Targeted dianthin is a powerful toxin to treat pancreatic carcinoma when applied in combination with the glycosylated triterpene SO1861

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Targeted cancer therapy provides the basis for the arrest of tumor growth in aggressive pancreatic carcinoma; however, a number of protein-based targeted toxins lack efficacy due to insufficient endosomal escape after being endocytosed. Therefore, we tested a fusion protein of the ribosome-inactivating protein dianthin and human epidermal growth factor in combination with a glycosylated triterpene (SO1861) that serves as an endosomal escape enhancer. In vitro investigations with the pancreatic carcinoma cell lines BxPC-3 and MIA PaCa-2 revealed no significant differences to off-target cells in the half maximal inhibitory concentration (IC50) for the fusion protein. In contrast, combination with SO1861 decreased the IC50 for BxPC-3 cells from 100 to 0.17 nM, whereas control cells remained unaffected. Monotherapy of BxPC-3 xenografts in CD-1 nude mice led to a 51.7% average reduction in tumor size (40.8 mm3) when compared to placebo; however, combined treatment with SO1861 resulted in a more than 13-fold better efficacy (3.0 mm3 average tumor size) with complete regression in 80% of cases. Immunohistochemical analyses showed that tumor cells with lower target receptor expression are, in contrast to the combination therapy, able to escape from the monotherapy, which finally results in tumor growth. At the effective concentration, we did not observe liver toxicity and saw no other side effects with the exception of a reversible skin hardening at the SO1861 injection site, alongside an increase in platelet counts, plateletcrit, and platelet distribution width. In conclusion, combining a targeted toxin with SO1861 is proven to be a very promising approach for pancreatic cancer treatment.

1. Introduction

Pancreatic ductal adenocarcinoma is the fourth most common cause of death from cancer in the USA; it was estimated that 48 960 new cases would be diagnosed and that 40 560 patients would succumb to the disease over the course of 2015 (Siegel et al., 2015). The identification of various molecular pathways that

Abbreviation
DMEM, Dulbecco’s modified Eagle’s medium; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HE, hematoxylin–eosin; IC50, concentration that inhibits cell survival to half maximal extent; KRAS, Kirsten rat sarcoma viral oncogene homolog; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Ni-NTA, nickel nitrilotriacetic acid; PBS, phosphate-buffered saline; RPMI medium, Roswell Park Memorial Institute medium; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; SO1861, an amphipathic glycoside of 1861 Da isolated from Saponaria officinalis.
are very prominent in pancreatic carcinoma has contributed to the development of targeted therapies including monoclonal antibodies and small-molecule inhibitors (Jones et al., 2008). In patients with advanced-stage disease, modest improvements in survival have recently been attained with the chemotherapy regimen FOLFIRINOX (folinic acid, 5-fluorouracil, irinotecan, and oxaliplatin) or albumin-bound paclitaxel (nab-paclitaxel) plus gemcitabine chemotherapy (Conroy et al., 2011; Von Hoff et al., 2013). The combination of gemcitabine and the epidermal growth factor receptor (EGFR; HER1; erbB1) tyrosine kinase inhibitor erlotinib gained regulatory approval following a 12-day improvement in median survival compared with gemcitabine alone in a large, randomized phase III trial (Moore et al., 2007). A factor limiting the efficacy of pancreatic cancer treatment is impaired drug delivery as a result of the unique desmoplastic response – the pervasive growth of dense fibrous tissue around the tumor – that occurs in pancreatic ductal adenocarcinoma (Garrido-Laguna and Hidalgo, 2015).

The EGFR is overexpressed in a variety of human tumors including pancreatic, non-small-cell lung cancer, breast, head and neck, gastric, colorectal, esophageal, prostate, bladder, renal and ovarian cancers (Salomon et al., 1995). Overexpression of EGFR and its ligands is frequently observed in pancreatic cancer and correlates with poor prognosis, disease progression, and metastasis (Oliveira-Cunha et al., 2011; Xie and Xie, 2015), supporting the prominent role of the EGFR family for malignant transformation, prevention of apoptosis, and drug resistance. The pancreatic islet cell is considered by some to be a signaling hub (Barker et al., 2013). Although the Kirsten rat sarcoma viral oncogene homolog (KRAS) is mutated and activated in >90% of pancreatic cancers, EGFR expression has also been shown to be essential for KRAS-driven pancreatic ductal adenocarcinoma (Eser et al., 2014; Morris et al., 2010).

Treatment with monoclonal antibodies has also gained importance in past decades. The antitumor effect of monoclonal antibodies is thought to be primarily mediated via antibody-dependent cell cytotoxicity, although complement-mediated lysis may also contribute. In addition to antibody-dependent cell cytotoxicity, these antibodies can be linked to toxins resulting in direct killing of the tumor cell after internalization or linked to a radioisotope and used as radiopharmaceuticals (Dodson et al., 2011).

Several clinical trials examining EGFR tyrosine kinase inhibitors are under way. A recent phase 3 trial of EGFR inhibitors has shown to be ineffective, including the use of the monoclonal antibody cetuximab in patients with late-stage pancreatic cancer. Nevertheless, the results of clinical trials that target EGFR seem to be promising because monotherapy with panitumumab provided significant clinical benefit in heavily pretreated patients without acquired resistance to prior cetuximab-based regimens (Pietrantonio et al., 2013), while the clinical relevance and cost-effectiveness remain debatable.

The first generation of targeted toxins, developed 35 years ago, employed chemical conjugates of antibodies with intact toxins, or toxins with attenuated cell-binding properties. Although they showed tumor regression in some patients with lymphoma, they were typically ineffective because the constructs were heterogeneous, nonspecific, and were too large to infiltrate solid tumors (Ghetie and Vitetta, 2001). Recombinant DNA techniques were applied in the production of third-generation targeted toxins to promote tumor specificity and penetration, and to reduce the cost and complexity of production. The cell-binding domain of the toxin is genetically removed and the modified toxin fused with a ligand or with DNA elements encoding the Fv portion of an antibody in these constructs. A variety of toxins, mainly from plants, fungi, or bacteria, have been characterized, structurally optimized for in vitro stability, activity, and safety, and evaluated in vivo in animal studies and clinical trials. Among them, ricin, Pseudomonas exotoxin, and diphtheria toxin are the most frequently used. However, the development of targeted toxins for the treatment of solid tumors poses challenges such as the generation of an immune response against the toxin moiety, poor tumor penetration, and reduced half-life. Targeted toxins with RNases as the cytotoxic moiety are recent examples aimed at reducing the immunogenicity (Chang et al., 2010; Huhn et al., 2001).

Cytosolic drug delivery is one of the major routes in cancer treatment (Yu et al., 2016). The toxins naturally consist of several domains—the cell-binding or cell recognition domain, the translocation domain, which enables release of the toxin into the cytosol, and the catalytic domain responsible for cytotoxicity. In the development of targeted toxins, the binding domain of these toxins is replaced by cancer cell-specific ligands. The cancer cell-specific ligands direct the internalization of the toxins via receptor-mediated endocytosis; however, in many constellations, the toxin remains ineffective as it is recycled back to the cell surface or transported to the lysosomes where it is degraded (Fuchs et al., 2016). This might be the reason why no antibody-targeted protein toxin has been approved for tumor therapeutic applications by the
authors to date. To overcome the insufficient endosomal escape, many strategies have been developed to weaken the membrane integrity of endosomal membranes by molecules that interact more or less directly with membranes (Fuchs et al., 2016). A common feature of these substances is that they are per se not target specific and distribute inside organisms with other kinetics than the targeted toxins. It is therefore obvious that the combination of these endosomal escape enhancers and targeted toxins can only be sufficiently tested in vivo; however, most of the published enhancers were only tested in vitro, which may explain lacking success of these strategies in clinical applications.

The aim of this study was to analyze the role of a targeted toxin consisting of the ribosome-inactivating plant toxin dianthin and human epidermal growth factor (HisDianthin-EGF) in combination with an amphipathic glycoside (SO1861) that serves as an endosomal escape enhancer in order to increase the cytotoxicity in EGFR-overexpressing pancreatic carcinoma cells. A xenograft model was developed to determine the therapeutic efficacy of the targeted toxin alone and the targeted toxin in combination with SO1861. As amphipathic glycosides are known to have the potential to induce hemolysis, one focus of side effect examination was laid on complete blood count analysis.

2. Materials and methods

2.1. Expression and purification of recombinant proteins

Plasmid DNA (HisDianthin-EGF-pET11d) (Weng et al., 2012) was transformed into chemically competent Escherichia coli NiCo21 (DE3) (New England Biolabs®, Inc., Frankfurt am Main, Germany) and grown in 3 mL lysogeny broth medium supplemented with 50 μg·mL⁻¹ ampicillin at 37 °C for 5 h at 200 rpm. These bacteria were used to inoculate 500 mL lysogeny broth supplemented with 50 μg·mL⁻¹ ampicillin for overnight culture at 37 °C. Subsequently, the culture volume was scaled up to 2 L and bacteria were grown until an optical density (A₆₀₀) of 0.9. Expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside at a final concentration of 1 mM. Cells were further grown for 3 h at 37 °C and 200 rpm. After centrifugation (5 min, 5000 g, 4 °C), cell pellets were resuspended in 20 mL phosphate-buffered saline (Dulbecco’s phosphate-buffered saline (PBS) with Ca²⁺ and Mg²⁺, pH 7.4) and stored at −20 °C. After thawing, HisDianthin-EGF was released by ultrasound device (Branson Sonifier 250, G. Heinemann). The solution was centrifuged (30 min, 4 °C) and adjusted to 20 mM imidazole concentration. The construct contained an N-terminal His-tag and was purified by nickel nitrolotriacetic acid chromatography (Ni-NTA agarose; Qiagen, Hilden, Germany). After elution with imidazole (20–250 mM), the eluates were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) (12%). Fractions containing HisDianthin-EGF were dialyzed against 2 L chitin binding domain buffer (20 mM tris (hydroxymethyl)-aminomethane/HCl, 500 mM NaCl, 1 mM EDTA, 0.1% Tween-20, pH 8.0) at 4 °C. Further purification by chitin column affinity chromatography served to remove bacterial proteins with binding activity for Ni-NTA agarose. After elution with chitin binding domain buffer, the fractions were analyzed by SDS/PAGE (12%). Fractions containing HisDianthin-EGF were dialyzed against 5 L PBS at 4 °C. His-tagged proteins were concentrated by Amicon centrifugal filter devices (10 kDa; Millipore, Eschborn, Germany). The protein concentration was determined by a bicinchoninic acid assay (Pierce, Rockford, IL, USA).

2.2. Isolation of SO1861

SO1861 was isolated from Saponaria officinalis L. Dried roots were finely ground and extracted by 90% methanol. The methanol was evaporated by vacuum distillation and cold acetone was added to the remaining aqueous extract. The resulting suspension was centrifuged and the pellet was dissolved in 20% methanol to 20 mg·mL⁻¹ raw material. SO1861 was isolated by semipreparative high-performance liquid chromatography using an UltraSep ES PHARM RP18E (7 μm, 250 × 8 mm) column from SepServ (Berlin, Germany) and a methanol/water (0.01% trifluoroacetic acid) gradient starting with 20% methanol to 80% methanol over 80 min. Flow rate was 1.5 mL·min⁻¹. Fractions of SO1861 were freeze-dried and analyzed by ESI-MS. The isolated SO1861 was subjected to high-performance thin-layer chromatography analysis using corresponding silica gel 60 F254 plates (Merek Chemicals GmbH, Darmstadt, Germany) and the upper phase of a glacial acetic acid/water/butanol (2 : 8 : 10) mixture as solvent. Derivatization was performed by sulfuric acid (10%) and plates were dried at 140 °C. Purity of SO1861 was assessed by densitometry using the CAMAG TLC 4 Scanner (CAMAG, Berlin, Germany). Absorption was measured at 600 nm. Scans are shown in the Supporting information (Fig. S4).

2.3. Determination of N-glycosylase enzymatic activity

The N-glycosylase activity of HisDianthin-EGF was determined by an adenine release assay where adenine...
residues were released from herring sperm DNA (Weng et al., 2012). Briefly, 600 nM of the targeted toxin or free toxin was mixed with 10 µL (100 µg) herring sperm DNA and the volume made up to 100 µL by acetate buffer (50 mM sodium acetate, 100 mM KCl, pH 4.0). The mixture was incubated for 1 h in a shaker at 50 °C. Controls were either incubated with 600 nM bovine serum albumin or only with acetate buffer and herring sperm DNA. After separation of released adenine and herring sperm DNA by centrifugation with filter membranes (molecular mass cutoff 3 kDa, 5000 g, 10 °C, 20 min; Sigma Aldrich, Hamburg, Germany), the absorbance of the flow-through was measured at 260 nm by a Nano Drop spectrometer (ND-1000; Pqlab Biotechnologie GmbH, Erlangen, Germany). Adenine release was calculated by the use of an adenine standard curve.

2.4. Cell culture
The human pancreatic carcinoma cell lines BxPC-3 and MIA PaCa-2 were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium and Dulbecco’s modified Eagle’s medium (DMEM), respectively. NIH-3T3 mouse embryonic fibroblast cells were cultured in DMEM. Medium was supplemented with 10% fetal bovine serum (BioChrom KG, Berlin, Germany), 100 U mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin (Merck Chemicals GmbH). Cells were cultured at 37 °C and 5% CO₂.

2.4.1. Viability assays
Cells were seeded in 96-well transparent round-bottomed plates (4000 cells per well) and grown for 24 h. Cells were then treated with culture medium either supplemented with SO1861 or not. The safely tolerated concentration of SO1861 on cells after 72 h was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Thereafter, viability of Dianthin (final concentration 1 µM to 1 µM) and Dianthin-EGF (final concentration 0.1 µM to 1 µM) either supplemented with SO1861 or not after 48 h was determined by MTT. Controls were treated only with culture medium or 0.5 µg·mL⁻¹ SO1861. Experiments with SO1861 variation were conducted at the determined IC₅₀ concentration of Dianthin-EGF obtained in the presence of 0.5 µg·mL⁻¹ SO1861 for each cell line (Table 1).

2.4.2. Real-time monitoring
Real-time monitoring of the cell growth after treatment with SO1861 in combination with Dianthin-EGF was carried out using the xCELLigence real-time cell analyzer (Roche, Basel, Switzerland). With this device, cell proliferation can be recorded continuously during the experiment by measuring the impedance of the cells that are attached on the surface of culture plates with interdigitated gold microelectrodes (e-plates).

NIH-3T3, MIA PaCa-2, and BxPC-3 cells were trypsinized by using trypsin/EDTA (0.25%). Meanwhile, the blank measurement (50 µL DMEM, or RPMI-1640 for BxPC-3) was performed on 96-well plates (16-well for BxPC-3) in the xCELLigence device. Thereafter, 4000 cells/100 µL were seeded per well. After 24 h when the impedance-based cell index reached between 0.5 and 1, Dianthin-EGF with and without SO1861 was added in triplicate (in duplicate for BxPC-3) in different concentrations (100 pM to 1 µM for single treatment and 10 pM to 100 nM in case of combination treatment with SO1861). After 120 h of real-time measurement, cytotoxicity was evaluated using the XCELLIGENCE software (RTCA 2.0; ACEA Biosciences Inc., San Diego, CA, USA).

All the in vitro experiments, unless otherwise mentioned in the legend, were performed in quadruplicate in three independent experiments, and the values are reported as their mean.

2.5. In vivo studies
All animal experiments were approved by the local authority according to national animal ethics.

Table 1. Specificity of Dianthin-EGF in the absence and presence of SO1861. The table shows IC₅₀ values calculated from corresponding cytotoxicity assays conducted as described in the methods section, and derived targeting indices and SO1861 enhancement factors. TI, targeting index = IC₅₀ (toxin)/IC₅₀ (targeted toxin); EF, enhancement factor = IC₅₀ (−SO1861)/IC₅₀ (+SO1861); D, Dianthin; DE, Dianthin-EGF.

| Cell lines | IC₅₀ (D) (nM) | IC₅₀ (DE) (nM) | TI D/DE | IC₅₀ (D + SO1861) (nM) | IC₅₀ (DE + SO1861) (nM) | TI D/DE + SO1861 | EF (D) | EF (DE) |
|------------|----------------|----------------|---------|-----------------------|-------------------------|--------------------|--------|--------|
| BxPC-3     | 10 900         | 100            | 109     | 390                   | 0.17                    | 2294               | 28     | 588    |
| MIA PaCa-2 | 100            | 30             | 3.3     | 40                    | 5.3                     | 7.5                | 2.5    | 5.7    |
| NIH-3T3    | 22             | 73             | 0.3     | 186                   | 110                     | 1.7                | 0.12   | 0.7    |
regulations (approval number G 0260/10 from LAGESo, Berlin, Germany). The recommendations of the animal welfare officer of the Charité, Berlin, were strictly adhered to during the entire duration of the experiment.

2.5.1. Acute toxicity study

Acute toxicity studies were performed on groups of 7-month-old male BALB/c mice weighing 30–35 g. The study comprised four groups each containing three mice. Doses of 40, 4, and 0.4 μg HisDianthin-EGF in 100 μL PBS were administered once per mouse intraperitoneally in three groups, and the fourth group was the nontreated control group. Thereafter, the mice were observed every 1 h on the first day and then twice every 24 h for their body weight and behavioral changes for 1 week. Animals were then sacrificed and organs were collected and prepared for histopathological analysis.

2.6. Xenograft studies

2.6.1. Development of a solid tumor xenograft model

To develop a tumor growth rate curve, 1.25 × 10⁶ BxPC-3 cells/100 μL in PBS/mouse were injected subcutaneously into the right flank of six 7-month-old male CD-1 nu/nu mice (28–35 g; Charles Rivers, Sulzfeld, Germany). Animals were observed daily for their body weight shifts and tumor development. After 13 days, a small palpable tumor was observed, although not measurable using vernier caliper. Two days later, tumor size was measured by caliper and the process was followed, twice weekly for 4 weeks. Animals were then sacrificed and tumor growth rate curve was established. The EGFR expression level was determined histologically for the grown tumors.

2.6.2. Efficacy of HisDianthin-EGF alone or in conjunction with SO1861 as compared to placebo

The in vivo efficacy experiments were carried out in 7-month-old CD-1 nu/nu mice, comprising five animals per group. The mice were housed in individually ventilated cages under a constant day and night cycle (12 h each) and had free access to animal feed (Sniff, Soest, Germany) and water. All animals were monitored daily for health and well-being during the entire experiment. In order to slightly elevate tumor growth, the tumor was inducted by a subcutaneous injection of 1.3 × 10⁶ BxPC-3 cells at the right flank of each animal with sufficient distance to the vertebral column. The cells were diluted in 100 μL PBS and injected with a 29-gauge needle.

2.7. Hematological analysis

Approximately 1.2 mL of blood was collected by cardiac puncture on the 25th day of the experiment in isoflurane-anesthetized mice. Blood was collected in an S-Monovette® 1.2-mL K3 EDTA sterile tubes and was sent to Labor 28 GmbH, Berlin, Germany, for complete blood count analysis, including red and white blood cell counts, platelets, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration.

2.8. Histopathological analysis

The animals were sacrificed by cervical dislocation. The tumors were collected along with the adjoining skin. In case that no tumor was macroscopically seen, the skin from the site of tumor cell injection was prepared. In addition, the spleen, liver, lung, heart, intestine, and kidneys were also collected. After formalin fixation, the organs and the tumor samples were embedded in paraffin for histological examination. The samples were cut into 4-μm slices and were stained with hematoxylin–eosin (HE) and immunohistochemically for the determination of the EGFR expression level (EGFR pharmDx™ Kit; DAKO, Hamburg, Germany). Ki-67 proliferation index (monoclonal antibody Ki-67P; fianova, Hamburg, Germany) was also determined for the tumor samples and detected with secondary reagents (K5005; DAKO). The tissues were
examined for any histopathological alterations, which might be due to toxin-induced injury using the criteria as published elsewhere (von Mallinckrodt et al., 2014).

2.9. Statistical analyses

All the statistical analyses were performed with the help of GRAPHPAD PRISM 6.0. Student’s t-test was utilized for the examination of significant difference in cytotoxicity assays. Paired and unpaired t-tests using GRAPHPAD software were used to evaluate significance level in body weight loss and dose–response curve studies in xenografts, respectively. The concentration that inhibits cell survival to half maximal extent (IC50) was calculated by the method of least squares using a sigmoidal four-parameter logistic regression.

3. Results

3.1. Purification and determination of enzymatic activity of HisDianthin-EGF

In a first step, the recombinantly expressed HisDianthin-EGF was purified by Ni-NTA chromatography. Obtained fractions were analyzed by SDS/PAGE (Fig. 1A). No substantial amounts of HisDianthin-EGF were found in the pellet obtained after ultrasonication, the flow-through of the column, and the washout. At 36 kDa, a clear band of HisDianthin-EGF was detected in the 125 mM imidazole eluate, but slight contaminations of bacterial proteins were also observed. As the 250 mM imidazole eluate also contained some HisDianthin-EGF, both fractions were dialyzed and then applied to a chitin column chromatography to remove contaminating proteins. SDS/PAGE revealed highly purified HisDianthin-EGF with no contaminants visible in the Coomassie stain (Fig. 1B). The identity was verified by western blotting (Fig. S1) and former preparations also by matrix-assisted laser desorption/ionization-time of flight (Gilabert-Oriol et al., 2013c). The total yield for HisDianthin-EGF was around 8 mg from 2 L of bacterial suspension. In an enzymatic activity assay for ribosome-inactivating proteins, recombinant HisDianthin-EGF released 42 pmol adenine-pmol toxin h^{-1} compared to 67 pmol-pmol^{-1}h^{-1} for ligand-free HisDianthin, indicating 37% loss in catalytic activity due to fusion to EGF.

3.2. Cytotoxicity of HisDianthin and HisDianthin-EGF augmented by SO1861

To first characterize efficacy and selectivity of HisDianthin-EGF on pancreatic cell lines and the enhancer effect of SO1861, cytotoxicity of HisDianthin-EGF

| Pellet | FT | kDa | W | 1 | 2 |
|--------|----|-----|---|---|---|
|        |    | 116 | 66.2 |   |   |
|        |    | 45  | 35 |   |   |
|        |    | 25  | 18.4 |   |   |
|        |    | 14.4 |   |   |   |

**A. Ni-NTA chromatography**

| 125 mM imidazole | 250 mM imidazole |
|------------------|------------------|
| Washouts         | Washouts         |
| W1               | W2               |
| kDa              | W1               |
|                  | W2               |
| 45               | 35               |
| 25               | 25               |

**B. Chitin column chromatography**

Fig. 1. SDS/PAGE (12%) of the pellet obtained after ultrasonication and purified fractions of proteins obtained after (A) Ni-NTA chromatography and (B) chitin column affinity chromatography. FT, flow-through; W, washout; lane ‘1’: 125 mM imidazole eluate; lane ‘2’: 250 mM imidazole eluate. Arrows point to HisDianthin-EGF.
in the absence and presence of SO1861 was determined on the EGFR-overexpressing pancreatic carcinoma cell lines BxPC-3 and MIA PaCa-2 as well as on off-target NIH-3T3 cells. The expression of EGFR in BxPC-3 cells was 1.7-fold higher than in MIA PaCa-2 cells (Fig. S5). The IC\textsubscript{50} of HisDianthin-EGF in the absence of SO1861 was found to be $1.0 \times 10^{-7}$ M for BxPC-3 (Fig. 2A) and $3.0 \times 10^{-8}$ M for MIA PaCa-2 (Fig. 2B) cells after 48 h. Similarly, NIH-3T3 cells revealed an IC\textsubscript{50} of $7.3 \times 10^{-8}$ M (Fig. 2C), indicating no relevant difference between target and off-target cells. Nevertheless, the targeting index shows that HisDianthin-EGF is specific for EGFR-expressing cells and the toxic effect on NIH-3T3 cells can be attributed to its intrinsic sensitivity to HisDianthin (see end of this section). For the combination treatment with SO1861, a safe concentration of the enhancer alone was determined first by a concentration variation in the range from 0.13 to 32 \mu g.mL^{-1} by end-point assays (Fig. S2) and in addition by real-time assays for BxPC-3 cells in the range from 0.2 to 10 \mu g.mL^{-1} (Fig. S6). A safe concentration was defined as a concentration that does not show any toxic effect and for that the twofold of this concentration shows more than 90% cell survival. In the presence of SO1861 (0.5 \mu g.mL^{-1}), a clear enhancement of the toxicity within 48 h was observed on target cells as compared to the off-target cell line (IC\textsubscript{50} of $1.7 \times 10^{-10}$ M for BxPC-3, $5.3 \times 10^{-9}$ M for

**Fig. 2.** SO1861-mediated augmentation of HisDianthin-EGF (DE). (A, D) BxPC-3, (B, D) MIA PaCa-2, and (C, D) NIH-3T3 cells were seeded in 96-well plates and grown for 24 h. Cells were then treated with (A–C) varying concentrations of HisDianthin-EGF in DMEM (MIA PaCa-2, NIH-3T3) or RPMI 1640 (BxPC-3) in the presence and absence of SO1861 (0.5 \mu g.mL^{-1}), or (D) at a fixed concentration of HisDianthin-EGF corresponding to the IC\textsubscript{50} shown in Table 1 and varying concentrations of SO1861. Cells were further incubated for 48 h. Viability was determined by an MTT assay. Each value represents the mean of three (two for panel (D)) independent experiments performed in quadruplicate. Single outliers defined as outside 3-fold standard deviation were omitted in (D). A statistical significant effect between single and combination treatments was observed in MIA PaCa-2 (Student’s t-test; $P < 0.05$) and BxPC-3 (Student’s t-test; $P < 0.01$) cell lines. The vertical line in (D) indicates the SO1861 concentration used in (A–C) to determine the IC\textsubscript{50} of HisDianthin-EGF and is thus the expected IC\textsubscript{50} for the variation in SO1861.
Mia PaCa-2 and 1.1 × 10^−7 M for NIH-3T3 cells, Fig. 2A–C). This clearly indicated a cytotoxic synergism between HisDianthin-EGF and SO1861 on pancreatic tumor cell lines. Notably, the synergistic effect is target cell specific, suggesting that HisDianthin-EGF is also internalized successfully in the absence of SO1861 but is ineffective due to insufficient endosomal escape, which is then triggered by SO1861 in the combined treatment. The corresponding enhancement factors are represented by the ratios of the IC_{50} values in the absence and presence of SO1861. We calculated 0.7 for off-target NIH-3T3 cells and 588 and 5.7 for BxPC-3 and MIA PaCa-2 cells, respectively (Table 1). A ratio close to 1 as observed for NIH-3T3 cells stands for no difference; that is, no substantial amount of targeted toxins has reached the endosomes, indicating receptor-dependent specificity. It is also important to mention that SO1861 itself does not have any selectivity to EGFR-expressing cells. When investigating the effect of an SO1861 concentration variation at the fixed IC_{50} concentration of HisDianthin-EGF (Table 1) for all cell lines, no significant difference can be observed for all cell lines independent of target receptor expression (Fig. 2D). Thus, the observation that SO1861 specifically enhances the effect of HisDianthin-EGF in target cells is solely attributed to the target cell specificity of HisDianthin-EGF. SO1861 only ensures that the toxin takes full effect (by mediating endosomal escape). Although high concentrations of SO1861 show better effects (Fig. 2D), these concentrations cannot be used for treatment as part of this effect is caused by the intrinsic toxicity of SO1861, which is not target cell specific (Fig. S2). Therefore, SO1861 is always used at a nontoxic concentration.

The specificity of HisDianthin-EGF for target cells was also shown by comparison with the ligand-free toxin HisDianthin alone. The IC_{50} values have been calculated from cytotoxicity curves were used to calculate the targeting index (IC_{50} of HisDianthin divided by IC_{50} of HisDianthin-EGF) (Table 1). The targeting index is much more better for BxPC-3 than for MIA PaCa-2 cells corresponding to the EGFR expression in these cell lines (Fig. S5). The index less than 1 in off-target NIH-3T3 cells might be traced back to the bigger size of the fusion protein, which most likely results in reduced unspecific uptake. The completely different IC_{50} values of HisDianthin for the examined cell lines reflect the different unspecific cytosolic uptake of HisDianthin and varying intrinsic sensitivity for this toxin. The enhancement factors of SO1861 for HisDianthin are greater than 1, indicating unspecific enhancement, but less than for HisDianthin-EGF (Table 1), indicating in total a gain in specificity as already shown for another ribosome-inactivating protein, HisSaporin-EGF compared to HisSaporin (Bachran et al., 2010b).

3.3. Real-time monitoring of HisDianthin-EGF cytotoxicity

The kinetics of HisDianthin-EGF cytotoxicity was evaluated in real-time in all three cell lines (Fig. 3). A gradual increase in the impedance signal (i.e., cell growth) compared to the normalized cell index was observed when cells were treated with the targeted toxin in the absence of SO1861 at 100 pm, 10 nm, and 1 μM except for BxPC-3 cells when treated with 1 μM where a cytostatic effect was observed (Fig. 3A,C,E). Cell growth was reduced beginning 30 h after toxin incubation (3 h for BxPC-3 cells at 1 μM). The reduction was dose dependent and continued until the end of the experiment (92 h after induction) but there was still cell proliferation at any time except for BxPC-3 cells at 1 μM. In contrast, a dose-dependent decrease in the normalized cell index (i.e., cell death) was observed with the combined application of SO1861 and HisDianthin-EGF (Fig. 3B,D,F), finally leading to complete cell death for MIA PaCa-2 cells 72–92 h after beginning of the treatment for concentrations of 100 nm, 10 nm, and 100 pm of the targeted toxin, while off-target NIH-3T3 cells only show a decreasing signal at 100 nm (Fig. 3B). BxPC-3 cells do not reach complete cell death in the observed time period; however, the signal is still decreasing at the end of the experiment and it must be taken into consideration that untreated BxPC-3 cells proliferate much faster than MIA PaCa-2 cells. The lowest concentration of 10 pm HisDianthin-EGF resulted only in a cytostatic effect 20 h after start of the incubation for MIA PaCa-2 cells and in no effect for BxPC-3 cells. The kinetics of the first observable effect was faster in the combination therapy (14–24 h after treatment start for concentrations down to 100 pm) compared to the monotherapy (30 h, except for BxPC-3 cells at 1 μM). The enhancer effect of SO1861 is target cell specific as cell growth of NIH-3T3 cells was not substantially more reduced than with the monotherapy (Fig. 3A,B) and cell proliferation was observed for all concentrations except for the highest concentration of 100 nm that resulted in cytostatic behavior.

3.4. Evaluation of effect levels by acute toxicity study

No acute toxic effects were observed in former studies for mice treated only with a comparable enhancer
(Saponinum album) up to 100 µg (Heisler et al., 2005). For HisDianthin-EGF alone, 0.4 µg per mouse was found to be nontoxic. The body weight remained constant throughout the week after single dose administration, while for 4.0 µg per mouse, a statistical significant but mild decrease in body weight was observed with no adverse effects as defined in Table S1. However, 40 µg dose caused moribundity accompanied with white ocular discharge in 100% population (Fig. S3). Furthermore, a decrease in more than 10% body weight within 48 h was also observed (Fig. 4). The animals were sacrificed (after 48 h for 40 µg dose and after 1 week for 0.4 and 4.0 µg doses) and organs were preserved for further toxicological analyses by immunohistochemistry.

3.5. Efficacy of HisDianthin-EGF alone and in combination with SO1861 as compared to placebo

The therapeutic effect of the combination therapy in tumor-bearing nude mice was determined for a period of 25 days comprising six treatment cycles. The total amount was therefore six times 0.35 µg HisDianthin-EGF; that is, 2.1 µg. Taking into consideration that 4 µg in a single dose did not result in adverse effects,
we assumed the applied dose to be safe. There was a 96.5% average reduction in the tumor volume for the group treated with the combination of HisDianthin-EGF and SO1861 compared to the placebo (tumor volume 84.5 mm$^3$ for placebo and 3.0 mm$^3$ for combination) and four of five mice showed complete regression (Fig. 5). The monotherapy with HisDianthin-EGF also caused a decrease in tumor volume (40.8 mm$^3$), which was a 51.7% average reduction when compared to placebo; however, treatment together with SO1861 resulted in a more than 13-fold better efficacy. As a nonsevere side effect, SO1861-induced skin hardening was observed at the back of the neck after two therapy cycles. The endosomal escape enhancer alone has no effect on tumor growth as shown in previous studies (Bachran et al., 2009).

No complete regressions were observed in the monotherapy, two mice had continuous tumor growth, and three had retarded tumor growth. The experiment was brought to an end after six therapy cycles. The endosomal escape enhancer alone has no effect on tumor growth as shown in previous studies (Bachran et al., 2009).

3.6. Histological and immunohistochemical analyses

To evaluate acute toxicity, histopathological analyses of isolated organs by HE stains were performed. Most of the organs did not reveal any alterations and were labeled as regular; especially, no alteration was seen in stomach, heart, and intestine of any mice. However, mild to moderate alterations like single-cell necrosis

and necrosis of groups of hepatocytes were observed in liver tissue of mice, in which the highest dose (40 μg per mouse) was administered (Fig. 7A). Nevertheless, no severe damage was observed.

Tumors isolated from the efficacy studies accomplished in CD1 nu/nu xenografts were immunohistochemically analyzed for the expression of EGFR and the Ki67 proliferation marker. All isolated tumors from the placebo group expressed high levels of EGFR (Fig. 7B); however, only a weak EGFR staining was examined in mice treated with the monotherapy (Fig. 7C), indicating that the monotherapy is only suitable to kill tumor cells with high EGFR expression, while tumor cells with lower EGFR expression escape from treatment and selectively proliferate. We also tested EGFR expression in cell culture experiments by western blotting, but were unable to detect significant differences after 24, 48, and 72 h. Due to the toxicity of the treatment, longer observation periods were not possible in cell culture. The different EGFR expression in tumors might have been developed in an evolutionary selection process during the long period of 4-week treatment.

A high Ki67 proliferation index (> 60%; Fig. 7D) was observed in all isolated tumors of the placebo and monotherapy group. This let us assume that the reduced tumor size in the monotherapy group can be attributed only to the killing of high EGFR-expressing tumor cells but not to a lower proliferation rate of the tumor cells with lower EGFR amount. In the

Fig. 4. Body weight loss observed after single application of different doses of HisDianthin-EGF. The values refer to the body weight just before treatment. Significance in body weight changes is indicated by * (P < 0.05) and ** (P < 0.01).

Fig. 5. Xenograft tumor volume evaluated by the use of a digital vernier caliper for placebo (PBS only), single (HisDianthin-EGF; 0.35 μg per treatment), and combination (HisDianthin-EGF; 0.35 μg per treatment + SO1861; 30 μg per treatment) therapy. Arrows point to the days of treatment. A statistical significant decrease in tumor volume was observed in the combination treatment for the curves as a whole (P < 0.05 versus single therapy and versus placebo).
combination therapy after six treatment cycles, only one of five mice still had a tumor that could be investigated for the growth capacity. This tumor only had a 50% Ki67 proliferation index (Fig. 7E).

3.7. Complete blood count analyses

No significant difference was seen in the non-platelet-derived parameters of blood as displayed in Table 2. In contrast, the platelet-derived parameters showed statistically significant alterations as depicted in Fig. 8.

A significant increase in platelet count was observed in case of the combination treatment ($P < 0.01$ versus healthy as well as placebo). Surprisingly, there was also a significant increase in platelet count observed for placebo when compared to healthy ($P < 0.05$), indicating that the puncture wound has some influence on the platelets. An increase in plateletcrit was also significant.
compared to placebo (P < 0.05 versus $^{\text{HiDianthin-EGF}}$; P < 0.0001 versus $^{\text{HiDianthin-EGF}}$ + SO1861).

A slight but significant difference in platelet distribution width was observed for monotherapy compared to the combination (P < 0.05), while the mean platelet volume showed no significant difference.

### 4. Discussion

Recombinant expression of targeted ribosome-inactivating proteins in chemically competent *E. coli* Rosetta (DE3) pLysS cells had already been described in previous studies (Gilabert-Oriol *et al.*, 2013c;
Heisler et al., 2005). The purity of proteins plays an important role and poly-histidine-tagged recombinant proteins that are isolated by immobilized metal affinity chromatography are often contaminated with significant amounts of endogenous E. coli metal-binding proteins. Several techniques were developed to solve this problem including the variation in the poly-histidine tags and the applied detergent (Grisshammer and Tucker, 1997), use of particular matrices such as nitrolotriacetic acid (Hochuli et al., 1987) and Co\(^{2+}\)-carboxymethylaspartate (Chaga et al., 1999), or by the use of particular E. coli strains such as NiCo21(DE3) and NiCo22(DE3) that allow removal of contaminating E. coli proteins by the use of a chitin column (Robichon et al., 2011). We aimed to minimize this contamination by using the NiCo21(DE3) E. coli strain considering it to be a superior alternative to BL21(DE3) (Robichon et al., 2011). Although this led to 2.7-fold decrease in enzymatic activity as compared to an earlier study described (von Mallinckrodt et al., 2014), the targeted toxin was now highly pure and retained sufficient enzymatic activity. The decrease in activity might be attributed to partly improper folding of HisDianthin-EGF expressed in NiCo21(DE3) compared to BL21(DE3). Nevertheless, to avoid the proinflammatory effects observed by bacterial contaminants (Pepys et al., 2005), more emphasis on purity was given.

EGFR-targeting toxins in synergism to the endosomal escape mechanism of SO1861 might have an important regulatory role in the control of tumor growth in xenografts bearing human adenocarcinoma pancreatic tumors. A strong evidence for this hypothesis is based on in vitro (Bachran et al., 2011; Gilabert-Oriol et al., 2015; Weng et al., 2012) as well as in vivo studies that were previously performed in BALB/c and nude mice demonstrating very strong synergistic effects of the combination therapy for mammary and colon carcinoma (Bachran et al., 2009; Gilabert-Oriol et al., 2013c; von Mallinckrodt et al., 2014). The aim of our study was to investigate whether the combinatory treatment with SO1861 is also suitable to treat pancreatic adenocarcinoma, which is characterized by aggressive growth and a very low overall five-year survival rate of less than 4% (Kleeff et al., 2006). When we compared saporin- and dianthin-based targeted toxins, we observed that dianthin-based products were much more stable (Gilabert-Oriol et al., 2015) and produced better protein yield (Gilabert-Oriol et al., 2013c) as compared to saporin-based targeted toxins (Gilabert-Oriol et al., 2013b). Moreover, there are hints that dianthin is less immunogenic than saporin (Strocchi et al., 1992). Therefore, we will conduct future investigations with the aim of clinical studies with dianthin-based products. Moreover, side effects of this therapy were not sufficiently pursued to date and we therefore investigated hematological parameters after mono- and combination therapy of HisDianthin-EGF in particular with regard to the known hemolytic properties of some glycosylated triterpenoids in general (Gilabert-Oriol et al., 2013a,c).

In the present study, EGF serves as a ligand to target EGFR molecules. Cytotoxicity was therefore evaluated in EGFR-overexpressing human pancreatic carcinoma cells (BxPC-3 and MIA PaCa-2). Higher expression of EGFR had been found in BxPC-3 cells in comparison with MIA PaCa-2 as demonstrated by western blot (Fig. S5) (Ali et al., 2005) and also by flow cytometry (Ioannou et al., 2011). In vitro, we found no significant difference between the cytotoxic effect on target and off-target cells when cells were treated in the absence of SO1861 (Fig. 2). Addition of SO1861 solely enhanced the cytotoxic effect on target cells, indicating that HisDianthin-EGF enters off-target cells via unspecific routes omitting late endosomes (explaining the absent enhancer effect of SO1861), while late endosomes are expectedly reached in target cells, but no increased cytotoxic effect compared to off-target cells is observed due to missing endosomal escape, which only occurs in the presence of SO1861 (Fig. 2A,B). HisDianthin-EGF-induced growth inhibition in the presence of SO1861 varied between different target cell lines. As the expression level of EGFR is high in BxPC-3 in comparison with MIA PaCa-2 (Fig. S5), it can be expected that the augmentation of the endosomal escape by SO1861 results in a stronger efficacy increase in BxPC-3 cells. Indeed, the difference in the cytotoxic effect between single and combination therapy proved to be more eminent in BxPC-3 cells, as portrayed by an IC\(_{50}\) of 100 nM in monotherapy and 0.17 nM in combination therapy within 48 h. Studies on saporin-based targeted toxins showed that the IC\(_{50}\) is strongly dependent on target receptor expression and can range from 2.5 nM to 206 nM in the absence of endosomal escape enhancers (Bachran et al., 2010b). For HisDianthin-EGF, the IC\(_{50}\) was only 0.45 nM on transfected cells that highly overexpress EGR (Weng et al., 2012) and greater than 10 nM for HisDianthin chemically conjugated to panitumumab, trastuzumab, or cetuximab (Gilabert-Oriol et al., 2015). The effect by endosomal escape enhancers depends on the cell line, the targeting moiety, target receptor expression, and the structure of the enhancer. Enhancement factors of more than one million were observed (Weng et al., 2012). The IC\(_{50}\) for the above-mentioned antibody-HisDianthin conjugates in the
presence of SO1861 was 1.5, 23, and 5.3 pm, respectively (Gilabert-Oriol et al., 2015). Real-time cytotoxicity studies also revealed the combination treatment to be more superior compared to monotherapy with only targeted toxin.

In our acute toxicity experiments, 0.4 μg HisDianthin-EGF per treatment via intraperitoneal administration resulted in no biological effect and no statistically significant weight loss. Histological analyses showed no organ toxicity at this dose. Increasing the dose to 4 μg caused a significant weight loss but no adverse effects. Further increase to 40 μg caused moderate alterations in the liver by necrosis of groups of hepatocytes. The liver is the main organ that is affected when immunoconjugates are administered intraperitoneally (Ito et al., 1991). As we only observed moderate liver toxicity at 40 μg, it can be most likely assumed that the treatment dose of 0.35 μg, less than a hundredth of a moderately toxic dose, particularly when applied subcutaneously, is much below the highest nonobserved adverse effect level. Moreover, it can be assumed that multiple treatments will not result in an accumulation effect as the endosomal escape enhancer is almost completely excreted after 4 h (Bachran et al., 2010a).

The combination of HisDianthin-EGF and SO1861 revealed a very strong synergistic effect when administered subcutaneously as SO1861 alone has no toxic effect at the applied concentration and the monotherapy with HisDianthin-EGF has a 13-fold weaker effect with respect to tumor reduction than the combination. Earlier experiments showed that intraperitoneal injection of a targeted toxin did not cause a significant decrease in tumor size when the endosomal escape enhancer is applied at the same route (Bachran et al., 2009). The reason for this appears to be the high concentrations of targeted toxins and enhancers at the same site resulting in inflammation and possibly toxin degradation. To avoid inflammation, SO1861 was administered s.c. at the back of the neck and HisDianthin-EGF s.c. at the vicinity of the tumor. This strategy was already successful in the past (Gilabert-Oriol et al., 2013c; von Mallinckrodt et al., 2014); however, it required careful choice of the length of the time period between SO1861 and toxin application (Bachran et al., 2010a). With respect to clinical trials, the use of a deimmunized form of dianthin might facilitate systemic applications as shown for a deimmunized form of another ribosome-inactivating protein, bouganin, in an immunotoxin with trastuzumab (Dillon et al., 2016). Another possibility is to substantially decrease the systemic concentration of SO1861 by modifying the enhancer to a targeted molecule.

Evaluation of the complete blood count revealed a significant change in the platelet-derived parameters (Fig. 8). In our study, we observed that augmentation in the efficacy of treatment (combination therapy > monotherapy > placebo > untreated) led to an unexpectedly increased platelet count. This was also seen in more than a half of advanced non-small-cell lung cancer patients at phase II clinical trials with a combination of gemcitabine and cisplatin (Zwitter et al., 2005), in studies performed with a combination of gemcitabine and vincristine (Zwitter et al., 2001), and in pancreatic cancer patients treated with gemcitabine alone (Świeboda-Sadlej et al., 2012). Thus, an increase in platelet count appears to be not uncommon, but we do not know the molecular background behind this.

Our immunohistochemical studies on tumors from xenografts showed high EGFR expression in all pancreatic tumors of untreated animals. This overexpression of EGFR in pancreatic cancer cells had also been studied by Korc et al. (1992). The reason for increased expression can be associated with either structural or numerical alterations of chromosome 7 (Korc et al., 1986; Shimizu et al., 1984). Over the decades, many bacterial- and plant-based targeted toxins have been developed with the goal of targeting cancers reliant upon EGFR overexpression (Simon and FitzGerald, 2016). The targeted toxin 425(scFv)-ETA, consisting of an anti-EGFR single-chain Fv antibody fused to a truncated Pseudomonas exotoxin, was strongly cytotoxic toward metastatic pancreatic cancer cells (L3.6pl) with an IC50 of 0.1 nM (Bruell et al., 2003). In mice injected with L3.6pl cells, multiple applications of 425(scFv)-ETA reduced the number of lung metastases from 56 per mouse to 0.28 per mouse (Bruell et al., 2005), indicating the strong potential of this targeted toxin. Similar targeted toxins using single-chain Fv antibodies derived from cetuximab and panitumumab resulted in IC50 values of 0.29 and 0.26 nM, respectively, on L3.6pl cells (Niesen et al., 2015). In contrast to dianthin, the truncated Pseudomonas exotoxin possesses its own natural translocation domain, which makes it independent from endosomal escape enhancers, explaining the similar IC50 values observed for the exotoxin and dianthin in the presence of SO1861.

Notably, we observed weak staining signals in tumors treated with the targeted toxin alone in comparison with placebo (Fig. 7). At least for some cancers, EGFR is a strong prognostic indicator associated with more aggressive disease and reduced survival (Oliveira-Cunha et al., 2011). Therefore, on the one hand, the weak expression after HisDianthin-EGF
treatment can indicate that the tumor is now less aggressive after eradicating the part of tumor cells with high expression level. On the other hand, this let us assume that a monotherapy with HisDianthin-EGF is not sufficient to kill tumor cells with lower EGFR expression resulting in selective survival of these cells and continuous tumor growth (Fig. 5). Another possibility is an adaptive decrease in EGFR expression in affected cells to develop resistance. The reason for the missing potential of HisDianthin-EGF is most likely that only a small portion of the bound and internalized targeted toxin is able to escape from the endosomes before degradation or recycling. This can be overcome in general by the use of endosomal escape enhancers (Fuchs et al., 2016) and for ribosome-inactivating proteins in particular by certain glycosylated triterpenoids such as SO1861 (Bachran et al., 2011; Weng et al., 2012). The present study indicates that, in the presence of SO1861, tumor cells with low EGFR expression are also eliminated, finally leading to complete remission in four of five cases without adverse events. Thus, combining a targeted toxin with SO1861 is proven to be a very promising approach for pancreatic cancer treatment. Long-term efficacy in mice for 60 days or more must still be investigated.

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Author contributions
HD performed all histological and immunohistochemical analyses. AW isolated and characterized SO1861 and analyzed the corresponding data. CB conducted and analyzed all other experiments and wrote the draft of the manuscript. XZ assisted in all animal experiments. MFM gave advice in glycosylated triterpenes and corrected the manuscript. HF designed and supervised the entire study, interpreted the results, and finalized the manuscript. All authors proofread the manuscript.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Western blot of HisDianthin-EGF after final purification.

Fig. S2. Determination of a safe SO1861 concentration.

Fig. S3. Images displaying dose dependent discernible physical traits of mice injected with HisDianthin-EGF.

Fig. S4. Densitometry scans after separation of the samples by high-performance thin-layer chromatography silica gel plates.

Fig. S5. Western blot of EGFR expression level in all applied cell lines.

Fig. S6. Real-time cell analysis showing the dose-dependent increase in cytotoxicity in BxPC-3 cells caused by SO1861 alone.

Table S1. Definition of adverse effect symptoms in toxicity studies.