy studies
Therapeutic efficacy studies were carried out using BT-474, SK-OV-3, and NCI-N87 subcutaneous xenografts expressing high levels of HER2. The tumors were initiated by subcutaneous injection of 5 × 10⁶ cells, which were suspended in 0.1 mL of 30% Matrigel solution (BD Biosciences), into the right forelimb of athymic mice. BT-474 growth was facilitated by implanting estrogen pellets.
(0.72 mg, 90-day release, Innovative Research of America) 24 hours prior to inoculation. Tumor dimensions were measured periodically using calipers, and their volumes were calculated using the formula: \(4/3 \times \pi \times \text{length} \times \text{width} \times \text{depth}/8\). To investigate the efficacy of HER2-Affitoxin on disseminated disease, an intraperitoneal tumor model using SK-OV-3-luc-D3 cells, expressing the firefly luciferase gene, was established by intraperitoneal injection of \(5 \times 10^6\) cells suspended in 0.5 mL of PBS. Tumor growth was monitored by bioluminescent imaging using the IVIS Lumina Imaging System (Caliper Life Sciences) 8 minutes post–intraperitoneal injection of \(\nu\)-luciferin (75 mg/kg).

HER2-Affitoxin or Taq-Affitoxin, diluted in 100 \(\mu\)L of saline, was administered by tail vein injection. Body weight was monitored during the treatment.

**Immunogenicity studies**

**Antibody production.** Female BALB/c mice were randomized into 3 groups receiving saline or HER2-Affitoxin (0.25 mg/kg). Four mice of each group were terminally bled by cardiac puncture 10 days after each dose injection. The development of anti-HER2-Affitoxin toxin antibodies was analyzed by ELISA using immobilized recombinant HER2-Affitoxin as an antigen. Five micrograms of purified recombinant protein was added to each well of a Nunc Maxisorp 96-well microtiter plate (e Bioscience) and adhered overnight at 4 °C. The unbound protein was washed away with PBS-T and blocked for 2 hours with PBS-T containing 1% bovine serum albumin (BSA). Plasma samples were serially diluted, and 100 \(\mu\)L of the resulting solution was added in triplicates to the appropriate wells. Following a 3-hour incubation, plates were washed 8 times with PBS-T/1% BSA, and 100 \(\mu\)L of a 1:2,000 dilution of peroxidase-conjugated rabbit anti-mouse IgG (Millipore) was added to each well. The plates were then incubated at room temperature for an additional 2 hours. Next, the plates were washed twice and color development was achieved using e-phenylenediamine (Sigma-Aldrich) prepared in a phosphate-citrate buffer with 30% hydrogen peroxide (Sigma-Aldrich). After the addition of 50 \(\mu\)L H2SO4 (R&D Systems) to each well, absorbance was measured at 490 nm. The relative generation of HER2-Affitoxin-specific antibodies over time was determined by calculating the absorbance ratio of the specific toxin divided by the absorbance from mice receiving saline alone.

**Proliferation assays.** Female BALB/c mice were randomized into 3 groups receiving saline and HER2-Affitoxin (1, 2, or 3 doses of 0.25 mg/kg each, 2 weeks apart). Spleens were harvested from 4 mice in each group 10 days after injecting each consecutive dose. Isolated splenocytes were resuspended in complete Dulbecco's modified Eagle's media (DMEM) containing 20% PBS and were restimulated with recombinant HER2-Affitoxin in an *in vitro* proliferation assay to assess recall responses to the toxin. Briefly, 300,000 splenocytes were plated into a 96 U-bottom plate (100 \(\mu\)L/well) in triplicates. Cells were stimulated with various concentrations of HER2-Affitoxin (0–125 \(\mu\)g/mL). Treatment with concanavalin A (5 \(\mu\)g/mL), lipopolysaccharide (1 \(\mu\)g/mL), and phorbol-12-myristate-13-acetate (PMA) + ionomycin (10 ng/mL + 0.5 \(\mu\)mol/L) served as positive controls for the proliferative ability of splenocytes. Wells were pulsed with 1 \(\mu\)Ci of \[^{3}H\]thymidine (Amersham Bioscience) during the last 16 hours of the 72-hour culture. The cells were then lysed using a Cell Harvester (Tomtech), and radioactivity was measured in a Wallac Trilux MicroBeta liquid scintillation counter (Perkin Elmer). The stimulation index (SI) for the samples was calculated by dividing the average counts per minute (CPM) of cells + stimulation by the average CPM of unstimulated cells.

**Results**

**Taq-Affitoxin**

This off-target Affitoxin analogue contains Affibody molecules directed against Taq polymerase (ZTaqS1-1 Affibody molecule) at its N-terminus (21). The sequence of both Affitoxins differs by only 12 amino acids located in the binding site of Affibody molecules (Supplementary Table S2). Like HER2-Affitoxin, Taq-Affitoxin was expressed in the soluble fraction of an *Escherichia coli* protein and purified almost to homogeneity after 2 chromatographic steps: nickel affinity and ion exchange (Fig. 1A). In addition, before injection into animals, both Affitoxins were subjected to an endotoxin removal protocol. HER2-Affitoxin and Taq-Affitoxin were immunoreactive to anti-Affibody and anti-*Pseudomonas* exotoxin A antibodies (Supplementary Fig. S1).

**In vitro studies**

Data obtained from a toxicity assay using NCI-N87 cells, which expressed a high level of HER2, showed that HER2-Affitoxin was significantly better at killing NCI-N87 cells compared with the off-target toxin. The IC50 values obtained from measurements of residual ATP levels following exposure to increasing concentrations of either HER2-Affitoxin or Taq-Affitoxin indicated that the former was 30,000 times more potent in inducing cell death than its off-target analogue (Fig. 1B).

Next, we tested the minimal exposure time for the toxin that is sufficient to eliminate HER2-expressing cells. Exposure to HER2-Affitoxin for as short as 1 minute, followed by drug removal and an additional 72-hour incubation period, resulted in the inactivation of nearly 90% of HER2-overexpressing NCI-N87 cells. In contrast, the whole population of cells treated with the Taq-Affitoxin remained fully viable after 30 to 60 minutes of exposure, and more than 6 hours of exposure was needed to obtain a cell death rate similar to that resulting from 1-minute exposure to HER2-Affitoxin. A similar toxicity pattern was observed for HER2-negative cells, MDA-MB-468, treated both with HER2-specific or HER2 nonspecific Affitoxins (Fig. 1C).

**Pharmacokinetics of HER2-Affitoxin and acute toxicity**

Pharmacokinetics data obtained by cell-based ELISA indicated that the half-life of HER2-Affitoxin in the
bloodstream is 8.69 ± 1.31 minutes (Supplementary Fig. S2) and 5.5 ± 0.53 minutes, as estimated by residual plasma toxicity (Supplementary Fig. S3). The initial concentration of HER2-Affitoxin in the plasma was 6.87 ± 0.52 μg/mL, which corresponded to the injected dose of the drug.

To assess its toxicity in BALB/c mice, HER2-Affitoxin was administered by bolus intravenous injection into the tail vein. As listed in Supplementary Table S3, a 100% mortality rate was recorded for mice injected with 4, 2, and 1 mg/kg of the drug. One of 6 mice survived the treatment with 0.625 mg/kg. All death cases were reported within 72 hours postinjection. No mortality was observed in groups treated with 0.5 or 0.25 mg/kg. The calculated LD50 value was 0.572 ± 0.051 mg/kg.

**Biodistribution of HER2-Affitoxin**

Images were taken 2 hours postinjection of fluorescently labeled HER2-Affitoxin into mice bearing BT-474 tumors and showed an accumulation of fluorescence in the kidneys and the liver. A significantly lower but still over-the-background signal was detected in the tumors (Fig. 2A and C). All animals showed similar accumulation patterns, confirmed by postmortem fluorescence analysis of dissected organs (Fig. 2B). Tumor-to-muscle ratio was approximately 4 for all tested mice (Fig. 2D). Similar biodistribution patterns were observed using the IVIS Lumina Imaging System (Supplementary Fig. S4). Affitoxin accumulation in the tumor was further confirmed by immunostaining BT-474 tumor sections extracted 2 hours post–HER2-Affitoxin injection (Fig. 2E), whereas no signal was detected in saline-injected animals (Supplementary Fig. S5A). Both samples showed positive staining for HER2 receptors (Fig. 2F and Supplementary Fig. S5B).

**Efficacy of HER2-Affitoxin**

Mice bearing subcutaneous BT-474 tumors were divided into 3 experimental groups treated with (i) saline, (ii) Taq-Affitoxin, and (iii) HER2-Affitoxin. The drugs were injected in 6 fractions every third day to the total dose of 1.5 mg/kg. The average initial volume of the BT-474 tumors was 400 mm³ and continued to grow in both the vehicle-treated control group (saline) and in mice injected with Taq-Affitoxin. By day 24, animals in these groups had to be sacrificed because tumor sizes were approaching the dimension, which, according to Animal Care and Use Committee recommendations and our protocol, mandates euthanasia. Conversely, mice treated with HER2-Affitoxin showed immediate reduction in tumor size. Three days after the first dose of HER2-Affitoxin injection, tumors shrunk on average to 60% of their initial size (Fig. 3A). By the end of the treatment, the remaining tumor volume in HER2-Affitoxin–treated mice was barely 5% of that measured before the treatment. Moreover, the animals injected with HER2-Affitoxin did not show tumor regrowth within the following 76 days (Fig. 3A). All animals tolerated the treatment well and showed only slight (~10%) weight loss.
After the course of treatment (6 x 0.25 mg/kg every third day) was completed, liver enzyme levels were analyzed to assess the potential hepatotoxicity of the drug. The results revealed that the average alanine aminotransferase (ALT) level in the plasma was approximately 3 times higher in drug-treated animals than in control animals, whereas for aspartate aminotransferase (AST), the ratio was less than 2. No significant differences in the alkaline phosphatase (ALKP) or bilirubin levels were observed (Supplementary Table S4).

It is particularly noteworthy that when mice bearing extremely large tumors (average volume above 1,000 mm³) received six 0.1-mg/kg doses of HER2-Affitoxin, the tumors responded immediately, and the tumor volume, after completion of the treatment, was on average reduced to 100 mm³. These tumors did not regrow during the following 2 weeks (Fig. 3B).

HER2-Affitoxin efficacy was also tested using ovarian cancer xenografts. Three doses of HER2-Affitoxin (0.25 mg/kg, every second day) administered to mice bearing SK-OV-3 tumors (volume < 100 mm³) resulted in a significant delay in tumor growth (P < 0.001). However, 1 month postinjection, the tumors started to regrow. Repeated treatment with HER2-Affitoxin (3 doses of 0.25 mg/kg each, administered every second day) resulted in further growth delay but, unlike the case of BT-474, it did not completely eradicate the tumors (Fig. 3C). In addition, unlike the case of BT-474 tumors, lower doses of HER2-Affitoxin failed to affect the SK-OV-3 tumor growth. As depicted in Figure 3D, treatment of mice bearing NCI-N87 tumors with HER2-Affitoxin at 0.5 and 0.25 mg/kg significantly delayed the tumor growth.

To assess the efficacy of HER2-Affitoxin in treating disseminated cancer, luminescent SK-OV-3 cells were injected into the peritoneal cavity of mice, and the treatment was initiated 1 week later. Two groups of mice received either HER2-Affitoxin or saline (Fig. 4A). Tumor progression was measured every 3 to 4 days with bioluminescence imaging and showed that HER2-Affitoxin significantly slowed the tumor progression (Fig. 4B and C). However, like in the subcutaneous model, the treatment failed to completely eradicate the tumors.

**Immunogenicity of HER2-Affitoxin**

To evaluate the immunogenicity of HER2-Affitoxin, immunocompetent mice received 3 doses of the protein 2 weeks apart (22). The development of HER2-Affitoxin-specific antibodies and the proliferative ability of splenocytes to HER2-Affitoxin were investigated 10 days postinjection of each dose. After the first and second administration, anti-HER2-Affitoxin antibody levels were similar to vehicle-only control animals. A considerable
In contrast, only limited antigen-specific proliferation, as measured in recall assays, was observed in mice after the first injection of HER2-Affitoxin, second or third dose of the drug did not lead to increase the stimulation index (Supplementary Fig. S6). The limited proliferative response to HER2-Affitoxin was not due to toxicity, as preliminary studies showed that HER2-Affitoxin did not appear to be toxic for splenocytes at tested concentrations. As shown in Supplementary Figure S7, HER2-Afitoxin at 500 ng/mL (≈10 nmol/L) reduced splenocytes viability by only 25%, whereas picomolar concentrations were sufficient to eliminate HER2-positive tumor cells (Fig. 1B). In addition, these cells proliferated rigorously in response to mitogens and PMA + ionomycin stimulations, showing the proliferating capabilities of the cells (data not shown).

Discussion

The amplified HER2/neu gene and/or the overexpressed protein have been identified in approximately 20% of invasive breast and non–small lung carcinoma, as well as in ovarian carcinomas and B-cell acute lymphoblastic leukemia (23, 24). Particularly in breast cancer, elevated HER2 status is associated with increased proliferation and survival of cancer cells and contributes to poor therapy outcomes and unfavorable prognosis (25, 26).

Although the clinical use of trastuzumab, a humanized HER2-targeted antibody (27–29), has significantly improved treatment outcome, a large fraction of tumors do not respond to antibody treatment or develop resistance to therapy (30). Resistant tumors have still been shown to express HER2; therefore, HER2 can be used as a target for directed delivery of other therapeutic agents (31). Because HER2-Affitoxin uses a mechanism of cytotoxicity that is distinct from that of trastuzumab, it is a potential alternative or complementary therapy for patients who do not benefit from the traditional therapeutic approach.

In this study, HER2-Affitoxin appeared to be well tolerated by animals at a dosage of 0.5 mg/kg and below. The calculated LD₅₀ value (0.572 ± 0.051 mg/kg) shows a similar potential to induce acute toxicity, as do monovalent and divalent disulfate-stabilized antibody fragments against HER2 fused to PE38 (dsFv-PE38), according to Bera and colleagues (32).
Biodistribution data obtained in our study showed that HER2-Affitoxin accumulated in the livers of treated animals. Analyzing liver enzyme levels to assess the potential hepatotoxicity of the drug revealed that only moderate toxicity was induced by HER2-Affitoxin treatment (e.g., mice receiving 1 dose of acetaminophen, 300 mg/kg, by oral gavage had an ALT level ~400 times higher than the control group’s level; ref. 33). Moreover, the treated mice did not show significant weight loss during the treatment and survived more than 2 months without any signs of organ dysfunctions.

Recently, Weldon and colleagues reported that deleting a significant portion of the domain II of PE38 resulted in a lysosome-resistant immunotoxin with 10-fold decreased nonspecific toxicity. This new toxin, called HA22-LR, retained excellent biological activity against CD22-positive leukemia cells and showed superior activity in animal models (34). It is likely that similar modifications to the PE38 portion of our HER2-Affitoxin molecule could result in comparable improvements.

HER2-Affitoxin showed a fast rate of clearance from the bloodstream after intravenous administration. The obtained values were comparable to those reported by Bera and colleagues for the anti-HER2, monovalent e23 dsFv-PE38 fusion (32). This observation, along with biodistribution data (Fig. 2), strongly suggests that the filtration through kidney glomerulus is the main clearance mechanism and seems to be consistent with the HER2-Affitoxin size (46 kDa). On the other hand, relatively fast clearance of the drug from the blood raised the question of whether the drug would be able to reach the tumor tissue and exert its cytotoxic effect. Our in vitro studies showed that HER2-Affitoxin has a high affinity to the receptor (17), which facilitates immediate binding to the cell surface,
followed by slower internalization, and, finally induced cell death. Indeed, HER2-Affitoxin killed nearly 90% of the receptor-positive cell population after 1 minute of exposure, whereas Taq-Affitoxin required much more time to exert the same effect. Similarly, cells with no receptor expression remained unaffected by both specific and nonspecific toxins at up to 1 hour of exposure (Fig. 1). These findings suggest that although HER2-Affitoxin is cleared relatively quickly from circulation, it should still be effective in vivo. Moreover, normal cells with low HER2 levels should remain unaffected by the toxin.

Biodistribution analysis conducted by NIR optical imaging confirmed that an HER2-Affitoxin load given intravenously could be successfully delivered to BT-474 tumors. Tumor immunostaining confirmed retention of HER2-Affitoxin on the cell membrane; however, distribution was not even throughout the tumor tissue (Fig. 2E). For comparison, BT-474 tumors extracted from mice injected with HER2-Affibody showed an even distribution of the signal throughout the tissue section (Supplementary Fig. S8). This observation is not surprising when the sizes of HER2-Affitoxin and HER2-Affibody are taken into consideration. Approximately 5 times smaller than HER2-Affitoxin, HER2-Affibody molecules show better diffusion, resulting in enhanced tumor penetration.

A high level of drug accumulation was found in the kidneys and liver. Retention in the latter organ may be due to the high dose injected into animals to visualize tumor accumulation; however, a slightly elevated level of the liver enzyme suggests that the liver is involved in HER2-Affitoxin metabolism. It has to be mentioned that HER2-Affibody molecules used for HER2-Affitoxin construction do not cross-react with murine receptors (Supplementary Fig. S9). Therefore, in murine models, neither the observed liver and kidney accumulations nor the toxicity can be mediated through HER2. Receptor-dependent toxicity and biodistribution studies of HER2-Affitoxin in normal tissues require the use of other animal models.

**In vivo** studies showed the therapeutic efficacy of HER2-Affitoxin. HER2-Affitoxin treatment not only resulted in delayed growth of SK-OV-3 and NCI-N87 tumors (Fig. 3C and D) but also led to complete remission of relatively large BT-474 subcutaneous tumors. Moreover, the latter tumors did not regrow 2 months following treatment (Fig. 4A). The observed tumoricidal activity of HER2-Affitoxin is mediated by HER2, as mice treated with a non–HER2-specific toxin (Taq-Affitoxin) exhibited the same tumor growth rates as vehicle-injected animals. Interestingly, both SK-OV-3 and NCI-N87 xenografts responded to HER2-Affitoxin treatment when tumor size was relatively small, whereas larger tumors remained unaffected by HER2-Affitoxin (data not shown). This might result from hampered delivery of HER2-Affitoxin, most likely due to poor drug penetration to the tumor tissue combined with the relatively short half-life of HER2-Affitoxin in circulation. Results from our optical imaging studies support this hypothesis. Even a well-responding BT-474 model showed a much lower accumulation of the toxin than HER2-Affibody molecules, which are approximately 5 times smaller and have a longer, 20-minute circulation half-life (Supplementary Fig. S10). On the other hand, BT-474 tumor tissue shows a much higher HER2-Affibody accumulation than what was observed for NCI-N87 or SK-OV-3 tumors, even though, according to our ELISA data, NCI-N87 and SK-OV-3 tumors have higher HER2 expression levels (Supplementary Fig. S10). In our previous work, we showed that, while HER2-Affitoxin binding is strictly correlated with HER2 expression levels, the drug response of the cells did not necessarily follow this pattern (17). This indicates that, even in vivo, other cell line–specific factors affect the toxicity of HER2-Affitoxin. The situation is even more complicated at the tumor model level where other factors, including the development of blood vessels and hydrostatic pressure of interstitial fluid, in the tumor tissue may limit the delivery and efficacy of Affitoxin (35). These microenvironmental characteristics might explain the difference in effectiveness of HER2-Affitoxin against large NCI-N87 and SK-OV-3 tumors.

A potential solution to the problem of limited delivery to the tumor is to combine HER2-Affitoxin treatment with tumor-penetrating peptides. In a recent report published by Sugahara and colleagues, small iRGD peptides binding to αv integrins significantly increased vascular and tissue permeability in a tumor-specific and neuropilin-1–dependent manner (36). Similar findings have been shown using treatments in combination with small molecules (doxorubicin), nanoparticles (nab-paclitaxel and doxorubicin liposomes), or monoclonal antibodies (trastuzumab). In this case, direct conjugation of the peptides to drugs is not necessary because enhanced drug penetration was observed with coadministration (36).

Immunogenicity studies showed that administering HER2-Affitoxin induces humoral immune response but not cellular-mediated immune response. Anti-HER2-Affitoxin antibodies were observed in treated mice. If these antibodies are neutralizing, their generation may limit the effectiveness of repeated treatment cycles. As our effort to improve HER2-Affitoxin therapeutic potency continues, we will consider introducing several mutations into the PE38 part of the protein. As described by Onda and colleagues, replacing hydrophobic amino acids within B-cell epitopes on PE38 resulted in a less immunogenic version of the drug, leaving cytotoxic and antitumor activities uncompromised (22, 37).

Overall, although further studies are needed to investigate the toxicity of HER2-Affitoxin in animals expressing “human-like” (Affibody-binding) HER2 in normal tissues, our data indicate that this molecule is a potent anticancer drug that might prove to be effective against solid tumors in humans. The fact that a dose as low as 0.1 mg/kg was enough to eradicate relatively large tumors, with only mild toxicity observed at higher doses, suggests a therapeutic window broad enough to provide effective and safe treatment of HER2-positive tumors. These promising data will have to be confirmed by clinical trials addressing the
obvious concerns about Affitoxin immunogenicity and possible liver and kidney toxicity.

Disclosures of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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