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Targeting Cancer with a Bi-functional Peptide: *In Vitro* and *In Vivo* Results

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Abstract. *Background: Current therapies to treat cancer, although successful for some patients, have significant side-effects and a high number of patients have disease that is either non-responsive or which develops resistance. Our goal was to design a small peptide that possesses similar functions to an antibody drug conjugate with regard to targeting and killing cancer cells, but that overcomes size restrictions. Materials and Methods: We designed a novel cancer-specific killer peptide created by fusion of the toxic peptide (KLAKLAK)2 with the cancer recognition peptide LTVSPWY. Results. This bi-functional peptide showed toxicity to breast cancer, prostate cancer, and neuroblastoma cell lines. Only low toxicity to non-cancer cells, colon cancer, lung cancer, and lymphoma cell lines was observed. In vivo injections of the bi-functional peptide caused tumor growth retardation compared to mice treated with control peptides. The bi-functional peptide caused retardation of MDA-MB-435S tumors in vivo and increased survival to 80% at day 100 after tumor implantation, whereas all control animals died at day 70. Previous reports showed that the recognition moiety LTVSPWY targets the tumor-associated antigen HER2. Here we found that our new peptide TP-Tox also excerts toxic effects on HER2-negative cell lines. Therefore, we searched for the molecular target of the bi-specific peptide using immunoprecipitation and mass spectrometry. Our data suggest a possible interaction with RAS GTPase-activating protein binding protein 1 (G3BP1). Conclusion: We designed a bi-functional peptide of 23 amino acids and demonstrated its ability to bind and kill several cancer cell lines in vitro and to strongly increase survival in breast cancer bearing mice in vivo. This novel toxin could be used in future cancer therapies and warrants further pre-clinical and clinical exploration.*

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Cancer is among the most common causes of death in the Western world. Cancer treatment classically involves surgical removal of the tumor, chemotherapy, or radiation therapy. Unfortunately, escape of malignant cells from the primary tumor prior to surgery, side-effects caused by off-target actions of drugs, non-responsiveness, and development of resistance to chemotherapy often jeopardizes therapeutic benefit (1). With the goal of reducing toxicity, targeted drug-delivery approaches have been pursued, including antibody toxins (2). The most commonly considered target for antibody-directed therapy is HER2, which is overexpressed in several types of cancer (3). Trastuzumab is the most frequently used antibody to target HER2-antigen and its combination with several antibody drug conjugates has been investigated (4-6). Since penetration into tissue can pose an impediment for successful drug delivery to each tumor cell, smaller compounds than antibody-based toxins have the potential for more efficient tumor penetration and increased efficacy (7).

Our goal was to design a small peptide that possesses similar functions to an antibody-drug conjugate (ADC) with regard to targeting and killing of cancer cells, but which overcomes the size restrictions. The toxic domain $(KLAKLAK)_2$ has been shown to disrupt negatively charged membranes (8). Mitochondrial membranes have a high content of anionic phospholipids and a large transmembrane potential, in contrast to the low potential of plasma membranes. Thus, these peptides preferentially disrupt mitochondrial membranes (9-12). Coupling of $(KLAKLAK)_2$ to a selective targeting domain leads to enhanced toxicity towards different tumor cells (13-16). For example

conjugation of (KLAKLAK)2 to the antibody against CD19 caused apoptosis of B lymphoid lines (17) and when linked to a tumor blood vessel homing motive it exhibited toxicity to angiogenic epithelial cells and had anticancer activity (18).

We chose LTVSPWY as the targeting domain, because it has been shown to bind to breast cancer but not to normal cells (19-23). The targeting peptide improved *in vivo* toxicity when coupled to the vitamin E analog α-Tos as compared to unconjugated α-Tos (24). We have joined the two peptides LTVSPWY and $(KLAKLAK)_2$ using a linker composed of two glycines (Figure 1) as described before (14).

This bi-functional peptide is referred to as targeted peptide and toxin (TP-Tox). To allow for peptide binding and uptake studies, fluoresceinisothiocyanate (FITC) was attached to the C-terminus of the peptide.

Materials and Methods

Cells and culture conditions. All cell lines were obtained from DKFZ (Heidelberg, Germany), Cell Lines Service (Eppelheim, Germany), or the ATCC (LCG Standards, Wesel Germany). Tet21N and Wac2 cells were kindly provided by Professor Dr. Manfred Schwab (DKFZ, Heidelberg, Germany). All cells were incubated at 37˚C in 5% CO_2 and 80% humidity.

The breast cancer cell lines MCF-7, MDA-MB-435S, MDA-MB-453, SKBR3, T47D, the bronchial cancer cell line A549; the pancreatic cancer cell lines Panc02, Panc1.98, DanG, PK9, Su86.86, and the human embryonic kidney cell line 293 were cultured in Dulbecco's modified eagle*s medium (DMEM) supplemented with L-glutamine (Invitrogen, Darmstadt, Germany), 2.5% heat-inactivated fetal calf serum (FCS; Invitrogen), 0.2% insulin (Invitrogen) and 1% penicillin/streptomycin (P/S; PAA Laboratories, Pasching, Germany). The kidney cancer cell line A704 was cultured in DMEM supplemented with 15% FCS, 1% P/S and 0.2% insulin. The bladder cancer cell line T24, the kidney cancer cell lines A498, Caki-2, the colon cancer's cell lines Colo201 and Colo205, and neuroblastoma cells Tet21N and Wac2, the prostate cancer cell line DU145, and lymphoma cells OciLy8 were cultured in RPMI medium (PAA Laboratories, Germany) supplemented with 1% P/S and with 2.5-5% FCS. The ovary carcinoma cell lines A2780 and A278003 were cultured in RPMI medium supplemented with 5% FCS, 1% P/S and 7.5% L-glutamine. The multiple myeloma OPM2 and U266 cells were grown in RPMI medium supplemented with 10 % FCS, 1% P/S and 1.5% Hepes (Invitrogen). Human peripheral blood lymphocytes (PBL) were isolated by low density gradient centrifugation from whole blood with Lymphocyte Separation Medium LSM 1077 (PAA Laboratories Pasching, Austria). Cells were cultured in RPMI medium with 300 U/ml interleukin-2 (IL-2; Immunotools, Friesoythe, Germany).

Determination of HER2 status by flow cytometry. Adherent cells were harvested using a 0.05% Trypsin-EDTA solution (Invitrogen), set at 2×10^5 cells in 200 μl PBS and incubated with an antibody to HER2 (1:250) (PE anti-human CD340 ERB/HER2, BioLegend, Fell, Germany) for 10 min on ice and washed twice with PBS. Cells were analyzed immediately using the BD FACS Canto II. T47D cells (HER2-positive) were used as positive control (data not shown). HER2 was not removed by trypsin treatment.

Peptide design and synthesis. All peptides were synthesized by and purchased from Thermo Fisher Scientific (Ulm, Germany) and delivered as lyophilised, trifluoracetic salts. 5-carboxyfluorescein was linked to the "N"-terminus of peptides. All peptides were dissolved in isotonic phosphate-buffered saline and stored at –20˚C. FITC-conjugated peptides were stored in the dark at 4˚C according to the manufacturer's instructions. p34 is an irrelevant peptide (20). It was used as control. TP is the recognition peptide alone. Tox ist he toxic peptide alone. TP-Tox is the targeting peptide consisting of targeting peptide and toxic peptide connected with a glycin-glycin linker.

Binding and cellular uptake of the peptides. Cells were cultured and harvested as described above. The cell pellet was resuspended in 1 ml medium and counted. To analyze peptide binding and uptake, 1×10^5 cells in 500 μl medium were seeded on coverslips in 24-well plates and allowed to adhere overnight in an incubator at 37˚C. The medium was changed and fresh medium containing 10 μM of FITC-labeled peptides was added. To analyze binding to peripheral blood lymphocytes, coverslips were incubated with 10 μg/ml poly-L-lysine (Sigma Aldrich, Munich, Germany) for four hours overnight and washed once with PBS. Cells (1x105) in medium containing 10 μM of FITC-labeled peptides were then seeded onto coverslips coated with poly-L-lysine as above.

After 15 min to 24 h, the cells were washed with medium and stained with 25 nM Mitotracker^R Red CMXRos (Invitrogen) in medium for 30 min at 37˚C. The medium was removed and the cells were fixed with 4% formaldehyde (Applichem, Darmstadt, Germany) for 15 min at 37˚C. After an additional washing with medium, cells were stained with 1 μg/ml (DAPI; Applichem) for 15 min at 37˚C. Coverslips were rinsed with PBS, embedded in fluoromount (Carl Roth, Karlsruhe, Germany) and kept at 4˚C until detection of peptide uptake by fluorescence microscopy. Cells were analyzed with a Leica DMI 4000 fluorescence microscope.

Cytotoxicity assay. Toxicity of the peptides was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) proliferation assay. Yellow MTT (Sigma Aldrich, Munich, Germany) is taken up by viable cells. The MTT is reduced to purple formazan in the mitochondria of living cells. Then purple formazan is dissolved in acidified isopropanol. The resulting purple solution is spectrophotometrically measured at 492 nm.

A total of 1×10^4 cells in 100 μl/well were seeded in 96-well plates and allowed to adhere overnight at 37˚C in an incubator. At different timepoints, the medium were removed and fresh medium containing various concentrations of the different peptides were added to the cells. After the indicated time points 10 μl medium containing 5 mg/mL MTT (1:100 dilution in medium) was added to the cells at 37˚C. After three hours 80 μl of medium was removed. Cells were lysed in 50 μl acidified isopropanol by shaking at 700 rpm for 10 min. The amount of reduced formazan was measured at 492 nm. The absorbance of untreated cells was set at 100% and the absorbance of treated cells was compared to that of untreated cells and the relative cell viability was calculated. Cells treated with 100 μM cycloheximide (Applichem) were used as positive control.

To analyze the mechanism of peptide uptake, antimycin A (Sigma Aldrich), chlorpromazine (Sigma Aldrich), cytochalasin D (Applichem), and verapamil (Sigma Aldrich) were used (25). Cells were co-incubated with peptide and 10 μM chlorpromazine, 5 μM cytochalasin D, or 10 μM verapamil at 37˚C, because the effect of these inihibitors on endocytosis is reversible (26-29). For the

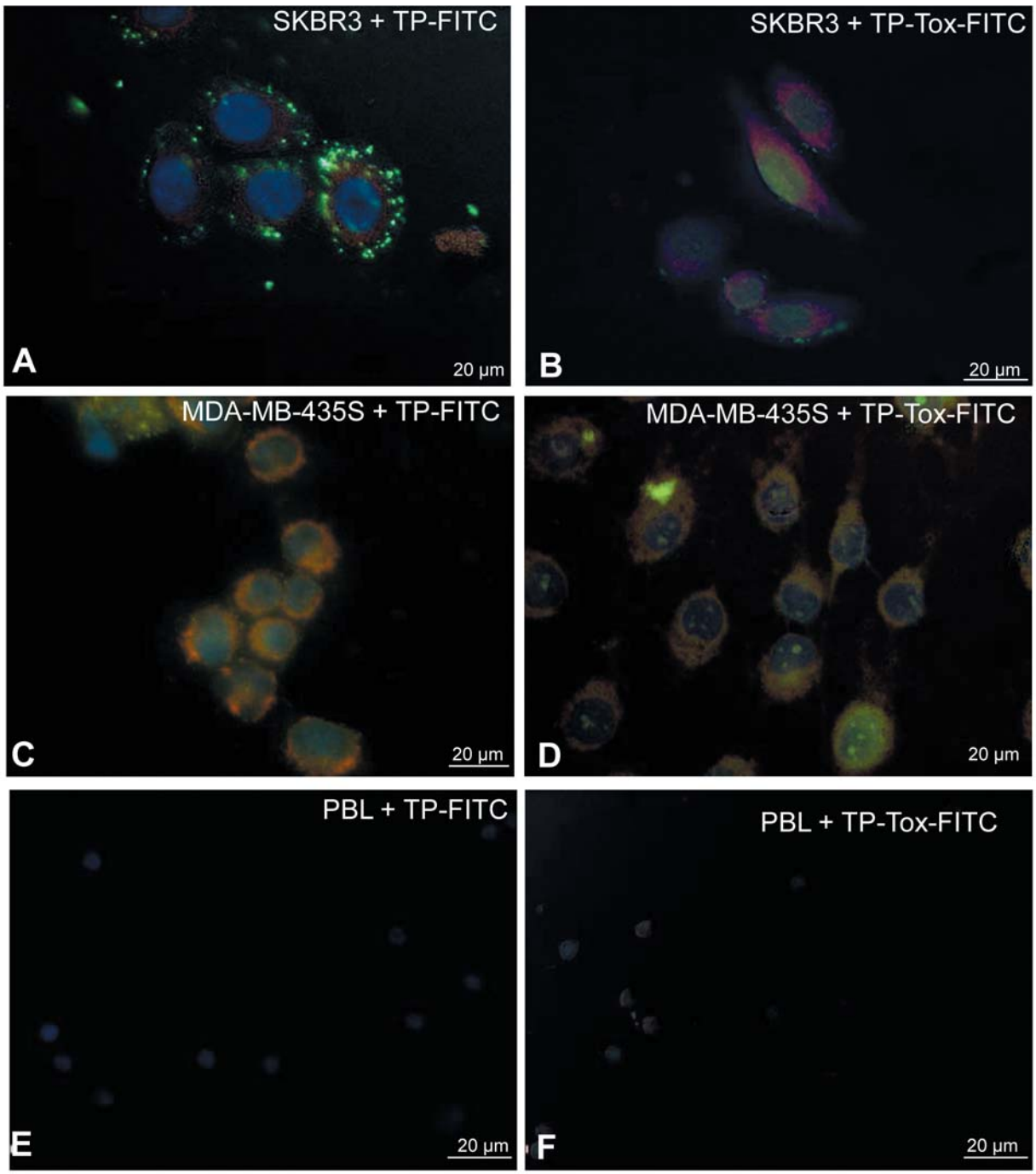

Figure 1. *(TP-FITC; LTVSPWY-C-FITC) binds cancer cells. [TP-Tox-FITC; LTVSPWY-(KLAKLAK)2-C-FITC] is taken up after 8 h. Cells were seeded on coverslips and allowed to adhere overnight. Cells were treated with 10 µM FITC-labeled peptides. After 8 h (MDA-MB-435S), medium was discarded, cells were washed with medium, stained with MitoTracker CMXRos, fixed and stained with staining solution. Bars-20 µM. A: SKBR3 + TP-FITC, B) SKBR3 + TP-Tox-FITC, C) MDA-MB-435S + TP-FITC; D) MDA-MB-435S + TP-Tox-FITC, E) PBL + TP-FITC, F) PBL + TP-Tox-FITC. Cells were analyzed by fluorescence microscopy.*

analysis with antimycin A, cells were pre-incubated with 10 µM antimycin A for 30 min at 37˚C. The effect of antimycin A is irreversible (26-29). The medium was removed and fresh medium containing peptides was added.

Isolation of primary tumor cells from tumor tissue. Fresh tumor tissue samples were cut in 1 to 3 mm^3 pieces and incubated in 1 mL of digest buffer (RPMI medium with P/S containing 300 µg/mL Hyaluronidase (Sigma Aldrich) 20 mg/mL collagenase IV (Sigma

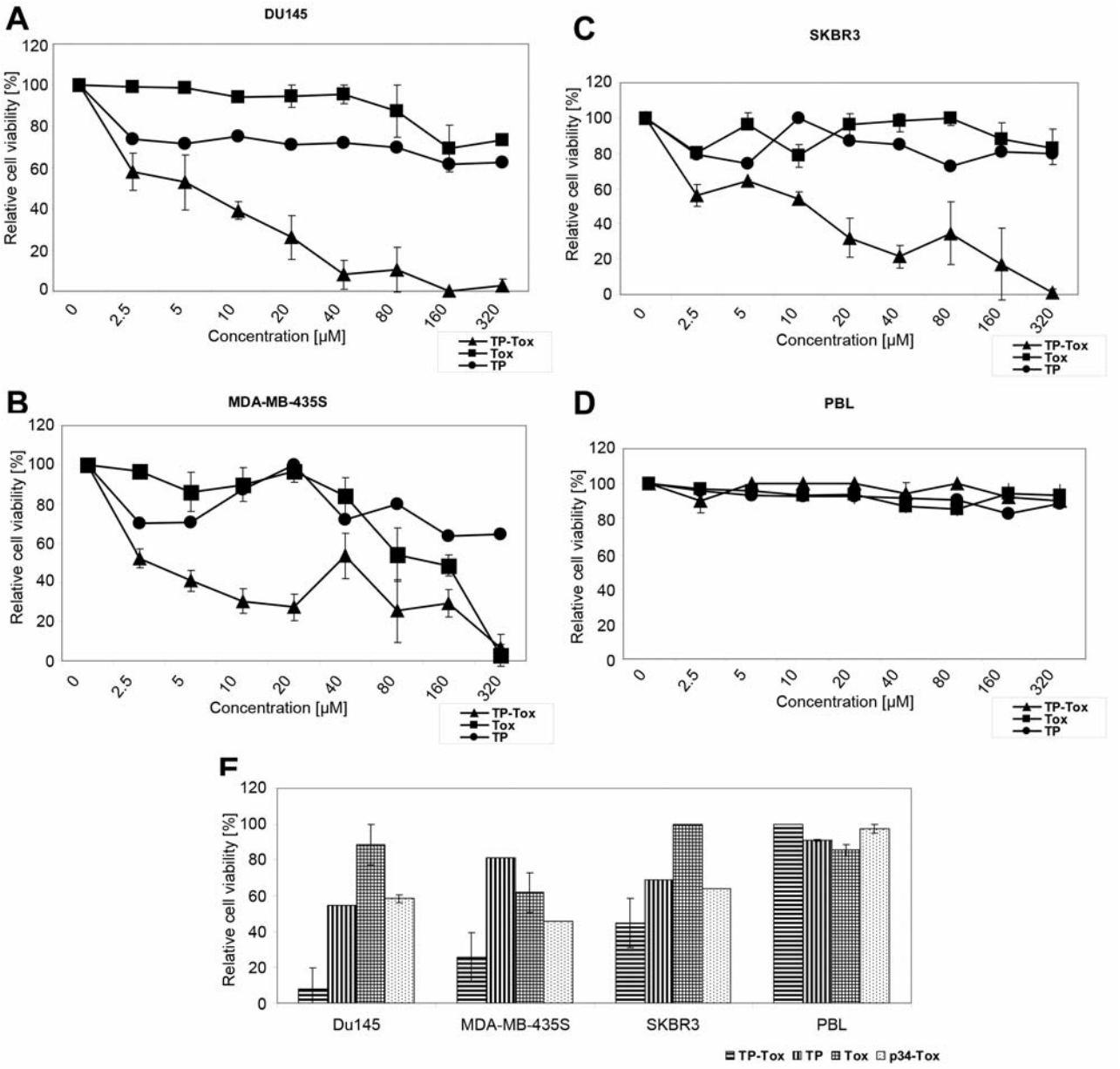

Figure 2. *Addition of the targeting peptide increases the cytotoxicity of the toxic peptide. Toxicity of the single peptides (TP, targeted peptide; Tox, toxin) and the combination of targeted peptide and toxic peptide (TP-Tox) was evaluated on different cell lines: DU145 (A), MDA-MB-435S (B), SKBR3 (C), and peripheral blood lymphocytes (PBL) (D). Cells were exposed to increasing doses of peptides for 24 h. E: Single peptides and an irrelevant peptide was used as control (p34-tox) and compared to toxicity at 80 μM for 24 h. Relative cell viability is shown at 80 μM of each peptide. Data are presented as mean±SD of duplicates of eight independent experiment. Two-tailed Student's t-test was used for statistical analysis. A p-value <0.05 was considered significant.*

Aldrich), DNase I (U/mL) (Fermentas, St. Leon-Roth, Germany) for 40 min at 37˚C in a sterile petri dish. After 40 min, the tissue solution was forced through a cell strainer, 100 μM pore size BD falcon (VWR, Langenfeld, Germany) and purged with RPMI medium. Cells were washed twice with PBS and resuspended in Mammary Epithelial Cell Medium containing 1% P/S (PromoCell, Heidelberg, Germany). Cytotoxicity was analyzed by MTT as described for the different cell lines (data not shown).

Co-immunoprecipitation. Cells were grown to 80% confluency in 75 cm^3 culture flasks. Medium was removed and cells were washed with cold PBS. Cells were then scraped in ice cold PBS, washed, and cell pellets were stored for one to two days at –20˚C.

Pellets were thawed on ice, lysis buffer was added [0.025 M Tris-HCl, 0.15M NaCl, 0.001 M EDTA, 1% NP-40, 5 % glycerol with 1% (PMSF)], and the cell suspension was sucked back and forth through a syringe several times and incubated for 10 min on ice.

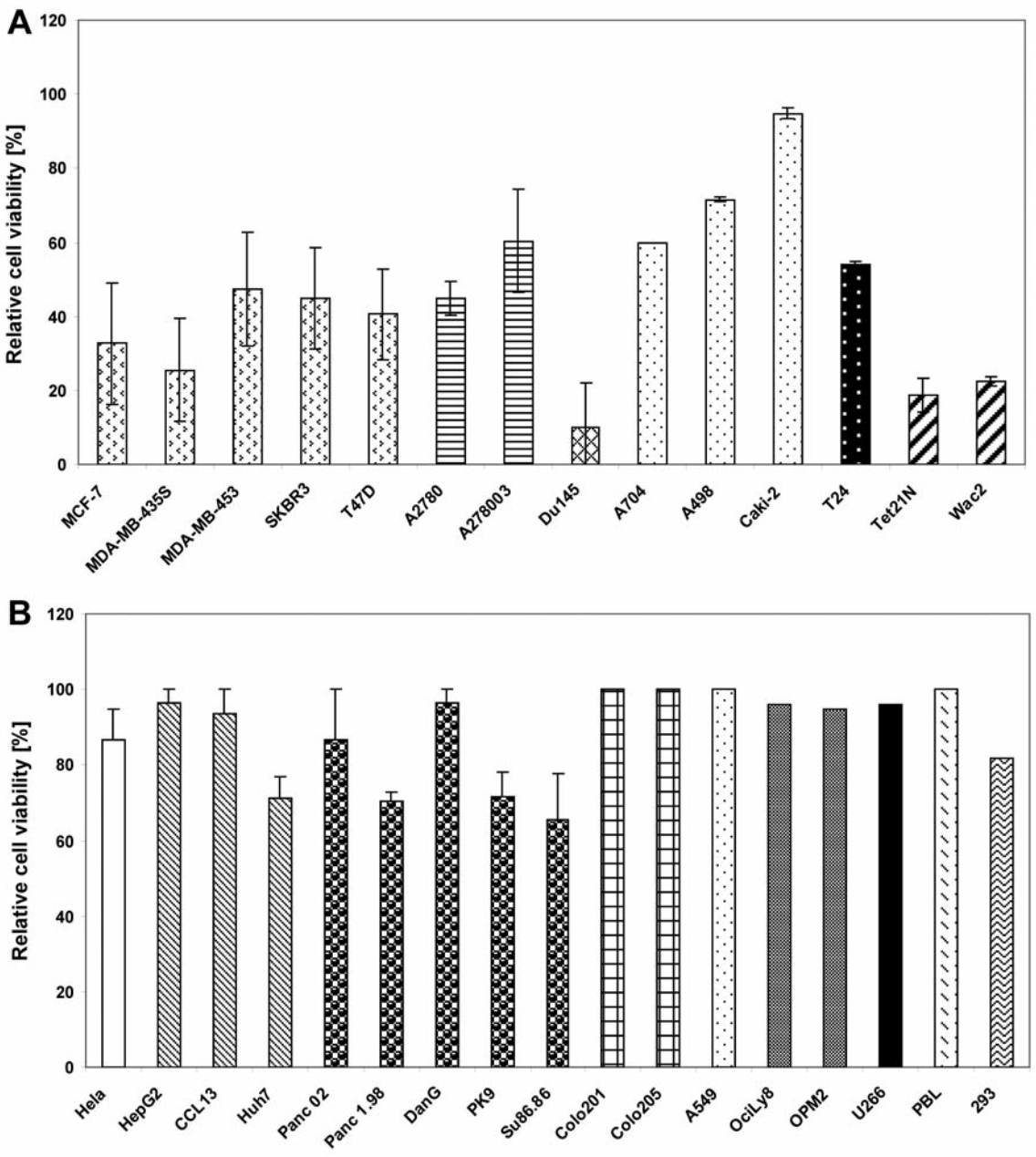

Figure 3. *Effect of TP-Toxin [LTVSPWY-GG-(KLAKLAK)2] after 24 h on different tumor cell types. Different tumor cell types were tested using the MTT assay. Toxicity of TP-Tox toxin at 80 μM was compared to healthy cells. A: Data of the indicated cell lines are shown as the mean±SD of six independent experiments. B: Data of the indicated cell lines are shown as mean±SD of duplicates of two independent experiments and duplicates of six independent experiments for PBL and 293 cells. Different sample sizes (n) were chosen for different cell lines. Student's t-test was used for statistical analysis. A p-value <0.05 was considered significant. C, D) EC_{50} of TP-Tox to the different tumor cell lines.*

Insoluble material was removed by centrifugation at 20000g for 15 min at 4˚C. The supernatant was used for immunoprecipitation. The protein concentration was determined with the Biorad $Protein_{DC}$ Assay (BioRad, Munich, Germany). 100 μl of protein G agarose bead slurry (Pierce Biotechnology, Thermo Fisher Scientific) per sample were mixed with 100 μl lysis buffer and centrifuged for 1 min at 5600× *g*. The supernatant was discarded and the agarose beads were washed with PBS three times, resuspended in 100 μl lysis buffer and incubated on ice for 30 min.

Protein lysates were precleared by incubating with mouse serum (1:200) for 30 min with rotation at 4˚C. Agarose beads (50 μl) were added and samples were incubated for 1 h rotating at 4˚C. To remove

the beads with nonspecific bound proteins, the samples were centrifuged for 1 min at 5600xg at 4˚C. The supernatant was incubated with 10 μM of the targeting peptide conjugated to FLAG (TP-Tox-GGG-DYKDDDK) for two to three hours. Agarose beads were incubated with anti-DYKDDDDK epitope tag monoclonal antibody, 1:200 dilution (MAI91878, Pierce Biotechnology, Thermo Fisher Scientific) for three hours. Beads were added to the samples and incubated rotating at 4˚C overnight. Samples were centrifuged for 1 min at 5600× *g* and beads were washed three times with PBS, resuspended in PBS and one volume of 4× loading buffer was added. Samples were incubated at 99˚C for 5 min and centrifuged for 1 min at 5600× *g*. The supernatants were then loaded onto a 4-12% NuPAGE Novex Bis-Tris gel (Invitrogen) and stained with Coomassie staining solution for 1 h according to the manufacturer's instructions. The gel was destained overnight with fixation buffer (10% acetic acid, 30% methanol). The protein was cut out and analyzed by mass spectrometry [LC-ESI-MS (HCT-Ultra)]. The analysis of the proteins by mass spectrometry was performed by Dr. Sebastian Franken.

Tumor xenograft model. To test toxicity of TP-Tox *in vivo*, the breast cancer cell line MDA-MB-435S was used. A total of 5×10^6 cells in 50 μl medium were co-injected with 250 μg peptide into the mammary gland fat pads of six week-old female nude mice (n=5) (Charles River, Sulzfeld, Germany). 250 μg of the respective peptides dissolved in 50 μl PBS were injected once per week for 90 days. Unconjugated peptides and an unspecific peptide (p34-tox) were used as controls. The tumors were measured with calipers every two to three days and tumor volume was calculated as follows: volume=length×width^2×0.52.

Statistical analysis. For statistical analysis, the results comprising the relative viability were expressed as the mean±standard deviation (SD). Statistical comparison between two groups of data was performed using unpaired Student's *t*-test. A *p*-value <0.05 was considered significant.

Results

Our goal was to design a small peptide that would possess similar functions to an ADC with regard to both targeting and killing of cancer cells, but which would overcome the size restrictions of ADCs. We have designed a novel cancer-targeting peptide-based toxin consisting of 23 amino acids. It contains two functional domains: a cancer-targeting domain and a toxic domain. We tested the toxicity of TP (LTVSPWY) alone, the toxin $(KLAKLAK)_2$ alone, the targeted peptide toxin (TP-Tox), and toxicity of an irrelevant peptide p34-tox, which was shown to be non-toxic against the various cell lines as well as non-cancer cells (19-21). All peptides are shown in Table 1.

Since LTVSPWY was originally described as a peptide which binds to HER2-expressing breast cancer cells (20, 30), we determined the HER2 status of the different breast cancer cell lines. MDA-MB-453, SKBR3, and T47D were HER2-positive while the breast cancer cell lines MCF-7, MDA-MB-435S, and the prostate cancer cell line DU145 did not show any HER2 expression and were used as negative controls (data not shown).

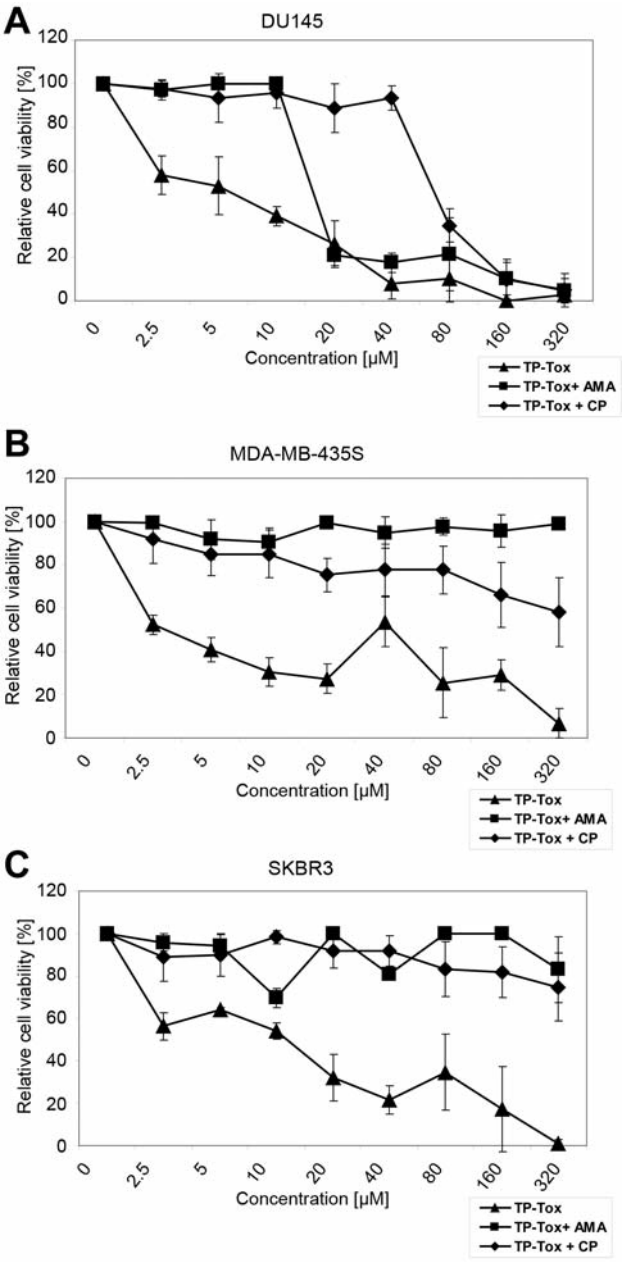

Figure 4. *TP-Tox is taken up via clathrin-mediated endocytosis. Cells were seeded for the MTT assay and allowed to adhere overnight. Medium was discarded and cells were pre-treated with 10 μM antimycin A (AMA) dissolved in medium, for 30 min before exposure to TP-Tox. 10 μM Chlorpromazine (CP) was given in combination with TP-Tox. Toxicity of TP-Tox is shown. A: DU145, B: MDA-MB-435S, C: SKBR3. Data are presented as the mean±SD of duplicates of four independent experiments. Two-tailed Student's t-test was used for statistical analysis. A p-value <0.05 was considered significant.*

Binding and uptake of the targeted peptide toxin TP-Tox. FITC-conjugated peptides were used to analyze binding and uptake of TP-Tox and the control peptides [TP-FITC: LTVSPWY-C-FITC, Tox-FITC: $(KLAKLAK)_2$-C-FITC] by

Table I. *Descriptions of the different peptides which were used in this study.*

Peptide	Sequence	Number of amino acids	MW Da
TP	LTVSPWY	7	865
Tox	KLAKLAKKLAKLAK	14	1524
TP-Tox	LTVSPWY-GG-KLAKLAKKLAKLAK	23	2485.1
p34-tox	HTSPLSV-GG-KLAKLAKKLAKLAK	23	2360
TP-FITC	LTVSPWY-C-FITC	7	1351.5
Tox-FITC	KLAKLAKKLAKLAK-C-FITC	14	1882.3
TP-Tox-FITC	LTVSPWY-GG-KLAKLAKKLAKLAK-C-FITC	23	2843.4
TP-FLAG	DYKDDDDK-GGG-LTVSPWY	18	2028

fluorescence microscopy. SKBR3 cells, which were originally used to identify the targeted peptide by phage display (19-21) served as positive controls. MDA-MB-435S cells and PBLs were used as negative controls. Cells were seeded onto coverslips and incubated with the different peptides. At different timepoints, cells were stained with MitoTracker, fixed, and stained with DAPI. TP-Tox-FITC (Figure 2 B and D) was taken up by SKBR3 (Figure 2 B) and MDA-MB-435S (Figure 2 D) cells after eight hours. Furthermore, we found a nuclear distribution of TP-Tox in MDA-MB-435S cells treated with TP-Tox–FITC (Figure 2 A and C). The green spots show partial localization in the nucleus (Figure 2 D). None of the FITC-conjugated peptides was taken up by PBLs (Figure 2 E and F).

To analyze the toxicity of the targeting peptide toxin, cells were incubated with increasing concentrations of TP-Tox, TP peptide and toxin peptide alone. Toxicity was analyzed at different time points (6 h, 8 h, 12 h, 16 h, 24 h, 36 h, 48 h and 72 h). The strongest effect of peptide on cell viability was found after 24 h. TP-Tox exerted its strongest toxicity towards the prostate cancer cell line DU145 (Figure 3 A).

The single-toxic peptide (Tox) showed minimal toxicity, but by fusing the targeting peptide to the toxin (TP-Tox), toxicity was strongly increased in all these cell lines.

EC_{50} of the toxin alone was greater than 40 μM, whereas the EC_{50} of the bi-functional peptide was 2.5 μM for DU145 (Figure 3 A), MDA-MB-435S (Figure 3 B) and SKBR3 (Figure 3 C) cells. No toxicity was observed towards PBLs (Figure 3 D). Figure 3 E summarizes the toxic effect of TP-Tox and the single peptides. Although both the Tox peptide and the targeting peptide had some activity by themselves the cytotoxic effect was strongly enhanced in the fusion peptide.

We found that the TP-Tox peptide exerted a cytotoxic effect on a number of tumor types independent of their HER2 expression status. Figure 3 shows a summary of the toxicity of TP-Tox (80 μM) towards different tumor types and peripheral blood lymphocytes. Similar toxicity, independent of the HER2 status of the cancer cells, was observed towards all breast cancer cell lines (Figure 4 A). The strongest toxicity towards breast cancer cells was observed for the HER2-negative cell line MDA-MB-435S. Likewise, considerable non-specific toxicity was observed using the ovarian carcinoma cell line A2780, and lower toxicity was observed for the platinum-resistant mutant A278003 (Figure 4 A). The strongest toxicity was measured on the prostate cancer cell line DU145 (Figure 4 A). Only a slight toxicity was observed on different kidney carcinoma cell lines (Figure 3 A). Low toxicity was measured on the urinary bladder cell line T24 (Figure 4 A). The neuroblastoma cell lines Tet21N and Wac2 were differently affected by TP-Tox (Figure 3 A). A similar effect was observed for the pancreatic tumor cell lines (Figure 4 B). No toxic effect on colon carcinoma cell lines Colo201, Colo205, lung carcinoma cell line A549, hepatocarcinoma cell lines Hep2G, Huh7, CCL13 lymphoma cells OciLy8, myeloma cells OPM2 and U266, and healthy cells such as PBL and hek293 was measured (Figure 4 B). The EC_{50} for all cancer cell types is summarized in Table II.

TP-Tox is taken up via endocytosis. To analyze the mechanism of peptide uptake, cells were pre-incubated with antimycin A for 30 min before addition of the peptide. Antimycin A is an inhibitor of nonspecific and receptor-mediated endocytosis at low peptide concentrations. Treatment of the cells with antimycin A reduced the toxic effect of TP-Tox on all three cell lines (Figure 4). Furthermore, we used chlorpromazine to inhibit clathrin-mediated endocytosis. Co-incubating cells with TP-Tox and chlorpromazine showed that toxicity of TP-Tox was strongly reduced (Figure 4). Cytochalasin D, an inhibitor of actin polymerization and verapamil, a calcium channel blocker, had no effect on toxicity (data not shown).

Identification of peptide-binding proteins. To identify the target to which TP-Tox binds, we performed immunoprecipitation using a FLAG-tagged targeting sequence (LTVSPWY-GGG-FLAG respectively LTVSPWY-GG-$(KLAKLAK)_2$-GGG-FLAG) as bait on protein lysates from MCF-7 and DU145

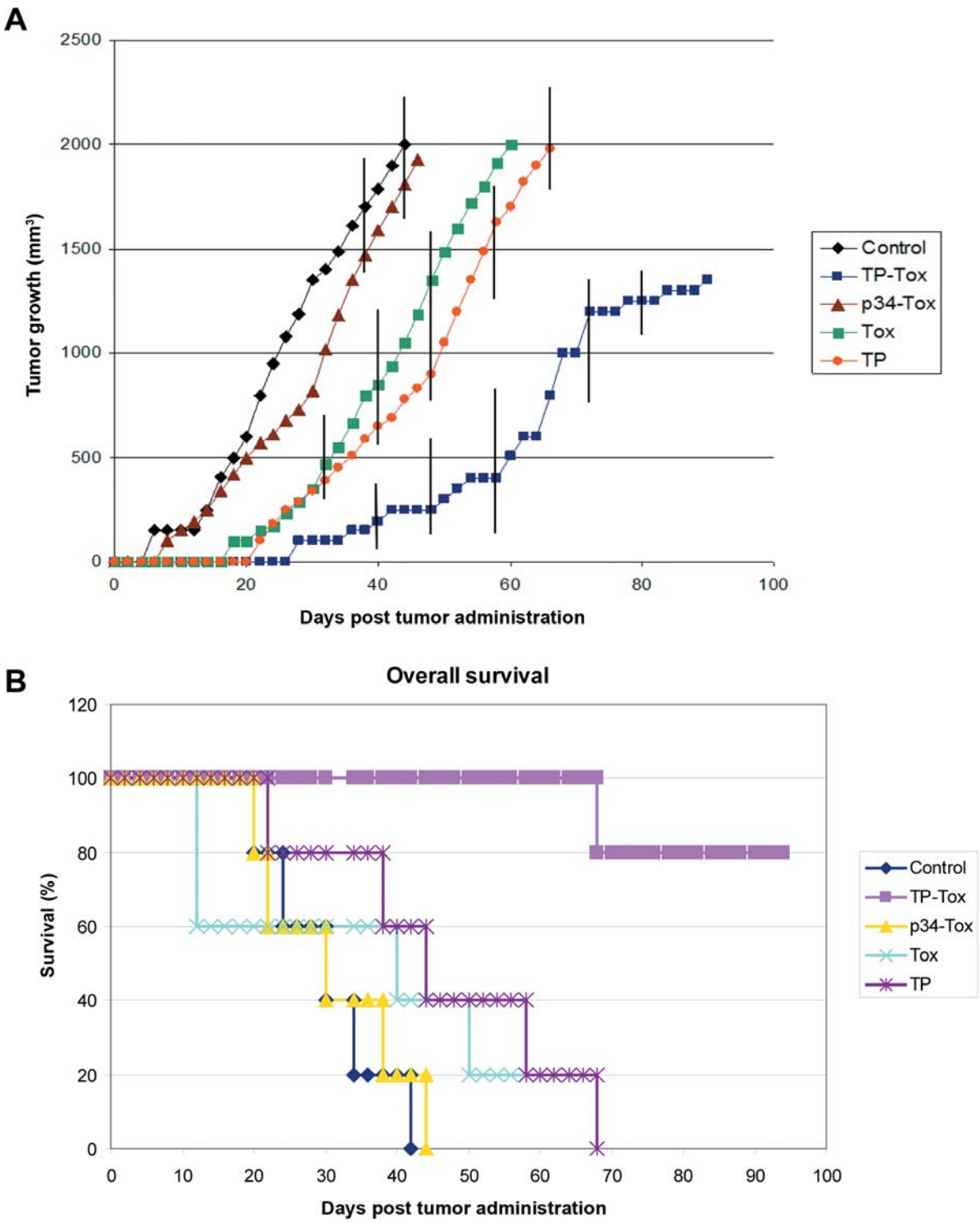

Figure 5. *TP-Tox retards tumor growth in vivo. Mice (n=5) were treated with 250 μg peptide/50 μl per mouse for two weeks, before tumor cells were injected. Unconjugated peptides and a non-specific peptide (p34-tox) were used as controls. The respective peptides (250 μg) dissolved in 50 μl PBS were injected once per week for 90 days. Control mice were injected with PBS. The tumors were measured with calipers every two to three days and tumor volume was calculated as follows: volume=length×width²×0.52. For statistical analysis, the results comprising the relative viability were expressed as the mean±standard deviation (SD). Different sample sizes (n) were chosen for different cell lines. Student's t-test was used for statistical analysis. A p-value <0.05 was considered significant. A: Tumor growth of mice which received weekly injections of PBS (control), TP-Tox, an irrelevant peptide p34-tox and the single peptides (TP and Tox). B: Overall survival of mice.*

cells. Since no toxicity of TP-Tox was measured on A549 cells and PBLs, these protein lysates served as negative control. The pulled down proteins were analyzed by mass spectrometry in comparison to the A549 cell and PBL control samples. Proteins identified in control cells were excluded as potential target. Table III summarizes the proteins which were regarded as potential binding partners for TP-Tox.

RAB; P20340 and ITSN1 Q6J334 are involved in endocytosis (26-29, 31, 32). RAS GTPase-activating protein binding protein (G3BP1, Q5U0Q1), DDX3X and DHX9 two ATP-dependent RNA helicases are involved in transcription (32–35).

TP-Tox inhibits tumor growth in vivo. The effect of the TP-Tox, single peptides and an irrelevant control peptide, respectively, on tumor growth *in vivo* was tested using MDA-MB-435S cells in which we observed the strongest cytotoxicity *in vitro*. Weekly intra-venous injections of 250 μg peptide was given to mice for 10 weeks (n=5). The control group received injections of PBS. After 10 days, an inhibition of tumor growth was observed in mice treated with the TP-Tox peptide compared to control mice (Figure 5A). Mice treated with either of the single peptides showed retardation of tumor growth, which was measurable starting from day 20, mirrored by an enhancement of overall survival (Figure 5 B). Eighty percent of mice treated with TP-Tox had survived by the end of the 100-day follow-up period, whereas mice in control groups died by day 70 after tumor onset. Mice in control groups (untreated mice, p34-tox) had a survival rate of 50% at day 30. Mice treated with the single peptides (TP, tox) had a survival rate of 50% at day 40 and day 45, respectively (Figure 6 B). The overall survival of mice treated with p34-tox was nearly identical to that of the control mice.

Table II. *Potential molecular targets of the bi-specific protein. Proteins identified by mass spectrometry from MCF-7, MDA-MB-435S, SKBR3 and DU145 cell lysates after binding and immunoprecipitation using FLAG tagged targeting peptide (LTVSPWY-GG-(KLAKLAK)2-GGG-FLAG). As described in Materials and Methods, protein lysates were incubated with LTVSPWY-FLAG peptide and Anti-FLAG beads over night at 4˚C. Beads were washed with PBS buffer and incubated at 99˚C for 5 min. Samples were centrifuged for 1 min at 5600 ×g and supernatant was loaded on a SDS gel. Bands were cut and used for mass spectrometry. (EC_{50}) values for (TP-Tox) measured by (MTT) assay after 24 h.*

Tumor cell type	EC_{50} (μM)
Breast cancer	
MCF-7	50
MDA-MB-435S	2.5
MDA-MB-453	80
SKBR3	2.5
T47D	60
Prostatic cancer	
DU145	2.5
Ovarian cancer	
A2780	80
A27003 (platin resitant)	160
Kidney cancer	
A498	120
A704	160
Caki-2	200
Bladder cancer	
T24	120
Neuroblastoma cancer	
Tet21N	60
Wac2	60
Hela	>320
Hepatocarcinoma	
Huh7	160
Hep2G	320
CCL13	120
Pancreatic carcinoma	
Panc02	120
Panc1.98	120
DanG	120
PK9	>320
Sum86	>320
Bronchial carcinoma	
A549	>320
Colon carcinoma	
Colo201	>320
Colo205	>320
Lymphoma cells	
OciLy8	>320
Myeloma cells	
OPM2	>320
U266	>320
Other cells	
PBL	>320
HEK293	240

Discussion

In the present study we designed a novel bi-functional peptide which we found to exert cytotoxicity towards breast and prostate cancer cells independent of their HER2 status. We showed that while the single peptides exerted minimal toxicity (Figure 2), the *in vitro* toxic potential was dramatically increased by coupling the targeted peptide to the toxic peptide to generate the bi-functional peptide. As previously shown, the toxic peptide exerts toxicity towards eukaryotic cells at concentrations greater than 300 μM (13) because of its positive charge, whereas TP-Tox has an EC_{50} of 2.5 μM, which is more than 100-times increased compared to the single-toxic peptide for DU145 cells. By coupling the targeted peptide to the toxic peptide, the positive charge was increased resulting in an increased toxicity (13). At high concentrations cationic peptides are taken up by the cells through the disruption of the plasma membrane integrity (13, 29, 36). This leads to a nonspecific toxic effect which could explain the observed effects of single peptides at high concentrations. In contrast, the bi-functional peptide TP-Tox is taken up by cells after six hours, and predominantly detected in the cytoplasm

Table III. *Potential molecular targets of the bi-functional protein. Proteins were identified by mass spectrometry from MCF-7, MDA-MB-435S, SKBR3 and DU145 cell lysates after binding and immunoprecipitation using FLAG-tagged targeting peptide [LTVSPW-GG-(KLAKLAK)2-GGG-FLAG]. As described in Materials and Methods, protein lysates were incubated with LTVSPWY-FLAG peptide and Anti-FLAG beads overnight at 4˚C. Beads were washed with PBS buffer and incubated at 99˚C for 5 min. Samples were centrifuged for 1 min at 5600 ×g and the supernatant was loaded on a SDS gel. Bands were cut and analyzed for mass spectrometry.*

Accession no.	DU145, MCF-7, MDA-MB-435S, SKBR3	MW
Q5U0Q1	RAS GTPase-activating protein binding protein 1 (G3BP1) MCF-7, MDA-MB-435S, SKBR3	54 kDa
Q9NRY4	Rho GTPase activating protein 35 (GRLF-1) DU145	170 kDa
Q53HU2	RACK1 (Receptor of activated protein kinase C1, guanine nucleotide-binding protein subunit beta 2 like 1) MCF-7, MDA-MB-435S, DU145	35 kDa
DDX3X	DDX3X (ATP-dependent RNA helicase), MCF-7, MDA-MB-435S	73 kDa

as a diffuse staining pattern, suggesting that it might have escaped the endosome and is therefore more toxic to the cells (Figure 2D: MDA-MB-435S) and finally reached the nucleus. Our results with small-molecule inhibitors suggest that the peptide is taken up by endocytosis. Chlorpromazine, which inhibits clathrin-mediumted endocytosis, completely inhibited toxicity of TP-Tox. In our target identification experiment many proteins which take part in the endocytotic pathway were identified as potential targets. Association with the actin filaments (Figure 2D: MDA-MB-435S) was supported by the immunoprecipitation results. We identified clathrin CHL1 in the control sample of A549 cells. Huntingtin-interacting protein HIP1 (O00291) and intersectin 1 (Q6J334, isoform 10) as accessory proteins of clathrin-mediumted endocytosis were also found in immunoprecipitation samples of the control lysates (PBL and A549). Both bind to actin and regulate cytoskeletal dynamics in proliferating cells (36, 37). Different members of (RAB) GTPases were found in A549 samples (RAB22) and RAB 5 was found in DU145 cells. RAB GTPases are known to be coordinators of vesicle trafficking (28). RAB5 is associated with clathrin-coated vesicles where it is essential for the assembly of clathrin-coated pits at the plasma membrane and vesicle motility along microtubules (38). Treatment of cells with cytochalasin D which is known to destroy the actin filaments had no effect on toxicity. These proteins support the results of cell treatment with endocytosis inhibitors. The peptide might be taken up by different cell lines but the intracellular target might be inactive due to methylation status (39). Toxicity of TP-Tox to tumor cell lines might be different due to differences in membrane composition (8, 9, 11, 37, 40). Subsequently the peptide might bind to an intracellular target such as RAS GTPase activating SH3 domain binding protein 1, for which a function in prostate and breast cancer has already been proposed (39). Both proteins were found in our MCF-7 pulldown. Furthermore, the target G3BP1 identified in the breast cancer cell line MCF-7 is involved in cytoskeleton regulation in proliferating cells and in transcription, which may explain the observed localization in the nucleus (41–43). A nuclear localization was observerved in a study with different breast cancer samples (43). G3BP1 was found in MCF-7 cells in a proteomic approach to identify new breast cancer biomarkers (44, 45). The identification of the G3BP1 as a binding partner for the targeting sequence supports the results of TP-Tox uptake *via* endocytosis. Earlier studies showed that RASGP as a potential downstream target of G3BP1 interacts with RHO proteins which take part in cytoskeletal reorganization (46, 47). Such a protein (RACK1) was identified in DU145, MCF-7 and SKBR3 cell lysates. The current study shows, that as a potential downstrean target, G3BP1 interacts with RASGAP through the SH3 domain of RASGAP. At low intracellular caspase activity (for example in MCF-7 cells) RASGAP is split into two fragments: an *N*-terminal 56 kDa fragment and a 64 kDa *C*-terminal fragment. Fragment *N* inhibits fragment C by activating anti-apoptotic pathways. Fragment C acts pro-apoptotically. It is possible that TP-Tox somehow interacts with the SH3 domain in the *N*-terminal fragment and by this promotes the apoptotic effect of the *C*-terminal fragment. A further possibility is that G3BP1 regulates transcription or RAS (48-50).

In addition to the observed toxicity *in vitro*, we have also shown a strong antitumor effect *in vivo*. Tumor growth was retarded when TP-Tox and tumor cells were co-injected compared to tumor growth of mice which were treated with the single peptides or the irrelevant peptide. The toxic effect was already measurable at day 20 and became clear at day 40. Mice which received TP-Tox peptide injections had a more than doubled life-span compared to mice which were injected with the irrelevant peptide.

Conclusion

We designed a bi-functional peptide of 23 amino acids and showed its ability to bind and kill several cancer cell lines *in vitro* and strongly increase survival in breast cancer-bearing mice *in vivo*. This novel toxin could be used in future cancer therapies and warrants further pre-clinical and clinical exploration.

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Competing Interests

There are no competing interests.

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