Target-specific cytotoxic effects on HER2-expressing cells by the tripartite fusion toxin $Z_{HER2:2891}$-ABD-PE38X8, including a targeting affibody molecule and a half-life extension domain

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Abstract. Development of cancer treatment regimens including immunotoxins is partly hampered by their immunogenicity. Recently, deimmunized versions of toxins have been described, potentially being better suited for translation to the clinic. In this study, a recombinant tripartite fusion toxin consisting of a deimmunized version of exotoxin A from *Pseudomonas aeruginosa* (PE38) genetically fused to an affibody molecule specifically interacting with the human epidermal growth factor receptor 2 (HER2), and also an albumin binding domain (ABD) for half-life extension, has been produced and characterized in terms of functionality of the three moieties. Biosensor based assays showed that the fusion toxin was able to interact with human and mouse serum albumin, but not with bovine serum albumin and that it interacted with HER2 (K_D=5 nM). Interestingly, a complex of the fusion toxin and human serum albumin also interacted with HER2 but with a somewhat weaker affinity (K_D=12 nM). The IC_{50}-values of the fusion toxin ranged from 6 to 300 pM on SKOV-3, SKBR-3 and A549 cells and was lower for cells with higher surface densities of HER2. The fusion toxin was found specific for HER2 as shown by blocking available HER2 receptors with free affibody molecule before subjecting the cells to the toxin. Analysis of contact time showed that 10 min was sufficient to kill 50% of the cells. In conclusion, all three regions of the fusion toxin were found to be functional.

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Abbreviations: ABD, albumin binding domain; BSA, bovine serum albumin; EF-2, elongation factor 2; Fv, variable fragment; HER2, human epidermal growth factor receptor 2; HSA, human serum albumin; IMAC, immobilized metal-ion affinity chromatography; K_D, equilibrium dissociation constant; MSA, mouse serum albumin; PBS, phosphate-buffered saline; scFv, single-chain variable fragment; PE38, truncated exotoxin A from *Pseudomonas aeruginosa*

Key words: affibody molecule, immunotoxin, albumin binding domain, PE38, HER2

Introduction

Immunotoxins, consisting of a plant or bacterial toxin coupled to an antibody or antibody fragment, selectively kill tumor cells by specifically recognizing molecular abnormalities on the malignant cell surface. A well-studied toxin is a truncated version of exotoxin A from *Pseudomonas aeruginosa* (PE38), where the natural cell-binding domain (1a) has been deleted (1). It delivers its cytotoxic effect in the cytosol by ribosylating elongation factor 2 (EF-2), preventing proper ribosomal function, which leads to cell death (1). Immunotoxins, including immunoglobulin-based targeting domains in various formats, such as Fv- and scFv of IgG, interacting with different cell surface receptors, have been found efficacious in pre-clinical as well as clinical studies (2). PE38 has also been coupled to targeting domains of non-immunoglobulin origin including designed ankyrin repeat proteins (3) and affibody molecules (4,5) and such fusion toxins has been found efficacious in pre-clinical models of cancer. PE38-based toxins are immunogenic, preventing multiple repeated injections, thus limiting the suitability in cancer treatment regimens. Recently, deimmuniztion efforts to remove B- and T-cell epitopes have been undertaken (6-10), which has successfully identified less immunogenic variants such as PE38X8 (7). Such toxins may potentially be more suited for clinical use.

The human epidermal growth factor receptor 2 (HER2) is a tyrosine kinase receptor that is often overexpressed in many types of cancer, including breast carcinoma (11). Overexpression of HER2 has been found to correlate with a poor patient prognosis and early relapse post-surgery (12). Since the expression levels of HER2 on normal tissues are relatively low, this receptor has been used to differentially target HER2-positive tumors. Current treatment regimens for advanced HER2-positive breast cancer may therefore include the HER2-specific antibody trastuzumab, marketed as Herceptin (13). Recently, an antibody-drug conjugate where trastuzumab has been functionalized with the toxin

emtansine (T-DM1) has also been approved for clinical treatment of patients with advanced HER2-positive breast cancer (14). Thus far, immunotoxins targeting HER2-positive tumors has not reached clinical use; however, based on the success of trastuzumab and T-DM1, it is likely that such immunotoxins could be developed if short-comings related to, for example immunogenicity can be overcome.

Affibody molecules are a class of folded, non-immunoglobulin based affinity ligands based on the triple helical B-domain of staphylococcal protein A (15). They are only 58 amino acids long and devoid of cysteins in their framework. Affibody molecules interacting specifically and with high affinity (in the low nanomolar to picomolar range) with several tumor cell markers including IGF-1R, EGFR, HER2 and HER3 have been described (16-19). In particular, the affibody molecule $Z_{HER2:2891}$ have been found to specifically interact with the HER2 receptor with an equilibrium dissociation constant (K_D) of 60 pM (20). $Z_{HER2:2891}$ is a derivative of $Z_{HER2:342}$ (18) that has increased melting point, stability, and overall hydrophilicity. The suitability of $Z_{HER2:2891}$ for use in man has been documented by a clinical study where HER2-positive tumors were visualized by radionuclide molecular imaging (21). When used for radionuclide molecular imaging, the small size of the affibody molecules enable very rapid clearance of unbound tracers. This provides a low background for the affibody-based imaging. A short residence time in circulation for affibody-based fusion toxins has similarly been shown, but in that case necessitated multiple injections (5). Thus, an extension of *in vivo* half-life of affibody-based fusion toxins would provide a clinical benefit. Streptococcal Protein G includes three motifs with high affinity for albumin from several species (22). One of them, the GA148-GA3 domain, consists of 46 amino acids and has a cysteine-free three-helix structure. This albumin binding domain (ABD) has been engineered to yield variants such as ABD_{035} with femtomolar affinity for human serum albumin as well as high affinity for serum albumin from rat, mouse and cynomolgus monkey (23). Inclusion of an albumin binding domain in a fusion protein causes a strong non-covalent interaction with serum albumin when injected into the blood (24,25). This has been shown to increase the serum half-life, both by increasing the molecular weight of the complex and by allowing serum albumin-mediated interaction with the neonatal Fc receptor (FcRn), leading to rescue from lysosomal degradation (26,27).

To further extend the potential of a deimmunized and truncated version of *Pseudomonas* exotoxin A (PE38X8) for targeted strategies, a novel tripartite fusion toxin, including PE38X8 fused to the HER2-binding affibody molecule $Z_{HER2:2891}$ and the half-life extension albumin binding domain ABD_{035} was evaluated. The interactions with HER2 and serum albumins from different species were analyzed and the cytotoxic potential was determined.

Materials and methods

General. All chemicals were from Merck (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. DNA modifying and restriction enzymes were from New England Biolabs (Ipswich, MA, USA).

Gene construction. An expression vector encoding His_6-$Z_{HER2:342}$-PE38 (4) was a kind gift from Jacek Capala. The gene enoding $Z_{HER2:2891}$-ABD-PE38X8 with the N-terminal amino acid sequence HEHEHE and $(S_4G)_3$ linkers connecting the three domains in the expression vector pET-26b(+) (Merck) was obtained from GenScript USA Inc. (Piscataway, NJ, USA). The construct was fitted with *Nde*I and *Bam*HI restriction sites surrounding $Z_{HER2:2891}$ and two *Nco*I restriction sites surrounding the ABD domain. The albumin binding domain (ABD) used was ABD_{035}, a version engineered for high affinity to human serum albumin (23). PE38X8 was a deimmunized version of PE38 with the following amino acid alterations: R313A, Q332S, R432G, R467A, R490A, R513A, E548S, K590S (7). The expression vector for Z_{Taq}-ABD-PE38X8 was created by PCR amplification of Z_{Taq} (28) followed by replacement with $Z_{HER2:2891}$ in pET-26b(+) encoding $Z_{HER2:2891}$-ABD-PE38X8, using the *Nde*I and *Bam*HI restriction sites. Expression vectors for $Z_{HER2:2891}$-PE38X8 and Z_{Taq}-PE38X8 were created by digestion of $Z_{HER2:2891}$-ABD-PE38X8 and Z_{Taq}-ABD-PE38X8, respectively, with *Nco*I followed by religation of the vectors. All constructs were verified by DNA-sequencing.

Protein expression and purification. *Escherichia coli* [Rosetta (DE3) pLysS] (Merck) was used for expression of the fusion toxins essentially according to the manufacturer's protocol. Cells harboring the expression plasmids were grown at 37˚C until OD_{600} reached 1.5 after which protein expression was induced by addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Protein expression was carried out for 2.5 h after which the cells were harvested by centrifugation and resuspended in 20 ml IMAC-loading buffer (300 mM NaCl, 50 mM Na-phosphate, pH 7.0) supplemented with Complete EDTA-free protease inhibitor cocktail (Roche Diagnostic, Basel, Switzerland). The cells were broken by sonication and the fusion toxins were purified from the supernatants by immobilized metal-ion affinity chromatography (IMAC) on a Ni-Sepharose 6 Fast Flow resin (GE Healthcare, Uppsala, Sweden) under native conditions with Imidazole elution according to the manufacturer's protocol. Eluted material was pooled and the buffer was changed to 20 mM Bis-Tris (pH 6.5) using PD-10 columns (GE Healthcare). The fusion toxins were further purified by anion exchange chromatography on 1 ml HiTrap Q HP columns (GE Healthcare) using 20 mM Bis-Tris (pH 6.5) as running buffer. Bound material was eluted by a NaCl-gradient from 0 to 0.6 M. Eluted material was pooled and further purified by gel filtration on a Superdex 75 column (GE Healthcare) with phosphate-buffered saline (PBS) as running buffer. The molecular masses of the fusion toxins, previously alkylated with 2-iodoacetamide, were determined by liquid chromatography electrospray ionization mass spectrometry (Agilent Technologies, Santa Clara, CA, USA). Purified, free targeting domain ($Z_{HER2:342}$), was a kind gift from Lisa Sandersjöö and John Löfblom (29).

Biosensor analysis. A Biacore 3000 instrument (GE Healthcare) was used for biosensor analysis. The extracellular domain of HER2 ($HER2_{ECD}$) (Sino Biological, Beijing, China) was immobilized on a CM5-chip by amine coupling in 50 mM sodium acetate buffer (pH 4.5). On a second CM5-chip, HSA, (Novozymes, Bagsvaerd, Denmark), MSA (Sigma-Aldrich),

and BSA (Merck) were immobilized in the same way. The final immobilization levels of $HER2_{ECD}$, HSA, MSA and BSA were 202, 869, 584 and 779 RU, respectively. Reference flow cells were created on both chips by activation and deactivation. HBS-EP [10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% Tween-20, (pH 7.4)] was used as running buffer and for dilution of the analytes. All experiments were performed at 25˚C with a flow-rate 50 μl/min. On- and off-rates were determined by BIAevaluation 4.1 software using a 1:1 Langmuir binding model.

Cell lines. The SKOV-3 and SKBR-3 cell lines were obtained from American Type Culture Collection (ATCC) through LGC Standards (Wesel, Germany) and were grown in McCoy's 5A supplemented with 10% fetal bovine serum in a humidified incubator at 37˚C in 5% CO_2 atmosphere. The A549 cell line was also obtained from ATCC and was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at the same conditions. HER2 expression levels were determined by incubating $2x10^6$ cells with Trastuzumab (Roche) (5 μg/ml) as primary antibody for 45 min followed by Alexa Fluor 488 conjugated goat anti-human antibody (Life Technologies, Carlsbad, CA, USA) (5 μg/ml) as secondary antibody for 45 min. The cells were subsequently analyzed on a Gallios flow cytometer (Beckman Coulter, Brea, CA, USA). 10,000 events were recorded and analyzed by Kaluza software (Beckman Coulter).

Cytotoxicity measurements. Approximately 5,000 cells/well were seeded in a 96-well microtiter plate and were allowed to attach overnight. Subsequently, the medium was replaced with fresh medium containing fusion toxins and other proteins followed by incubation for 72 h unless noted otherwise. Cell viability was determined using a cell counting kit-8 (CCK-8) (Sigma-Aldrich) according to manufacturer's protocol with determination of A_{450} in each well. The obtained absorbance values were analyzed by GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

Results

Construction of fusion toxins. Four fusion toxins were investigated in this study, consisting of a deimmunized variant of PE38 coupled to an affibody molecule binding to human epidermal growth factor receptor-2 ($Z_{HER2:2891}$) or to a control affibody molecule not expected to interact with any human protein (Z_{Taq}) and with or without the half-life extension albumin binding domain (ABD) (Fig. 1A). A tag with the amino acid sequence HEHEHE was added to the N-terminus to allow purification by immobilized metal-ion affinity chromatography (IMAC) (30). The characteristics of the fusion toxins were compared to $Z_{HER2:342}$-PE38, a fusion toxin consisting of the wild-type PE38 domain coupled to $Z_{HER2:342}$, a predecessor to $Z_{HER2:2891}$, with an N-terminal His_6-tag.

Production and initial characterization of the fusion toxins. The proteins were expressed in *Escherichia coli* followed by purification using three sequential chromatographic steps: IMAC, anion exchange chromatography and gel filtration. The chromatograms recorded during the gel filtration are displayed in Fig. 1C and show single, essentially symmetrical peaks, indicating the absence of multimer formation. Proteins eluted after the final gel filtration step were analyzed by SDS-PAGE, showing pure proteins with the expected molecular weights (Fig. 1B). More accurate measurements of the molecular masses were obtained by mass spectrometry and the results showed the expected molecular masses with <2 Da error (Fig. 1D and Table I).

Table I. Characterization of fusion toxins.

	Theoretical MW (Da)	Detected MW (Da)	Measured K_D (nM)[a]
$Z_{HER2:342}$-PE38	45,978.1	45,979.2	2[b]
$Z_{HER2:2891}$-PE38X8	46,714.6	46,715.4	5
$Z_{HER2:2891}$-ABD-PE38X8	53,899.6	53,901.4	5
Z_{Taq}-PE38X8	46,493.3	46,494.2	NB[c]
Z_{Taq}-ABD-PE38X8	53,678.3	53,679.3	NB

[a]The measured interaction between the fusion toxins and $HER2_{ECD}$. [b]From ref. 4. [c]NB, not binding.

Biosensor analysis of the interaction between the fusion toxins and serum albumins. The inclusion of an ABD in the fusion toxins potentially allowed interaction with serum albumin. To investigate the interaction with serum albumin from different species, the fusion toxins were injected over flow cells with immobilized HSA, MSA or BSA. The two toxins including the ABD interacted with HSA and to a lesser extent MSA (Fig. 2A and B). They did not interact with BSA (Fig. 2C). As expected, the three fusion toxins lacking the ABD did not interact with any of the serum albumins.

Biosensor analysis of the interaction between the fusion toxins and HER2. Fig. 3A displays an overlay of sequential injections of the three Z_{HER2}-containing and one Z_{Taq}-containing (control) fusion toxins over a flow cell with immobilized $HER2_{ECD}$. As expected, the three fusion toxins which contain $Z_{HER2:342}$ or $Z_{HER2:2891}$ interact with HER2 and the control does not. To investigate a possible interference in the fusion toxin/HER2 interaction, when the fusion toxin is in complex with serum albumin, $Z_{HER2:2891}$-ABD-PE38X8 was also injected over the HER2-flow cell after pre-incubation with an excess of HSA (Fig. 3B). $Z_{HER2:2891}$-ABD-PE38X8 was found to interact with HER2 while in a complex with HSA. As a control, Z_{Taq}-ABD-PE38X8 was also pre-incubated with HSA and injected over the flow cell with immoblilized HER2, which showed no interaction. The affinity between $Z_{HER2:2891}$-ABD-PE38X8 and HER2 was determined in the absence and presence of HSA by injecting serial dilutions of the fusion toxins (Fig. 3C and D). The resulting equilibrium dissociation constants were 5 and 12 nM in the absence or presence of HSA. In addition, the affinity between $Z_{HER2:2891}$-PE38X8 and HER2 was determined similarly and the equilibrium dissociation constant was found to be 5 nM.

Determination of the cytotoxicity of the fusion toxins on cells expressing different levels of HER2. The relative expression

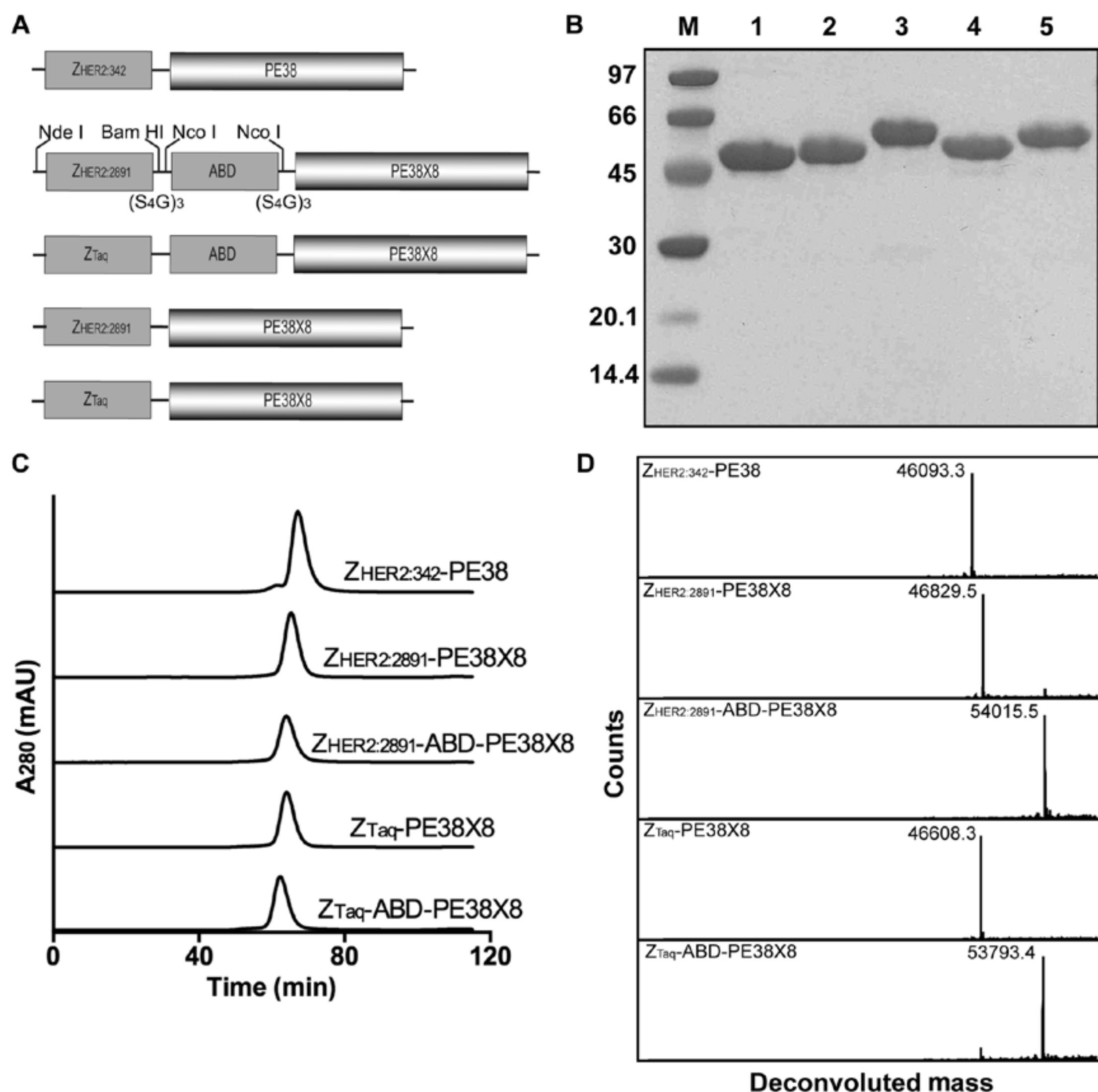

Figure 1. Construction and initial characterization of the fusion toxins. (A) Schematic representations of the genes encoding the fusion proteins are shown. Restriction endonuclease sites used during construction and linkers connecting the three domains, with the amino acid sequence $(S_4G)_3$, are indicated on $Z_{HER2:2891}$-ABD-PE38X8. (B) The purified fusion toxins were analyzed by SDS-PAGE on a 10% gel. Numbers to the left indicate the molecular weight of the marker proteins in kDa. 1, $Z_{HER2:342}$-PE38; 2, $Z_{HER2:2891}$-PE38X8; 3, $Z_{HER2:2891}$-ABD-PE38X8; 4, Z_{Taq}-PE38X8; 5, Z_{Taq}-ABD-PE38X8. (C) The chromatograms recorded during gel filtration of the fusion toxins are displayed. (D) The spectra of alkylated proteins recorded during mass spectrometry analysis.

Table II. Cytotoxicity of fusion toxins.

		IC_{50} (pM)				
Cell line	HER2 expression level	$Z_{HER2:342}$-PE38	$Z_{HER2:2891}$-PE38X8	$Z_{HER2:2891}$-ABD-PE38X8	Z_{Taq}-PE38X8	Z_{Taq}-ABD-PE38X8
SKBR-3	High	0.16 (0.12-0.23)[a]	2.1 (1.5-2.9)	5.6 (4.0-7.8)	22,000 (14,000-32,000)	1,400 (900-2,100)
SKOV-3	High	5.1 (4.1-6.4)	6.9 (5.5-8.8)	25 (20-32)	24,000 (19,000-30,000)	19,000 (15,000-24,000)
A549	Moderate	50 (35-71)	160 (110-230)	300 (210-430)	32,000 (22,000-45,000)	37,000 (26,000-52,000)

[a]Range (95% confidence interval).

levels of HER2 on SKOV-3, SKBR-3 and A549 cells were determined by flow cytometry. SKOV-3 and SKBR-3 have relatively high expression levels of HER2 in contrast to A549, which has a moderate expression level (Fig. 4A, C and E). To determine the cytotoxicity of the fusion toxins, the cell lines were incubated with serial dilutions of the fusion toxins for 72 h, after which cell viability was measured and plotted as a function of the logarithm of the toxin concentration (Fig. 4B, D

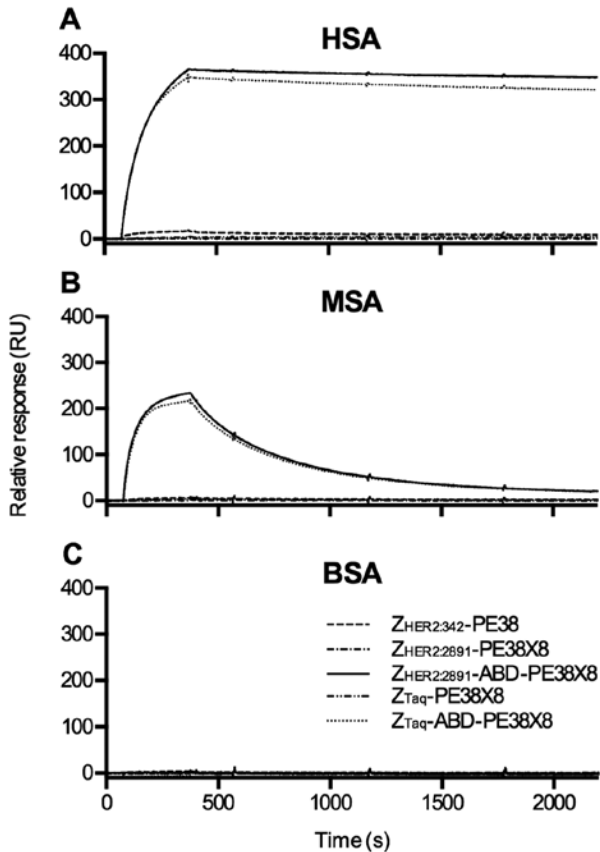

Figure 2. Biosensor analysis of the interactions between the fusion toxins and serum albumins. The fusion toxins (200 nM) were sequentially injected over flow cells with immobilized (A) human serum albumin (HSA), (B) mouse serum albumin (MSA) or (C) bovine serum albumin (BSA). The recorded sensorgrams are displayed.

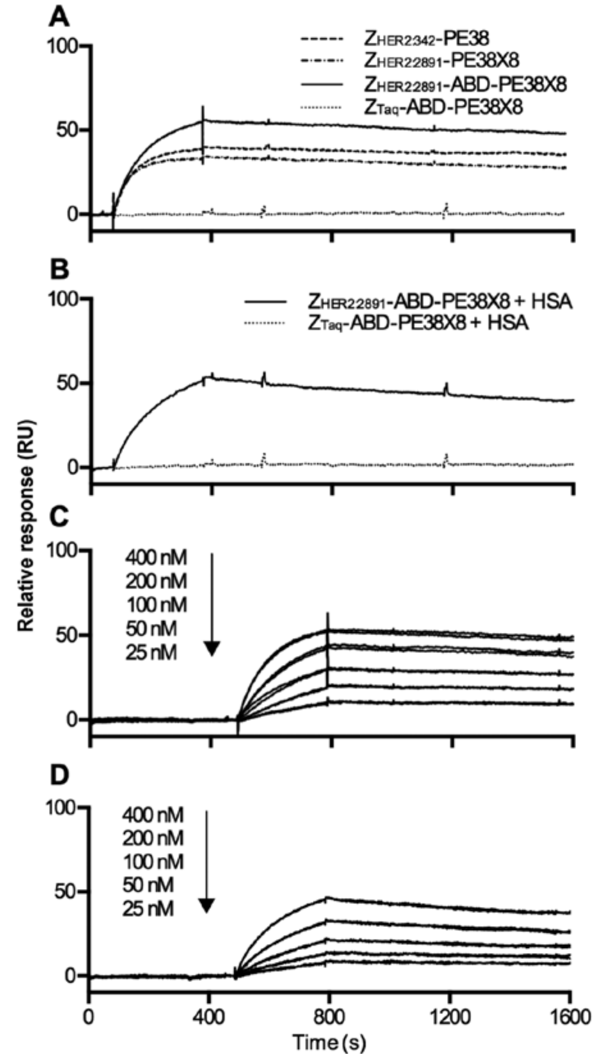

Figure 3. Biosensor analysis of the interactions between the fusion toxins and HER2. (A) The fusion toxins (400 nM) were injected over a flow cell with immobilized $HER2_{ECD}$. (B) $Z_{HER2:2891}$-ABD-PE38X8 (400 nM) and Z_{Taq}-ABD-PE38X8 (400 nM) were pre-incubated with HSA (400 nM) for 3 h followed by sequential injection over a flow cell with immobilized $HER2_{ECD}$. (C) A dilution series of $Z_{HER2:2891}$-ABD-PE38X8 was injected over a flow cell with immobilized $HER2_{ECD}$. The experiment was repeated once and an overlay of the sensorgrams from both experiments is displayed. (D) Different dilutions of $Z_{HER2:2891}$-ABD-PE38X8 was pre-incubated with HSA (400 nM) for 3 h and injected over a flow cell with immobilized $HER2_{ECD}$. The experiment was repeated once and an overlay of the sensorgrams from both experiments is displayed.

and F). IC_{50} values were determined from the viability plots and are displayed in Table II. On SKOV-3 cells, the two fusion toxins including $Z_{HER2:2891}$ and the control including $Z_{HER2:342}$, had IC_{50} values ranging between 5 and 25 pM which were 4,000-1,000 times lower than the IC_{50} values of the control fusion toxins including Z_{Taq}. The IC_{50} values of His_6-$Z_{HER2:342}$-PE38 and $Z_{HER2:2891}$-PE38X8 were similar and 5-fold lower than the IC_{50} value of $Z_{HER2:2891}$-ABD-PE38X8, indicating that inclusion of ABD lowers the cytotoxicity somewhat. SKBR-3 cells have a similar density of HER2 on the cell surface and similar IC_{50} values for the fusion toxins were measured on this cell line. Here, a slightly lower IC_{50} value was measured for His_6-$Z_{HER2:342}$-PE38 compared to $Z_{HER2:2891}$-PE38X8, indicating that PE38X8 is slightly less cytotoxic compared to PE38. Since a similar decrease in cytotoxicity was not found on SKOV-3 cells, the result suggests that the difference is dependent on the cell line. The A549 cell line has a lower HER2 density than SKOV-3 and SKBR-3 and consequently the IC_{50} values for the fusion toxins including $Z_{HER2:2891}$ or $Z_{HER2:342}$ was higher, ranging from 50 to 300 pM. The IC_{50} values for the control fusion toxins including Z_{Taq}, were similar for A549, SKOV-3 and SKBR-3, except for Z_{Taq}-ABD-PE38X8 on SKBR-3 cells, which was lower. In combination, these results indicate that the fusion toxins interacting with HER2 are more cytotoxic to cells with a higher surface density of HER2 than cells with a lower, further proving that the fusion toxins are HER2 specific.

Analysis of toxin specificity. To further investigate the specificity of the fusion toxins, SKOV-3 cells were incubated with $Z_{HER2:2891}$-ABD-PE38X8 after the cells had been pre-incubated with an excess of free targeting domain ($Z_{HER2:342}$) which was expected to block available HER2 receptors on the cells, or with an excess of control protein (transferrin), which was not expected to interact with the HER2 receptor. Fig. 5A shows that $Z_{HER2:342}$ and transferrin does not affect cell viability by themselves. However, pre-incubation of the cells with $Z_{HER2:342}$ rescues the cells from $Z_{HER2:2891}$-ABD-PE38X8 cytotoxicity.

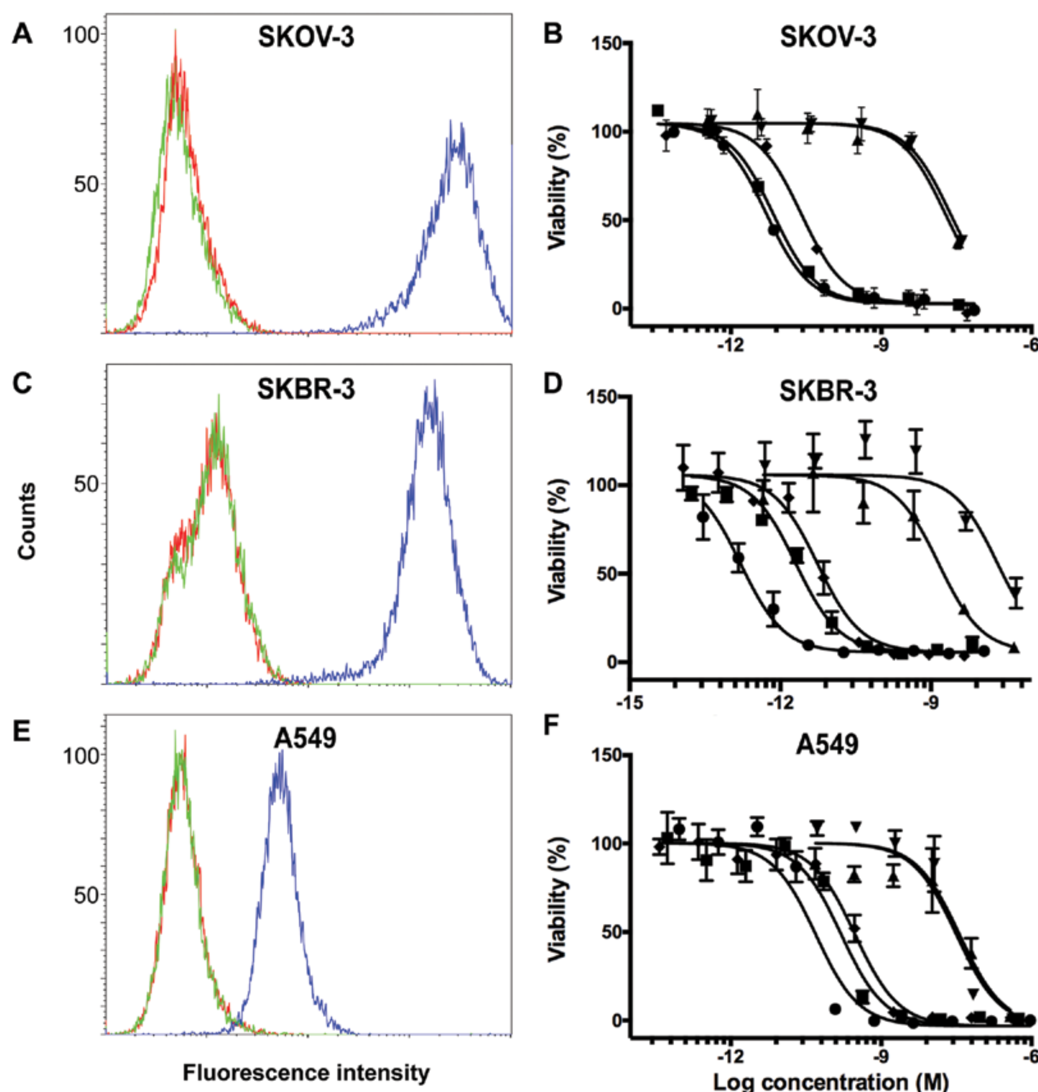

Figure 4. Analysis of relative HER2 expression on SKOV-3, SKBR-3 and A549 cells and determination of the cytotoxicity of the fusion toxins. (A, C and E) The relative expression levels of HER2 on SKOV-3, SKBR-3 and A549 cells, respectively, were determined by flow cytometry. Blue lines indicates spectra after incubation with primary and secondary antibody, red lines indicate control spectra recorded after incubation with secondary antibody only and green lines indicate control spectra recorded of the cells without incubation with any antibody. The cytotoxicities were analyzed by incubating serial dilutions of the fusion toxins with SKOV-3, SKBR-3 and A549 cells. (B, D and F) Cell viability are plotted on the y-axis as a function of fusion toxin concentration on the x-axis. The viabilities of cells cultivated in growth media without addition of any toxin were set to 100% for each panel. Each point was measured in triplicates and the error-bars correspond to 1 SD. $Z_{HER2:342}$-PE38 (•); $Z_{HER2:2891}$-PE38X8 (■); $Z_{HER2:2891}$-ABD-PE38 (♦); Z_{Taq}-PE38X8 (▼); Z_{Taq}-ABD-PE38X8 (▲).

Pre-incubation with the control protein transferrin does not rescue cells similarly, showing that the cytotoxic potential of $Z_{HER2:2891}$-ABD-PE38X8 is HER2-dependent. The cytotoxic potential of $Z_{HER2:2891}$-ABD-PE38X8 in combination with the free targeting domain $Z_{HER2:342}$ was further investigated by pre-incubating SKOV-3 cells with increasing concentrations of $Z_{HER2:342}$ followed by addition of $Z_{HER2:2891}$-ABD-PE38X8. The results showed that cell viability increases with increasing concentration of $Z_{HER2:342}$ (Fig. 5B), further corroborating the finding that the cytotoxicity of $Z_{HER2:2891}$-ABD-PE38X8 is dependent on the level of free HER2 on the cell surface.

Analysis of the effect of contact time on cytotoxicity. To investigate the influence of contact time on cytotoxicity, SKOV-3 cells were incubated for different amounts of time with a concentration of $Z_{HER2:2891}$-ABD-PE38X8 expected to reduce cell viability close to 0 after incubation for 72 h, followed by measurement of cell viability. The results are plotted in Fig. 6 and show that cell viability is reduced with increasing exposure time. The longest exposure time of 1,440 min (24 h) reduces cell viability close to 0. A 50% reduction of cell viability is found after exposure for 10 min.

Discussion

The use of immunotoxins for treatment of cancer has been hampered partly by the immunogenicity of the toxin part, which may lead to formation of neutralizing antibodies after only few injections (31). Deimmunized toxins such as PE38X8, where mouse B-cell epitopes have been removed, may be more suited for immunotoxin construction since formation of neutralizing antibodies is significantly lower while the potency

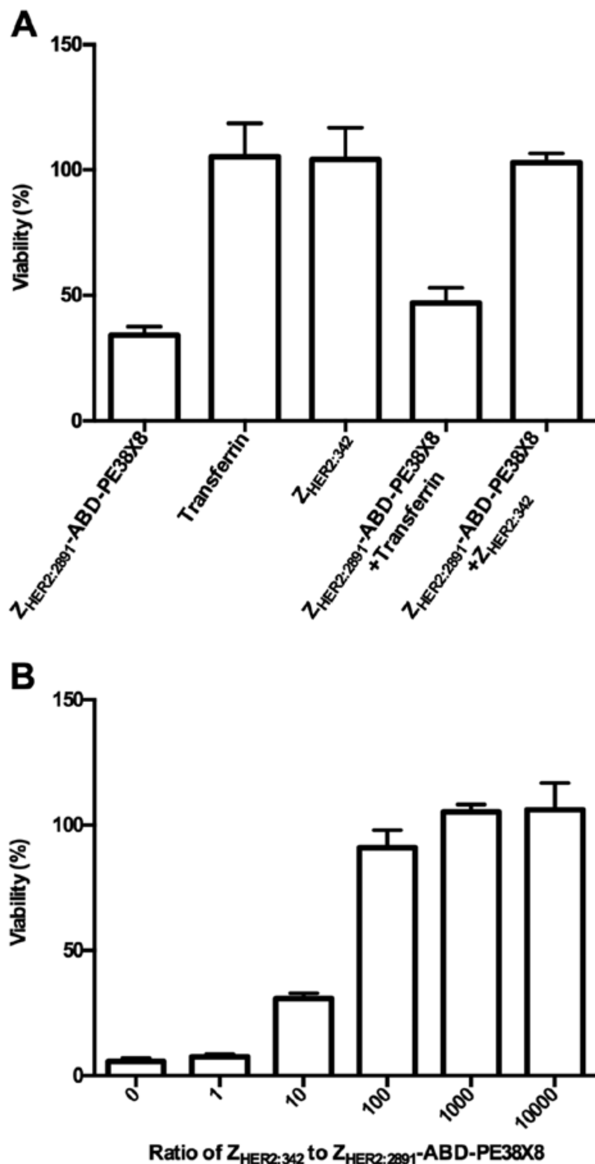

Figure 5. Analysis of toxin specificity. SKOV-3 cells were incubated with different proteins for 72 h followed by determination of cell viability. The viability of cells cultivated in growth medium without addition of any toxin was set to 100% for each panel. (A) From left to right, incubation with 25 pM $Z_{HER2:2891}$-ABD-PE38X8; 100 nM transferrin; 100 nM $Z_{HER2:342}$; cells were pre-incubated with 100 nM transferrin for 5 min followed by addition of 25 pM $Z_{HER2:2891}$-ABD-PE38X8; cells were pre-incubated with 100 nM $Z_{HER2:342}$ for 5 min followed by addition of 25 pM $Z_{HER2:2891}$-ABD-PE38X8. (B) Cells were pre-incubated with an excess of $Z_{HER2:342}$ for 5 min followed by addition of 500 pM $Z_{HER2:2891}$-ABD-PE38X8. Numbers below the panel correspond to the different molar ratios between $Z_{HER2:342}$ and $Z_{HER2:2891}$-ABD-PE38X8. Each data-point corresponds to the average measured viability of three independent experiments. The error-bars indicate 1 SD.

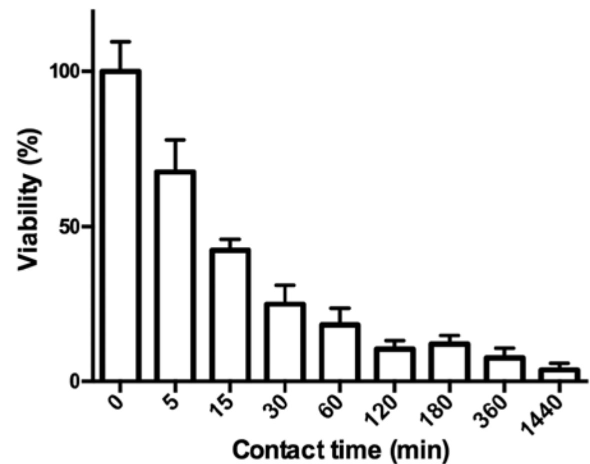

Figure 6. Analysis of the correlation between contact time and cytotoxicity. SKOV-3 cells were incubated with $Z_{HER2:2891}$-ABD-PE38X8 for the times indicated on the x-axis. The corresponding cell viabilities were measured and plotted on the y-axis. The viability of SKOV-3 cells cultivated in growth medium without addition of any toxin was set to 100% (contact time=0). Each data-point corresponds to the average measured viability of three independent experiments. The error-bars indicate 1 SD.

of the toxin is intact (7). However, for use in humans, further engineered versions, where for example also human B-cell epitopes have been removed, could prove even more useful, despite their lower potency (8). Deimmunized PE38-variants, such as PE38X8, have not yet been evaluated in combination with non-immunglobulin based targeting domains and one of the goals of this study was to compare PE38X8 with PE38 using a HER2-interacting affibody molecule ($Z_{HER2:324}$ or the derivative $Z_{HER2:2891}$) as targeting domain. The results showed that $Z_{HER2:2891}$-PE38X8 had a comparable toxicity to $Z_{HER2:342}$-PE38 on SKOV-3 cells but was slightly less toxic for SKBR-3 and A549 cells. In a previous study, PE38X8 was found to be slightly more toxic than PE38 to Raji cells (7). Thus, the difference in toxicity of PE38X8 and PE38 appears to be cell line-dependent. Inclusion of an albumin binding domain (ABD) for half-life extension also slightly lowered the cytotoxicity on all three cell lines. The tendency of lower toxicity of $Z_{HER2:2891}$-ABD-PE38X8 and $Z_{HER2:2891}$-PE38X8 is possibly a consequence of their slightly lower affinity for HER2 (5 nM) in comparison with $Z_{HER2:342}$-PE38 (2 nM). The lower affinity is in turn a consequence of the lower affinity between HER2 and $Z_{HER2:2891}$ (60 nM) in comparison with HER2 and $Z_{HER2:342}$ (22 pM) (18,20).

The affinity of $Z_{HER2:2891}$-ABD-PE38X8 was 200-fold weaker compared to the affinity between $Z_{HER:2891}$ and HER2 (20). A similar reduction in affinity has previously been reported for $Z_{HER2:342}$-PE38 (4). Despite this reduction, the difference in cytotoxic potency of $Z_{HER2:2891}$-ABD-PE38X8 and the control Z_{Taq}-ABD-PE38X8 on SKOV-3 and SKBR3 cells, which express high levels of HER2, was 1,000-fold which suggests a rather wide therapeutic window.

The half-life of $Z_{HER2:342}$-PE38 was previously found to be only 9 min (5), which is less than the contact time needed to kill 50% of cells *in vitro* (4). Inclusion of an ABD in the current construct is likely to increase the serum half-life significantly, since a strong interaction with both human and mouse serum albumin was detected (Fig. 2). A longer serum half-life may lead to a higher *in vivo* efficacy, although a longer serum half-life also gives the immune system a longer time to interact with the fusion toxin to form neutralizing antibodies. An important issue is that interaction with the comparatively large serum albumin does not impart HER2-binding and as a consequence cytotoxicity. Interestingly, $Z_{HER2:2891}$-ABD-PE38X8 appeared to be able to interact with HER2 while in a complex with HSA (Fig. 3B). This suggests

that $Z_{HER2:2891}$-ABD-PE38X8 should be cytotoxic also when in complex with serum albumin.

Off-target toxicity has been an obstacle when developing immunotoxins and fusion toxins. A particular problem with early constructs including *Pseudomonas* exotoxin A was damage to the vasculature, which set the limiting dose (32). Fortunately, with the development of PE38, damage to the vasculature has been reduced. Also of importance is that the construct does not unspecifically accumulate in healthy organs. In a study by Zielinski *et al* (5), where $Z_{HER2:342}$-PE38 was used to treat xenografted tumors in mice, some unspecific accumulation of the fusion toxin in the liver was observed. A combination of free $Z_{HER2:342}$ with an N-terminally placed His_6-tag, as in $Z_{HER2:342}$-PE38, has previously been found to lead to unspecific uptake in the liver (30). A solution was to modify the His_6-tag to the amino acid sequence HEHEHE, which was found to reduce liver accumulation significantly. In the currently investigated fusion toxin, $Z_{HER2:2891}$-ABD-PE38X8, a HEHEHE-tag was placed in the N-terminus instead of the His_6-tag present in $Z_{HER2:342}$-PE38. Even though half of the histidines were substituted with glutamic acid, $Z_{HER2:2891}$-ABD-PE38X8 could still be efficiently purified by IMAC. It is possible that this substitution may lead to lower unspecific uptake in the liver in future *in vivo* studies.

In conclusion, a tripartite fusion toxin based on a deimmunized version of PE38 was successfully constructed. All three parts were found to function properly. Future experiments will reveal if this fusion toxin also have the ability to kill HER2-expressing malignant cells *in vivo*.

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