Tumor-specific targeting of pancreatic cancer with Shiga toxin B-subunit

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Abbreviations: Gb3 – globotriaosyl ceramide; GSL – glycosphingolipid; STxB – Shiga toxin B-subunit

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ABSTRACT

Pancreatic carcinoma is one of the most aggressive tumor entities, and standard chemotherapy provides only modest benefit. Therefore, specific targeting of pancreatic cancer for early diagnosis and therapeutic intervention is of great interest. We have previously shown that the cellular receptor for Shiga toxin B (STxB), the glycosphingolipid globotriaosyl ceramide (Gb₃ or CD77) is strongly increased in colorectal adenocarcinoma and their metastases. Here, we report an up-regulation of Gb₃ in pancreatic adenocarcinoma (21 of 27 cases), as compared to matched normal tissue (n=27). The mean expression was highly significantly increased, from 30 ± 16 ng Gb₃ per mg tissue in normal pancreas to 61 ± 41 ng Gb₃ per mg tissue (mean ± SD, p = 0.0006), as evidenced by thin layer chromatography. Up-regulation of Gb₃-levels did not depend on tumor stage or grading, and showed no correlation with clinical outcome. Tumor cells and endothelial cells were identified as the source of increased Gb₃ expression by immunocytochemistry. Pancreatic cancer cell lines showed rapid intracellular uptake of STxB to the Golgi apparatus, following the retrograde pathway. The therapeutic application of STxB was tested by specific delivery of covalently coupled SN38, an active metabolite of the topoisomerase I inhibitor Irinotecan. The cytotoxic effect of the STxB-SN38 compound in pancreatic cancer cell lines was increased more than 100-fold compared to Irinotecan. Moreover, this effect was effectively blocked by competing incubation with non-labeled STxB, demonstrating the specificity of the targeting. Thus, STxB constitutes a promising new tool for specific targeting of pancreatic cancer.
INTRODUCTION

Pancreatic carcinoma is one of the most aggressive tumor entities with a median survival of 6 months and a 5-year survival of less than 5% (1). Curative resection often is not possible due to late diagnosis of the tumor, and the current standard chemotherapy with gemcitabine has increased the overall survival only modestly (2, 3). The targeting of pancreatic cancer for early diagnosis, e.g. by imaging methods, and for therapeutic purposes, e.g., by specific delivery of cytotoxic compounds to pancreatic tumor cells, is therefore of great clinical interest. The non-toxic B-subunit of the bacterial Shiga toxin (STxB) is a promising new option for diagnosis and therapy of gastrointestinal tumors. We and others showed previously that the expression of the cellular receptor of Shiga toxin, the glycosphingolipid Gb3 (CD77), is significantly increased in colorectal carcinoma and their metastases (4, 5). Studies on genetic mouse models for human digestive cancer showed that STxB is suitable for non-invasive tumor imaging (6). STxB is taken up rapidly by tumor cells that express the receptor Gb3 on their surface membrane, and STxB then follows the so-called retrograde route (7). The retrograde route bypasses the late endocytic pathway and avoids the degrading environment of the lysosomes. Instead, STxB is transported from the endosome to the trans-Golgi network, then to the Golgi apparatus, and is transported from there to the endoplasmic reticulum (ER) (8-10).

It is feasible that STxB has naturally evolved to withstand inactivation by intestinal fluids. Moreover, STxB has only low immunogenicity in humans (11, 12). Functionalized STxB can be used efficiently to target gastrointestinal tumors in animal models (6), as well as short-term primary cultures from human colon cancer and liver metastases (5). Moreover, a recent study confirmed the findings on Gb3 overexpression in colon cancer, and reported an up-regulation of Gb3 expression in human pancreatic cancer patients (13). In the present study, we have quantified Gb3 expression in human pancreatic adenocarcinoma by lipid extraction and thin-
layer chromatography, as well as by immuno-fluorescence microscopy on tissue sections, and tested pancreatic cancer cells for the uptake kinetics of functionalized STxB. Moreover, human astrocytoma xenografts in nude mice are eliminated rapidly, completely, and with long-term efficacy using verotoxin, supporting the concept of therapeutic use of Shiga toxin-like toxins (14). Recently, successful coupling of STxB to SN38 (7-ethyl-10-hydroxycamptothecin), the active metabolite of the topoisomerase inhibitor Irinotecan (CPT-11), has been reported (15). SN38 is amenable to biochemical modification, making it a useful candidate drug for a proof of principle approach for STxB-mediated tumor targeting. Furthermore, SN38 is several orders of magnitude more active than Irinotecan, but its hydrophobicity and insolubility in most physiologically compatible and pharmaceutically acceptable solvents limits its clinical application (16, 17). Irinotecan has already been reported in several clinical trials as second-line chemotherapy of advanced pancreatic cancer, either as single agent or in combination with gemcitabine or other agents (2, 18-20). Anti-cancer cytotoxicity was reported, even though no obvious survival benefit was reported so far. The STxB-SN38 compound has been demonstrated previously to possess highly increased cytotoxicity as compared to Irinotecan, when tested on a colorectal cancer cell line (15). Moreover, the STxB-SN38 compound showed high receptor-specificity, since the cytotoxic effect relied on an intracellular uptake that is mediated by the receptor Gb3. The cytotoxic effect was inhibited when the glycosphingolipid Gb3 was blocked (15). Therefore, STxB-SN38 could be a highly useful tool for targeted chemotherapy with greatly enhanced cytotoxicity and low side effects. The aim of this study was therefore to analyze the potential diagnostic and therapeutic use of STxB on pancreatic carcinoma. Taken together, we report a significantly increased expression of Gb3 in pancreatic cancer, as compared to matched normal tissue samples. In addition, we have successfully tested a new compound for targeted chemotherapy, STxB-SN38, on pancreatic cancer cell lines.
MATERIALS AND METHODS

Patient Collective

Samples were obtained from 28 patients with pancreatic carcinoma admitted to our Surgical Department in 2003 to 2006 (16 male and 12 female, median age 68 years, range 42 - 83). Informed, written consent regarding the use of tissue samples had been obtained previously, with approval of the local ethics committee. Median follow-up after surgery was 16.9 months. During this period, three patients died due to unrelated causes, 17 died due to cancer progression or disease recurrence. Tumors were classified according to the International Union Against Cancer (UICC), as summarized in Table I (21). Histology-guided sample selection was performed by a pathologist to identify tumor samples and to exclude cancer cell contamination in the control samples. All samples were snap frozen in liquid nitrogen immediately after resection and stored at –80°C until use.

Reagents and Antibodies

To allow for chemical coupling of fluorophores or cytotoxic drugs to a defined acceptor site in STxB, a Cys residue was added to the C-terminus of the wild-type protein. The recombinant mutant STxB-Cys protein was produced in endotoxin-free form as described (6). Antibodies and reagents used were: anti-golgin p97 (Molecular Probes, Karlsruhe, Germany), anti-STxB (22), anti-CD31 (BD Biosciences Pharmingen, San Diego, CA), DAPI, Ki67, Staurosporine (from Sigma-Aldrich, Munich, Germany), Calnexin, and anti-Vimentin (both from Santa Cruz Biotechnology, Heidelberg, Germany). Secondary antibodies coupled to fluorophores were purchased from Jackson Immunoresearch (West Grove, PA), and cell culture reagents from Invitrogen (Karlsruhe, Germany). Irinotecan was obtained from the Pharmacy of the Klinikum rechts der Isar. SN38 was coupled to STxB as described (15).

Indirect Immunofluorescence
Immunostaining was performed as described before (6). Briefly, after paraformaldehyde-fixation (PFA), cell lines on cover slips were permeabilized with 0.1% Triton X-100, blocked with PBS containing 2% BSA, and primary antibodies in blocking buffer were added before counterstaining with secondary antibodies. At this concentration, Triton X-100 did not alter the Gb3 detection on cryosections. STxB was purified from bacteria as previously described (23). Covalent coupling of STxB to fluorochrome Cy3 (Amersham Biosciences, Little Chalfont, UK) was carried out according to the supplier's instructions. For staining of endogenous Gb3, tissue cryosections were fixed with 3% paraformaldehyde for 20 min and incubated with STxB-Cy3 for 30 min at a final concentration of 10 μg/ml, in PBS containing 0.2 % BSA. For counterstaining against the nuclear antigen Ki67, 0.1% Triton X-100 was used on cryosections. For image acquisition, epifluorescence or confocal microscopes (Zeiss, Goettingen, Germany) were used. Images were processed using Adobe Photoshop Software CS3 (San Jose, CA) and colocalization was tested with ImageJ version 1.42q (NIH, Bethesda, MD).

**Quantification of Gb3 expression by thin layer chromatography**

Gb3 expression was quantified as previously described (5, 24-26)(additional details in supplementary material). Briefly, unfixed tissue of pancreatic tumors and adjacent tissue were collected immediately after surgery by an experienced pathologist. Tissues were weighed and mechanically homogenized in 1 ml of aqueous buffer. The established pancreatic and colorectal cancer cell lines were collected from cell culture dishes, washed, and analyzed accordingly. The material in aqueous buffer was injected immediately into 3.75 ml of chloroform/methanol (1:2). After mixing, 1.25 ml of chloroform and 1.25 ml of water were added. The hydro-alcoholic phase was washed once with 1.5 ml of chloroform. The combined chloroform phases were dried under nitrogen, and lipids were saponified at 56°C for 1 h in 1 ml of methanol/KOH. The saponification reaction was once again extracted as described above, and the chloroform phase was washed once with methanol/water (1:1). The isolated
neutral glycolipids were spotted on high-performance thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany) and separated with chloroform/methanol/water (65:25:4). Dried plates were soaked in 0.1% polyisobutylmethacrylate in hexane, floated for 1 h in blocking solution, followed by incubation with STxB (20 nM), primary polyclonal antiserum against STxB, and secondary horseradish peroxidase-coupled anti rabbit antibodies. Reactive bands were revealed with enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, UK).

**Cell culture and STxB uptake assays**

Pancreatic (DanG, MiaPaCa2, BxPC3) and colorectal (DLD1 and HT29) cancer cell lines were kept in DMEM medium with fetal calf serum (10% for BxPC3, 7% for all other cells), 1% Pen/Strep, and 1% glutamine. STxB covalently labeled with the fluorophore Cy3 was added at final concentration of 2.5 μg/ml as published earlier (5). After various time points, cells were fixed with 3% paraformaldehyde and analyzed by immunofluorescence as specified (see above). Cell lines were authenticated by the German cell line repository (DSMZ, Braunschweig, Germany).

**Cytotoxicity assays**

Cell growth analysis was performed using the Cell Proliferation Kit II (XTT) from Roche Diagnostics (Mannheim, Germany), the amount of dye corresponding to the number of metabolically active cells. The cell lines were split into 6-well-plates (at 1x10^5 cells / well) and grown for 3 days. Treatment with Irinotecan or STxB-SN38 was carried out for 6h in cell medium. Cells were washed with DMEM, and substituted with fresh medium for an additional 48h. Cells were then seeded onto 96-well-plates (in triplicates at 0.5 x 10^4 cells / well, and 1 x 10^5 cells per well) in medium containing 0.5% FCS (fetal calf serum). After 12-18 hours, the XTT solution was added in medium with 3% FCS, and the cells were incubated for 30 hours. The amount of formazan was quantified with a Mithras LB 940 microplate reader (Berthold Technologies, Bad Wildbad, Germany) at 450–500 nm. Mean values of five
independent analyses are shown. A competition assay was performed with either 5 μM STxB-SN38, or 50 μM unlabeled STxB, or 50 μM of unlabeled STxB 5 minutes, prior to treatment with 5 μM STxB-SN38.

**Statistical analysis**

Statistical analyses were performed using SPSS (version 16.0, Chicago, IL, USA), Graph Pad Prism 5, and GraphPad InStat3 (Graph Pad Software, La Jolla, CA, USA). Data are presented as mean ± standard deviation or where specified as median (range). The comparison of Gb3-expression in pancreatic adenocarcinoma and corresponding non-diseased pancreas was done using paired t-test. For this calculation the 27 pairwise complete observations were used. For evaluating the influence of Gb3-expression on survival time, Cox regression was calculated. Mann-Whitney U tests were used for evaluating the correlation of Gb3-expression with histopathological data. All statistical comparisons were done at a 0.05 level of significance. The IC50 values were calculated using GraphPad Prism 5, a general-purpose curve fitting and scientific graphics program.
RESULTS

Gb3-Expression is increased in pancreatic cancer

Previously, we have shown that the glycosphingolipid Gb3 is over-expressed in human colorectal cancer (5). Here, we have analyzed surgically resected pancreatic adenocarcinoma and corresponding non-diseased pancreas by lipid extraction and thin layer chromatography. Gb3 expression was detectable in all tumors tested (n=28, Table I). Average median expression was 54.9 ng Gb3/mg tissue as compared to 29.0 ng Gb3/mg tissue in normal pancreas (n= 27). The mean difference of 31.2 ng Gb3/mg tissue was highly significant (CI: [14.8, 47.7], p= 0.0006). In a direct comparison of matched tissue samples from individual patients, we could detect an increased Gb3 expression in 78% (21 of 27) of the tumors as compared directly to the surrounding normal pancreas (Figure 1A, Suppl. Figure S1) In 48% (13 of 27) of the samples, the Gb3 level was higher than the calculated median expression of 54.9 ngGb3/mg tissue. In addition, established pancreatic cancer cell lines were tested, and all showed Gb3 expression (Figure 1B). However, Gb3 expression in cell lines was lower than in primary patient samples. Thus, Gb3 was clearly up-regulated in pancreatic adenocarcinoma, in good accordance with recent observations (14). HT29 colon cancer cells, which have been described to bind STxB (16) had 40.9 ng Gb3/ mg wet weight (not shown), whereas DLD1 colon cancer cells completely lacked Gb3. Our statistical analysis showed no correlation between Gb3 expression and histopathological or survival data. In fact, the hazard ratio for the level of Gb3-expression and survival time was 1.005. The correlation with histopathological data involved the tumor size ("pT", p=0.78) and lymph node metastasis status ("pN"-category p=0.52) as well as grading ("G", p=0.21), lymphangiosis (p=0.19), and angioinvasion (p=0.48) (supplementary table SI). For further testing on differential expression, the parameter "Gb3-delta" was devised, a calculation based on the level of Gb3 in carcinomas minus Gb3 expression level in normal tissue for each of the 27 matched pairs. In normal
pancreatic tissue, Gb3 was detectable by lipid extraction and by STxB overlay on cryosections, albeit at lower levels than in carcinoma samples. However, comparison of Gb3-delta with histopathology failed to show significant association (supplementary table II). Thus, Gb3 seems to be broadly expressed in pancreatic neoplasm, irrespective of grading or tumor stage.

**Immunocytochemistry reveals tumor cells and endothelia as the source of increased Gb3 expression**

Analysis of tissue sections indicated that pancreatic cancer cells, but not stroma components, were the major source of up-regulated intratumoral Gb3 levels. Previous work demonstrated that endothelial, immune, and enteroendocrine cells express Gb3 (5, 6, 27). Moreover, tumor cells of epithelial origin, as well as tumor-associated blood vessels and stroma are the source of increased Gb3 expression in colorectal adenocarcinoma (5). In the present study, tissue from patients with high (n=3) or low Gb3 expression (n=3) was analyzed by immunohistochemistry (Fig. 2), and in parallel, by thin layer chromatography. In spite of different sensitivities both detection methods were in good accordance. In normal pancreas, Gb3 expression was mainly detected in endothelia (Fig. 2 A-C). In tumors with low Gb3 expression, stromal components like blood vessels were the major source of Gb3 expression (Fig. 2 D-F). However, in tumors with high Gb3 expression, duct-like cancer cells were strongly stained with fluorescently labelled STxB (Fig. 2 G-L). Tumor-associated endothelial cells were only weakly stained for STxB (Fig. 2 F and J). No association of tumor cell growth with Gb3 expression was detected in carcinoma with Gb3-overexpression (Suppl. Table III). However, in carcinoma with low Gb3 expression, the few cells that were positive for Gb3 were negative for the proliferation marker Ki67 (Suppl. Table II).

**Intracellular uptake of STxB in pancreatic cancer cells**

Next, we investigated if pancreatic cancer cells actively take up STxB along the retrograde route. Incubation of pancreatic cancer cell lines with fluorescently labeled STxB-Cy3 at 4°C
allowed binding to the receptor in the absence of cellular uptake, and resulted in STxB staining at the plasma membrane (Fig. 3). Upon incubation at 37° C, STxB-Cy3 was rapidly internalized and co-localized with the Golgi marker Golgin p97, or with the endoplasmic reticulum marker Calnexin (Fig. 3, Suppl. Fig. S2 and S3), indicating that STxB followed the retrograde route to the Golgi apparatus. Two days after the initial uptake, STxB was still detectable in punctate structures, co-localizing with the Golgi in DanG and MiaPaCa2 cells. However, no obvious co-localization of STxB could be detected with Golgi structures or ER in BxPC3 cells after 48h (Suppl. Fig. S2). In accordance with our findings on colorectal cancer cells, we did not observe an induction of apoptosis by STxB in any of the cell lines tested, as evidenced by staining for cleaved Caspase-3 (data not shown). The cell line DLD1, which lacks Gb3, did not show STxB binding and uptake (Fig. 3). The percentage STxB-positive cells was quantified after 60 minutes: 19% of BxPC3, 20% of DanG, 35% of MiaPaCa2, compared to 56% for HT29 colon cancer cells (positive control), and 0% of DLD1 cells (negative control; not shown). Supplementary Fig. 3 shows the distribution of STxB-uptake in analyzed cell lines at the tested time points, distinguishing between plasma membrane-, Golgi- and vesicular staining.

**STxB specifically delivers a topoisomerase type I inhibitor to pancreatic cancer cells**

Since STxB was taken up efficiently by pancreatic cancer cells, we investigated the feasibility of STxB-mediated chemotherapy with a topoisomerase I inhibitor coupled covalently to STxB (15). SN38 is linked to STxB via a cleavable linker arm that allows for SN38 release from STxB in membrane of the endoplasmic reticulum or Golgi apparatus. On pancreatic cancer cells, the cytotoxic effects of this compound were compared to the effects of the standard drug Irinotecan (Fig. 4). The left column indicates cell growth in response to increasing concentrations of Irinotecan, the right column shows cell growth after treatment with STxB-SN38. All pancreatic cancer cell lines showed significant growth inhibition upon treatment with Irinotecan, and cell growth was more strongly inhibited by STxB-SN38 (right column),
with IC$_{50}$ values in the sub-micromolar range (Suppl. Table II, and suppl. Fig. S4). The IC$_{50}$ (inhibitory concentration 50%) is the concentration that exerts half the specific cytotoxicity. Moreover, even when cells were treated with the chemotherapeutic agent for only 15 minutes, STxB-SN38 showed a 20-fold greater efficacy than Irinotecan (Suppl. Fig. S5). Irinotecan had a cytotoxic effect on DLD1 cells which lack the receptor Gb$_3$, whereas STxB-SN38 did not significantly inhibit cell growth (Fig. 4). At highest concentrations of STxB-SN38, a reduction of cell growth was observable for DLD1 cells, which may be due to non-specific pinocytosis. Figure 5 shows the results of a competition assay for MiaPaCa2 cells. Treatment of cells with unlabeled STxB induced only a slight change in cell growth, whereas STxB-SN38 had a pronounced cytotoxic effect. After prior treatment with a 10-fold molar excess of unlabeled STxB, the effect of STxB-SN38 was significantly diminished. Even though the competition with unlabeled STxB did not completely block the cytotoxic effects of STxB-SN38, this indicates that the effect of STxB-SN38 is mediated specifically by the Gb$_3$ receptor.
DISCUSSION

In the present work, we demonstrate the use of the non-toxic B-subunit of bacterial Shiga toxin (STxB) for specific targeting of human pancreatic cancer cells. Pancreatic cancer is one of the most aggressive tumor entities and represents the fourth leading cause of cancer related death in Western countries. So far, the only curative therapy is surgery, but due to late diagnosis most patients do not qualify for this treatment. The standard chemotherapy with gemcitabine and chemotherapeutic combinations improves cancer related symptoms, but enhances survival only modestly (2, 20). Therefore, new options for diagnostic and therapeutic approaches are urgently needed. Previously, we were able to show that STxB allows efficient targeting of colorectal carcinoma, due to an increased expression of its specific receptor, the glycosphingolipid Gb3 (or CD77) (5, 6). The binding kinetics of STxB to the receptor Gb3 are complex. The pentameric STxB has three binding sites per monomer, thus, up to 15 Gb3 molecules per pentamer are predicted to bind to STxB (7, 28). When bound to Gb3, STxB is internalized and transported to the Golgi apparatus and the endoplasmic reticulum via the retrograde pathway, avoiding degradation in the lysosomes (8-10). The protein is detectable in cancer cells for as long as five days (5, 7, 22, 23).

Consistent with recent data, we detected increased levels of Gb3 in 78% of pancreatic carcinoma compared to non-diseased pancreas (n=21 of 27 cases) (13). Distler and co-workers reported overexpression of Gb3 in 62% of pancreatic carcinoma (n=21), based on a newly developed mass spectrometry method (13). We employed a thin layer chromatography approach, which yielded an absolute quantification of Gb3 levels, based on wet weight of the tissue samples analyzed (5). The median expression of Gb3 for non-diseased pancreas was 29 ng per mg of tissue, as opposed to 55 ng Gb3 per mg tumor tissue. However, the data reported here show differences to previous reports, which may be explained by the relatively small sizes of the patient collectives. The difference in expression between normal and carcinoma
tissue was statistically highly significant in our case (p=0.0006), but not in a previous study (13). Gb3 expression has been reported to lack correlation with any clinico-pathological parameter, except with tumor differentiation (13). This indicated high levels of Gb3 in less differentiated tissue. However, we could not confirm a correlation between tumor grading and Gb3 expression on our collective (p=0.21). Furthermore, our analysis provided no evidence for a correlation of Gb3 expression with clinical or pathological parameters. Survival analysis showed no significant difference concerning Gb3 levels. This is in accordance with our earlier findings obtained on colon cancer (6), and indicates that elevated Gb3 expression is a frequent phenomenon in malignant gastrointestinal tumors, irrespective of stage and differentiation. The pathophysiological role of up-regulated Gb3 expression in tumors is still unclear (29, 30). Recently published data show that cisplatin induces Gb3 expression in cancer cells, and that Gb3 expression is linked to acquired cisplatin-resistance (31). Moreover, increased Gb3 levels were correlated to increased expression of MDR1/PgP (Multidrug resistant 1/ permeability glycoprotein), a glycoprotein involved in the biosynthesis of glycolipids such as Gb3 (29). Gb3 and MDR1/PgP partially co-localize, with Gb3-containing lipid rafts being crucial for intracellular MDR1/PgP surface trafficking. Furthermore, elevated levels of Gb3 were described in drug resistant cancers (32, 33) and a functional interplay between MDR1/PgP and membrane Gb3 was suggested. Importantly, carcinoma cells were the major Gb3-source within the tumors. In contrast, the stroma was essentially negative for Gb3. Therefore, the large stroma content which is frequently found in pancreatic cancer may lead to an under-estimation of the actual Gb3 levels. Interestingly, not all tumor cells within one sample were positive for Gb3. This heterogeneous expression pattern is in accordance with earlier findings, reporting an association of the invasive status of colorectal cancer cells with high Gb3 expression (4). Even though Gb3 expression may be restricted to a subset of pancreatic cancer cells, a therapeutic intervention based on vectorized STxB could still yield a clinical benefit, e.g., by eliciting an
anti-tumoral immune response caused by dying tumor cells. In the present study, an association of the proliferative status of cancer cells with Gb3 levels was tested. However, there was no clear correlation of Gb3 content with the proliferation marker Ki67. The Gb3 levels found in normal tissue are most likely due to endothelial cells, immune cells, and myofibroblasts, which have been reported to express Gb3 (24, 34, 35). We validated the endothelial expression via CD31 staining of tissue sections. Endothelial cells in normal tissue were positive for Gb3, intratumoral blood vessels were not strongly marked. However, we and others described a strong Gb3 expression in tumor-associated neovascularization in human bladder cancer and colorectal carcinoma (35-38). Our findings indicate a less pronounced expression of Gb3 in intratumoral blood vessels in pancreatic cancer, which may result from organ specific variances concerning tumor vascularization. However, it cannot be excluded that Gb3 may also be expressed by further cells at levels that are below the detection threshold in immunocytochemistry.

Here, we could validate an intracellular uptake along the retrograde pathway in pancreatic cancer cells, whereas no uptake of STxB was observed in DLD1 cells lacking the Gb3 receptor. Co-localization of fluorescently labeled STxB and the Golgi-marker Golgin p97 occurred within 60 minutes, and intracellular STxB was still detectable after 2 days, even though cell-specific differences were observable at late timepoints. Therefore, targeted chemotherapy mediated by STxB may be feasible on pancreatic cancer. As proof of principle, we utilized a previously established compound that consists of a topoisomerase type I inhibitor, coupled covalently to STxB (16). SN38 is the active metabolite of Irinotecan, which inhibits the DNA topoisomerase I (16, 17, 39). Irinotecan is activated by hydrolysis to SN38, but only 2% to 8% of Irinotecan is converted to SN38 in the liver and the tumor cells, which requires higher dosage to obtain therapeutic efficacy (40, 41). SN38 is far more efficient than Irinotecan, but due to its hydrophobicity and low stability in serum, its clinical use is limited (14, 40, 41). In vitro, the cytotoxicity was successfully demonstrated for lung, ovarian,
colorectal, and gastric cancer, and several trials investigate the clinical use of the agent (16, 17, 39, 32-43). Covalent coupling to STxB could be an effective means to stabilize SN38 (15), and has the advantage of greatly enhanced specificity. Only cells which express the Gb3 receptor are targeted, and consequently, the clinical side effects could be expected to be greatly diminished. Endothelial cells or kidney epithelia express Gb3 and might therefore be negatively affected by a cytotoxic STxB-based compound (44, 45). However, although Gb3 levels and Shiga toxin (STx) binding does not change depending on patients age, the effects, in particular the hemolytic uremic syndrome, appear age dependent. Therefore the renal damage caused by STx and conclusively in case of therapeutic usage of STxB the possible renal side effects cannot be estimated yet and should be evaluated (46).

The standard therapy for pancreatic cancer is gemcitabine (2, 3, 47, 48). However, Irinotecan is currently analyzed as first-, second-, and third-line therapy for advanced and metastatic pancreatic cancer in gemcitabine refractory cases, and showed slight but objective responses as single agent, or in combination (FOLFIRI) (21, 49, 50) . We therefore decided to test STxB-SN38 as a novel therapeutic compound on pancreatic cancer cells. As positive control, STxB-SN38 was tested on the Gb3-expressing colon cancer cell line HT29 (15). Proliferation assays demonstrated cytotoxicity of STxB-SN38 on HT29 cells (not shown). To validate the receptor-specificity, tests were performed on DLD1, a Gb3-negative colon cancer cell line. Reduced cytotoxicity was observed for STxB-SN38, whereas the cells responded to Irinotecan. All pancreatic cancer cell lines tested were sensitive to treatment with Irinotecan or STxB-SN38. Importantly, STxB-SN38 had a significantly stronger cytotoxic effect than Irinotecan; the IC50 values for STxB-SN38 were enhanced over 100-fold as compared to Irinotecan, in accordance with earlier findings on colorectal cancer cells (15) (Fig. 4, Table 2). However, there was no positive correlation between the Gb3 expression levels in the cell lines and sensitivity towards STxB-SN38. This may indicate that additional parameters, like regulation of apoptotic pathways, may determine the cellular sensitivity to the cytotoxic
compound. Moreover, a competition assay with a ten-fold molar excess of unlabeled STxB in competition with STxB-SN38 demonstrated that the cytotoxicity is mainly mediated by Gb3-triggered uptake. Taken together, STxB-mediated tumor targeting offers a promising new approach to diagnose or treat pancreatic cancer.
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TABLE I: PATIENT COLLECTIVE AND CLINICAL DATA

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**Figure Legends**

**Figure 1:** Gb₃ is overexpressed in pancreatic carcinoma as compared to non-diseased pancreas. (A) Gb₃ was quantified by thin layer chromatography from tissue extracts of pancreatic carcinoma (n=28) and matched normal tissue (n=27). The difference in Gb₃ expression was highly significant (p=0.0006). (B) Gb₃ is expressed in pancreatic cancer cell lines (DanG, MiaPaCa2, BxPC3), but is undetectable in the control cell line DLD1 (colorectal cancer).

**Figure 2:** Immunocytochemical analysis of Gb₃ expression. Hematoxylin/Eosin (HE) stained sections show non-diseased pancreas (A), and pancreatic carcinoma (D, G). Fluorescently labeled STxB-Cy3 (shown in red) was used on consecutive sections (B, E, H), nuclear staining is shown in blue (DAPI). (A-C) Non-diseased tissue. Note the co-localization of STxB-Cy3 and CD31-positive blood vessels (green), high-power view in (C). (D-F) Pancreatic carcinoma with low Gb₃ expression. (Co-staining for blood vessels with anti-CD31 (green) reveals minor expression of Gb₃ in blood vessels, and duct-like pancreatic cancer cells are weakly positive for Gb₃ staining (high-power view in F). (G-J) Pancreatic carcinoma with high Gb₃ expression. Co-staining for blood vessels reveals minor expression of Gb₃ in blood vessels, whereas duct-like pancreatic cancer cells are strongly positive for Gb₃ staining (high-power views in I and J). (K) Association of Gb₃ expression with cell proliferation: double-positive cells are stained both for STxB as well as for Ki67 (arrow), whereas other proliferating cells are negative for Gb₃ expression (arrowhead). (L) Gb₃ expression is confined to the bona-fide pancreatic cancer cells (upper left part of the panel). The mesenchymal marker Vimentin (green) shows no co-localization with STxB. Panels A, B, D, E, G, H, and L: magnification 200x (size bar: 100 μm). All other panels: magnification 400x (size bar: 20 μm).
Figure 3: Analysis of intracellular uptake kinetics of STxB in pancreatic cancer cells (BxPC3, DanG, MiaPaCa2) and a colon cancer cell line (DLD1). The white bars indicate 20μm. STxB-Cy3 (red), Golgi-staining with anti-golgin p97 (green) and nuclear staining (blue; overlay: nuclear staining combined with STxB-Cy3 staining). Incubation with STxB was carried out on ice for 15 min, leading to distinct plasma membrane staining for STxB. After incubation with STxB at 37°C for 15 min, co-localization with the Golgi apparatus is visible (arrow). After incubation with STxB at 37°C for 60 minutes, prominent co-localization with Golgi structures occurs. After 48 hours in culture, STxB is still detectable within tumor cells. However, part of the STxB is found in intracellular vesicles that are clearly distinct from the Golgi apparatus (arrowhead). Note that Gb3-negative DLD1 cells show no uptake.

Figure 4: STxB-SN38 inhibits cell growth in pancreatic cancer cells. Results of cell proliferation analysis (n=5 independent tests for each cell line and condition). Left panel, relative cell growth, determined at fixed time points after treatment with increasing concentrations of Irinotecan. Right panel, cell proliferation after treatment with increasing concentrations of STxB-SN38.

Figure 5: The cytotoxic effect of STxB-SN38 is mediated by the receptor Gb3. XTT analysis of MiaPaCa2 cells after competing incubation with STxB-SN38 and un-labeled STxB (n=3 assays). Mock-treatment with unlabelled recombinant STxB ("STxB-WT" at 50 μM) had only minor effects, whereas treatment with STxB-SN38 (5 μM) leads to significant growth inhibition. Five minutes of pre-incubation with 50 μM STxB-WT, before treatment with STxB-SN38 (5 μM), significantly reduces the cytotoxic effects of STxB-SN38 (one asterisk: significance p<0.05, two asterisks: high significance p<0.001).
Figure 2
Figure 3

48h 37°C

60min 37°C

15min 37°C

15min on Ice

BxPC3 63x
BxPC3 63x
STxB-staining only
DanG 63x
MiaPaCa2 63x
DLD1 40x
Figure 4

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# Molecular Cancer Therapeutics

## Tumor-specific targeting of pancreatic cancer with Shigatoxin B-subunit

Matthias Maak, Ulrich Nitsche, Larissa Keller, et al.

*Mol Cancer Ther* Published OnlineFirst July 25, 2011.

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