Gastric Adenocarcinomas Express the Glycosphingolipid Gb3/CD77: Targeting of Gastric Cancer Cells with Shiga Toxin B-Subunit

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Abstract

The B-subunit of the bacterial Shiga toxin (STxB), which is nontoxic and has low immunogenicity, can be used for tumor targeting of breast, colon, and pancreatic cancer. Here, we tested whether human gastric cancers, which are among the most aggressive tumor entities, express the cellular receptor of Shiga toxin, the glycosphingolipid globotriaosylceramide (Gb3/CD77). The majority of cases showed an extensive staining for Gb3, (36/50 cases, 72%), as evidenced on tissue sections of surgically resected specimens. Gb3 expression was detected independent of type (diffuse/intestinal), and was negatively correlated to increasing tumor-node-metastasis stages (P = 0.0385), as well as with markers for senescence. Gb3 expression in nondiseased gastric mucosa was restricted to chief and parietal cells at the bottom of the gastric glands, and was not elevated in endoscopic samples of gastritis (n = 10). Gb3 expression in established cell lines of gastric carcinoma was heterogeneous, with 6 of 10 lines being positive, evidenced by flow cytometry. STxB was taken up rapidly by live Gb3-positive gastric cancer cells, following the intracellular retrograde transport route, avoiding lysosomes and rapidly reaching the Golgi apparatus and the endoplasmic reticulum. Treatment of the Gb3-expressing gastric carcinoma cell line St3051 with STxB coupled to SN38, the active metabolite of the topoisomerase type I inhibitor irinotecan, resulted in >100-fold increased cytotoxicity, as compared with irinotecan alone. No cytotoxicity was observed on gastric cancer cell lines lacking Gb3 expression, demonstrating receptor specificity of the STxB–SN38 compound. Thus, STxB is a highly specific transport vehicle for cytotoxic agents in gastric carcinoma. Mol Cancer Ther; 15(5); 1008–17. ©2016 AACR.

Introduction

With only 30% 5-year survival in advanced stages, gastric cancers are among the most aggressive tumors (1). The most frequent type, arising from the glandular epithelium of the stomach mucosa, is gastric adenocarcinoma, which displays highly heterogeneous histologic differentiation patterns (2). The two major types of gastric carcinoma are the intestinal and diffuse type (Lauren classification). The intestinal type frequently shows disorganized tubular structures, whereas diffuse-type cancer cells are scattered, mucus-secreting, and typically poorly differentiated (3). Currently, surgical resection is the only option for curative treatment and represents the standard therapy for locoregional gastric carcinoma. However, surgery alone is recommended only for early stages (1). Most tumors are locally advanced at diagnosis, requiring perioperative systemic therapies, which are associated with toxic side effects and relatively low response rates (1). Therefore, targeted therapy of gastric cancer, by specific delivery of cytotoxic compounds to gastric cancer cells, is urgently required.

We and others showed previously that gastrointestinal tumors, like colorectal cancer and pancreatic ductal adenocarcinoma, show an overexpression of the neutral glycosphingolipid globotriaosylceramide in tumor cells, but also in normal as well as tumor-associated vasculature (Galα1-4Galβ1-4Glcα1-1Cer, Gb3/CD77; refs. 4–8). The terminal disaccharide of this lipid is the receptor of the bacterial Shiga toxin. Its nontoxic B-subunit (STxB) is a promising tool for targeted therapy, as STxB specifically binds to tumor cells that express its cellular receptor Gb3 on the outer leaflet of the plasma membrane (9). Upon binding, STxB is rapidly taken up, following the "retrograde transport" route from the endosome via the trans-Golgi network and the Golgi apparatus to the endoplasmic reticulum (10). Thereby, STxB bypasses the late endocytic pathway and avoids lysosomal degradation. Shiga toxin is produced by Shigella dysenteriae (serotype I), and Shiga-like toxins (or verotoxins) by enterohemorrhagic Escherichia coli strains (11, 12). Shiga toxin is comprised of one toxic A-subunit, and five nontoxic B-fragments that form the B-subunit (STxB), and serve as a highly specific delivery tool. One STxB binds...
to up to 15 Gb3 molecules (12, 13), which leads to lipid rearrangements in membrane microdomains (14), tubular membrane invagination, and eventually to intracellular uptake (15).

Evolutionarily acquired properties of STxB, like high receptor specificity and affinity, fast intracellular uptake, high stability against proteolytic degradation (16) and low immunogenicity in humans (17,18), predestine STxB for biomedical applications. The decisive requirement for application of STxB is the cell-specific expression of Gb3 in tumor cells: Gb3 is expressed by Burkitt and centrofollicular lymphoma and solid tumors like testicular seminoma, breast, ovary, pancreatic, and colon cancer (5,6,8,19–26).

The potential for diagnostic use of STxB conjugates was successfully demonstrated by endoscopic, as well as by PET and ultrasound imaging in xenografts of human tumors (27), and in genetic mouse models of digestive cancer (28). The coupling of STxB to SN38 (7-ethyl-10-hydroxycamptothecin), the active metabolite of the topoisomerase I inhibitor irinotecan (CPT-11), was successfully shown (29). The STxB-SN38 conjugate has highly increased cytotoxicity as compared with irinotecan, when tested on Gb3-expressing colorectal or pancreatic cancer cell lines (29). SN38 is orders of magnitude more active than irinotecan, but its hydrophobicity and insolubility in most physiologic solvents preclude it from clinical application (30). The parental compound, irinotecan, has been reported for second-line chemotherapy of gastric carcinoma (31,32).

Here, we report Gb3 expression in patient samples and established cell lines of gastric carcinoma. Moreover, intracellular uptake kinetics of fluorescently labeled STxB were established for gastric carcinoma cell lines. A STxB-SN38 conjugate showed high receptor specificity and over 100-fold greater cytotoxicity on gastric cancer cells, as compared with the clinically established drug irinotecan. Taken together, our study describes for the first time a specific therapeutic targeting of gastric carcinoma cells with the conjugate STxB-SN38.

### Materials and Methods

#### Patient collective

Informed written consent had been obtained with prior approval of the ethics committee of the Faculty of Medicine of the TUM (#1926/2007). Tissue samples were obtained surgically from 50 patients with gastric carcinoma admitted to our Surgical Department in 1990 to 2005 (clinical data in Supplementary Table S1). Endoscopic samples of ten tumor-free patients with type B/C gastritis were collected (Supplementary Table S2), and tested for presence of *H. pylori* by rapid urease test, as well as PCR. Tumors were classified according to the International Union Against Cancer (AJCC/UICC), summarized in Table 1. Histology-guided sample selection was performed by a trained pathologist to identify tumor samples, as well as controls for nondiseased gastric mucosa, essentially as indicated in (33). Samples were snap frozen in liquid nitrogen immediately and stored at −80°C.

#### Reagents and antibodies

The recombinant variant STxB/Cys was produced in endotoxin-free form as described previously (28). Antibodies and reagents were purchased from the indicated suppliers: antigolgin p97 (Molecular Probes), anti-von Willebrand factor (DakoCytomation), anti-pepsinogen A (Acris), anti-chromogranin A (Epitomics), DAPI [2-(4-Carbamimidoylphenyl)-1H-indol-6-carboximidamide], anti-Ki67, and anti-calnexin antibodies (Sigma-Aldrich), and BSA-Cy3 (Linscott). Secondary antibodies

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**Table 1.** Correlation of Gb3 expression with clinicopathological data in gastric cancer

<table>
<thead>
<tr>
<th>Clinical/pathologic parameter</th>
<th>No. of cases (%)</th>
<th>Gb3 expression scorea</th>
<th>P</th>
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<tr>
<td></td>
<td></td>
<td>0</td>
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<tr>
<td>Lauren classification</td>
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<td>Diffuse type</td>
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<td>Histologic type</td>
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<tr>
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<td>Tubular carcinoma</td>
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<td>Moderately diff. (G2)</td>
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<tr>
<td>Undifferentiated (G4)</td>
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<td>pT1/2</td>
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<tr>
<td>pM1</td>
<td>12 (24%)</td>
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<td>7</td>
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<tr>
<td>Mean postoperative survival (months)</td>
<td>35.8</td>
<td>36.0</td>
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</table>

*aGb3 score: 0, no STxB staining; 1, weak to intermediate STxB staining; 2, strong STxB staining in major areas of the section.

*bns, not significant, pairwise comparison.
were purchased from Jackson Immunoresearch (West Grove), cell culture reagents were from Invitrogen. Rituximab was obtained from the Pharmacy of the Klinikum rechts der Isar. SN38 was coupled to STxB as described previously (29).

**Immunofluorescence microscopy**

Immunostaining was performed as described previously (28). Briefly, after paraformaldehyde-fixation, cells 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/Cys was purified from bacteria as described previously (34). Covalent coupling of STxB/Cys to the fluorophore Cy3 (Amer sham Biosciences) was carried out according to supplier’s instructions. Gb3 was stained on 3% paraformaldehyde fixed cryosections by incubation with STxB-Cy3 for 30 minutes at a final concentration of 10 μg/mL in PBS containing 0.2% BSA. For counterstaining against Ki67, 0.1% Triton X-100 was used for permeabilization. Immunofluorescence staining was detected with a Zeiss Axiosvert 200M microscope with an AxioCam MRm CCD camera (Zeiss). Images were captured using Axiovision software (version 4.8.2, Zeiss) and image files were imported into Adobe Photoshop version 12.0.4 software (Adobe). STxB staining on tissue sections was quantified by three independent observers, blind to sample identity (P. Emanuel Geyer, M. Maak, K.-P. Jansen). A scoring system was established, ranging from no detectable STxB staining (0 points), weak to intermediate staining (1 point), and strong staining (2 points; Supplementary Fig. S1).

**Cell culture and STxB uptake assays**

Cell lines were acquired from ATCC or DSMZ since 2013, respectively, and periodically tested for mycoplasma infection. To avoid contamination and phenotype changes, cells were kept as frozen stocks and cultured consecutively for 4 weeks maximum in DMEM with FCS (7%), 1% penicillin/streptomycin, and 1% glutamine. Gastric cancer cell lines of diffuse/poorly differentiated type: AGS and KATO III, GP220 MKN-45 NUGC4, cell lines of nondiseased tissue was done using paired t test. In all cases, STxB-labeling was restricted to the abumin al parts of gastric glands, and no major differences were observed between different parts of the stomach. On the basis of anatomic localization and position within the gastric mucosa, as well as by IHC, the Gb3-expressing cell types corresponded to both parietal and chief cells (Fig. 1). This was confirmed by comparing sections stained with hematoxylin and eosin (Fig. 1A), to parallel cryosections stained with fluorescently labeled STxB (Fig. 1B–E). Blood vessel endothelial cells, identified by staining against von Willebrand factor, did not show

**Senescence staining for cultured cells and cryosections**

Senescent cells were stained by enzymatic activity of senescence-associated β-galactosidase with the Promocine Senescence Detection Kit (PromoCell). Tissue cryosections, or cells grown on coverslips for various time points, were washed briefly with PBS, and fixed following the manufacturer’s instruction for 15 minutes at room temperature. The staining solution was added (940 μL staining solution, 10 μL staining supplement, 50 μL 20 mg/mL X-Gal in DMSO), and incubated for 48 hours at 37°C.

**Flow cytometry**

A total of 2 × 10^6 cells were seeded in 10-cm cell culture dishes, cultivated for 24 hours, and counted. Staining was carried out for 5 × 10^6 cells in 1 mL medium, with either 20 nmol/L STxB-Cy3, staining control with 20 nmol/L BSA-Cy3, or without staining, for 15 minutes at 37°C. Cells were harvested by centrifugation, resuspended in 0.5 mL PBS with 0.5% (v/v) BSA, 0.01% (w/v) NaN₃, and passed through a cell strainer immediately before flow cytometry using a FACSCalibur device (BD Becton Dickinson) with CellQuest Pro software (BD Biosciences). Doubles, dead cells, and debris were excluded, and histograms were analyzed with the software FlowJo 8.8.2 (Tree Star, Ashland).

**Statistical analysis**

Analyses were performed using SPSS (version 16.0). GraphPad Prism 5, and GraphPad InStat3 (Graph Pad Software). Data are presented as mean ± SD or, where specified, as median (range). Comparison of Gb3 expression in carcinoma and corresponding nondiseased tissue was done using paired t test. Influence of Gb3 expression on survival time was analyzed by Cox regression. Mann-Whitney U tests were used for correlation of Gb3 expression with histopathologic data. All statistical comparisons were done at a 0.05 level of significance.

**Results**

**Gb3 expression in nondiseased gastric tissue**

To establish the expression pattern of Gb3 in normal stomach mucosa, we analyzed surgically resected gastric tissue (10 cases, two samples each, from antrum and corpus regions), from tumor-free patients. Samples were stained with fluorescently labeled STxB, according to methods established previously (8). In all cases, STxB-labeling was restricted to the abumin al parts of gastric glands, and no major differences were observed between different parts of the stomach. On the basis of anatomic localization and position within the gastric mucosa, as well as by IHC, the Gb3-expressing cell types corresponded to both parietal and chief cells (Fig. 1). This was confirmed by comparing sections stained with hematoxylin and eosin (Fig. 1A), to parallel cryosections stained with fluorescently labeled STxB (Fig. 1B–E). Blood vessel endothelial cells, identified by staining against von Willebrand factor, did not show
Gb3 expression (Fig 1B). Gb3 expression in chief cells was shown by counterstaining of tissue sections with labeled STxB and specific antibodies against pepsinogen-A (Fig 1C; double-positive cells in yellow). However, not all pepsinogen-A positive cells were positive for STxB-Cy3. A part of presumed chief cells, located at the very bottom of gastric glands, did not show detectable Gb3 expression (Fig. 1C, inset). STxB-staining was restricted to a narrow zone located distally above these cells, consisting of double-positive cells stained both for pepsinogen A and STxB (i.e., chief cells), as well as Gb3-positive and pepsinogen A-negative cells (i.e., parietal cells; Fig. 1C). Cells in the isthmus, identified by the proliferation marker Ki67, were negative for STxB (Fig. 1D). Only a fraction of gastric neuroendocrine cells, identified by an antibody against chromogranin A, were positive for Gb3 (Fig. 1E). The foveola part of the mucosa, stained by lectin UEA, was also negative for STxB (Fig. 1F). Taken together, expression of Gb3 was detected in nondiseased stomach mucosa, anatomically restricted to a narrow band of cells at the bottom of glands, most likely corresponding to a subset of chief and parietal cells. More superficial areas of the gastric mucosa, the proliferating compartment and stroma cells were negative for Gb3 expression. Cholesterol depletion following published protocols allowed detection of Gb3 expression in blood vessel endothelia (Supplementary Fig. S1; ref. 35). After investigation and Gb3 expression in nondiseased gastric mucosa, endoscopically derived samples of 10 patients with clinically confirmed type B/C gastritis were analyzed (Supplementary Fig. S1; Supplementary Table S2). Two patients were diagnosed positive for H. pylori infection, but none of the patients had neoplastic malignancies. In all cases, the STxB staining pattern was confined to the anatomically restricted zone observed in nondiseased gastric mucosa, and no elevated Gb3 expression was observed.

Gb3 is strongly expressed in the majority of gastric carcinoma

Next, we analyzed surgically resected tissue samples of gastric carcinoma (n = 50). The collective consisted of n = 25 cases of the intestinal type, and n = 25 cases of the diffuse type, according to the classification by Laurén (ref. 3; details in Supplementary Table S1). Samples were studied in a blinded fashion, following a robust scoring system (Supplementary Fig. S1). The analysis revealed strong Gb3 expression in bona fide cancer cells of intestinal (Fig. 2A), as well as diffuse-type carcinoma (Fig. 2B). 36 of 50 (72%) human gastric carcinomas were classified as Gb3-expressing, and 7 cases showed very high Gb3 levels. In intestinal-type carcinoma, cancer cells arranged in glandular structures showed STxB-staining (Fig. 2A). In diffuse-type carcinoma, dedifferentiated and scattered cancer cells were positive for Gb3 (Fig. 2B). Gb3 was often expressed only in parts of the tumors. In rare cases, Gb3 staining was completely absent from tumor cells, and only detectable at low levels in blood vessel-associated endothelia (Fig. 2C). Cholesterol depletion revealed Gb3 expression in most tumor-associated blood vessels, albeit at varying levels (Supplementary Fig. S1). For control, the scoring system was applied to normal tissue from n = 20 patients. Around 45% of normal tissues were devoid of STxB staining (9/20), as compared with 28% STxB-negative tested carcinomas (14/50; χ² test for trend, df = 4.536; P = 0.0332). Both proliferating (Ki67-positive) as well as nonproliferating cancer cells were positive for Gb3, in accordance to findings for other tumor entities (Fig. 2D). Controls with BSA-Cy3 demonstrated the specificity of the STxB-staining (Fig. 2E).

Next, Gb3 expression was analyzed according to clinicopathologic parameters (Fig. 2F and G; Table 1). The frequency of Gb3-expressing tumors did not differ significantly between intestinal or diffuse-type tumors. Postoperative survival of patients with Gb3-negative or Gb3-positive tumors was indistinguishable...
A significant reduction of Gb3 expression was observed with increasing tumor progression. Gb3 levels were negatively correlated to increasing tumor–node–metastasis (TNM) stage \((P = 0.0385, \text{stage I/II compared with metastasized stage III/IV tumors})\). Moreover, Gb3 levels were negatively correlated to tumor size \((T\)-category, \(P = 0.0267\); Table 1). Gb3 expression in gastric carcinoma cell lines Expression of Gb3 was further confirmed in gastric cancer cell lines (details in Supplementary Table S3). The number of Gb3-expressing cells was quantified by flow cytometry, and independently by fluorescence microscopy in cell lines from intestinal \((n = 5)\) and diffuse-type \((n = 5\); Fig. 3). In the intestinal-type lines St.23132, St.3051, St.2957 and the diffuse-type line NUGC4, more than half of all cells expressed Gb3 (Fig. 3A). Intermediate to low Gb3 expression was observed for NCI-N87, MKN-7, GP220, and MKN-45, whereas lines KATO III and AGS were completely negative for Gb3. As control, uptake in colorectal cancer cells \((\text{CaCo2})\) was monitored (Supplementary Fig. S2). There was a trend toward higher Gb3 expression in gastric cancer cell lines from intestinal type, as opposed to diffuse-type cancer \((P = 0.06593)\). Of note, several cell lines with weak Gb3 levels \((\text{GP220, MKN-7, and MKN-45})\) showed Gb3 expression, confined to isolated postmitotic cells (Supplementary Fig. S2).

Figure 2.
Gb3 expression in gastric cancer of intestinal (A, D, E), or diffuse type (B, C). Left row: hematoxylin and eosin staining, middle and right row: STxB (red), anti-vWF antibody (green), and nuclei (DAPI, blue) on consecutive sections, unless stated otherwise. A, intestinal-type lesion with carcinoma cells arranged in glandular structures. Middle (overview): Gb3 expression in cancer cells (arrow), no expression in tumor-associated blood vessels (arrowhead). Right: higher enlargement. B, gastric-type cancer showing partial Gb3 expression. C, example for lesion of gastric type essentially negative for Gb3. Right: minor STxB-staining in blood vessel endothelia. D, intestinal-type carcinoma. Proliferation marker anti-Ki67 (green) reveals no obvious correlation with STxB-staining (red). E, control for specificity; intestinal-type carcinoma, stained either for STxB and anti-vWF (middle panel), or for fluorescently labeled BSA and anti-vWF (right panel). Left and middle row, and control staining in E: 100-fold; size bars, 100 \(\mu\)m. Right row: 400-fold; size bars, 25 \(\mu\)m. F, quantitative examination of Gb3 expression. No difference was observed between the fraction of Gb3-expressing tumors of intestinal \((n = 25)\) or diffuse type \((n = 25)\). G, Gb3 expression is downregulated with increasing TNM stage. Metastatic tumors (UICC-stage III and IV) showed significantly decreased Gb3 levels, compared with locally restricted lesions \((P = 0.0385)\).

Cancer cell senescence may be associated with downregulated Gb3 expression Gb3 levels strongly decreased in St3051 cells during prolonged cell culture periods (96 hours), analyzed as fraction of STxB-positive cells by flow cytometry (Supplementary Fig. S3). The decrease in Gb3 levels was accompanied by a strong increase in activity of senescence-associated \(\beta\)-galactosidase, which has not been described to enzymatically degrade Gb3 (Supplementary Fig. S3). Essentially, the same observations were made for HCT116 colon cancer cells. Addition of fresh serum or glucose to the culture medium at 96 hours did not lead to increased Gb3 biosynthesis. However, complete replacement of culture medium induced a rapid increase in Gb3 levels. Moreover, Gb3 expression and senescence markers were mutually exclusive in parallel tissue sections of gastric carcinoma, indicating that senescence, or loss of proliferative capacity, may inhibit Gb3 synthesis in cancer cells (Supplementary Fig. S3). STxB is taken up along the retrograde transport route Intracellular transport of STxB was investigated by incubation of Gb3-expressing gastric carcinoma cells with fluorescently labeled STxB. Incubation with STxB-Cy3 for 15 minutes at 4 \(^{\circ}\)C resulted in cell surface binding, no intracellular uptake was observed (Fig. 4A). Upon incubation for 15 minutes at 37 \(^{\circ}\)C, STxB-Cy3 was largely taken up (Fig. 4B), colocalizing in part with the Golgi marker golgin p97. After incubation for 1 hour at 37 \(^{\circ}\)C,
complete internalization and perinuclear localization in and around the Golgi was visible (Fig. 4C). Upon a 48-hour chase period, after an uptake for 1 hour, STxB was still detectable in intracellular compartments marked by anti-golgin p97 (Fig. 4D). Thus, STxB was taken up rapidly, following the retrograde transport route. Importantly, binding and uptake of STxB to the cell surface of gastric cancer cells was maintained even at low pH (Supplementary Fig. S4). In contrast, no uptake of STxB was detected in Gb3-negative AGS cells (Supplementary Fig. S5). To exclude unspecific fluid-phase uptake effects, the Gb3-positive cell line St3051 was incubated with fluorescently labeled BSA. Even large molar excesses (1 μmol/L BSA-Cy3, in contrast to 20 nmol/L of STxB-Cy3), did not lead to detectable staining for BSA-Cy3 (Supplementary Fig. S5).

**STxB–SN38 can be used for targeted therapy on gastric cancer cells**

Uptake in gastric cancer cells was exploited for targeted therapy with a STxB conjugate with SN38, the active metabolite of irinotecan. On the basis of our previous experiments, cell lines with high (St3051) or absent (AGS) Gb3 expression were chosen and incubated with different concentrations of STxB–SN38 (IC50:64 μmol/L; summarized in Supplementary Table S4). The cytotoxicity of STxB–SN38 clearly dependent on the presence of the STxB-receptor Gb3: the cell line AGS had a similar sensitivity toward the parental drug irinotecan, as compared with St3051 cells, but AGS cells were far less sensitive toward STxB–SN38 (IC50:21 μmol/L). In addition, nonvectorized SN38, which is precluded from clinical use due to its toxicity, was tested. As expected, SN38 had far greater toxicity than irinotecan (by 2 orders of magnitude) in all cell lines, but was in the same range of activity as STxB–SN38 (Supplementary Fig. S6).

**Discussion**

Here, we demonstrate the use of the nontoxic B-subunit of bacterial Shiga toxin (STxB) for specific targeting of human gastric cancer, the fourth most common cancer worldwide. Gastric cancer treatment relies on surgical resection and multimodal therapy with cytotoxic agents (1). However, the therapy response in general is limited, despite pronounced toxicity of chemotherapy. Therefore, targeted approaches with higher efficiency and reduced side effects offer new intervention strategies for gastric cancer. The nontoxic B-subunit of the bacterial Shiga toxin (STxB) may be used as an efficient and specific tool for tumor targeting. The cellular STxB receptor, the neutral glycosphingolipid globotriaosylceramide Gb3 (Galα1-4Galβ1-4Glcβ1-1Cer), or CD77, is overexpressed in colon carcinoma (6, 21), pancreatic (5, 8), and breast cancer (25). Moreover, STxB can be functionalized and coupled to specific agents for noninvasive or endoscopic tumor imaging, which has successfully been demonstrated in preclinical models (28,36). In the current study, we analyzed whether STxB could be used for targeted therapy in gastric cancer.

However, the expression pattern and regulation of glycosphingolipids in gastrointestinal pathologies are still far from being understood (37). On the basis of the analysis of nondiseased gastric mucosa, we found expression of Gb3 in an anatomically well-defined narrow region at the distal bottom of glands, corresponding to a part of the chief, parietal, and enteroendocrine cell

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**Figure 3.**

Gb3 expression in gastric carcinoma cell lines, quantified by flow cytometry and fluorescence microscopy. A, four cell lines expressed Gb3 strongly in >33% of cells, four lines at intermediate to low levels (between 15% and 1% of cells), two were negative. B, flow cytometry–based quantification of Gb3 expression in St3051 cells (histogram: STxB-Cy3, red line; BSA-Cy3: blue; unstained: green). C, fluorescence microscopy, incubation of St3051 cells with STxB (red), nuclei (DAPI, blue), D, cell line AGS is Gb3-negative (histogram: STxB-Cy3, red line; BSA-Cy3, blue; unstained, green), E, fluorescence microscopy, incubation of AGS cells with STxB (red), nuclei (DAPI, blue). C and E, enlargement, 200-fold; size bar, 50 μm.
populations, as judged by cellular localization, histomorphologic appearance and counterstaining against pepsinogen A and chromogranin A, respectively. Of note, the Gb3 synthase gene seems to be dispensable for stomach development and function (38). Proliferating cells in the isthmus, lectin-marked surface epithelia, as well as mesenchymal stroma cells, did not show Gb3 expression. Therefore, as opposed to intestinal epithelia, where Gb3 expression is restricted to enteroendocrine cells and not detectable in the bulk of epithelial cells (28), there is physiologic expression of Gb3 in human nondiseased gastric mucosa. Several cell types and tissues express Gb3, among them myeloid immune cells, which are insensitive to the toxin (39). Gb3 expression has been found in children in the kidney, in accordance with Shiga-toxin being the disease agent for the childhood hemolytic-uremic syndrome (40). In adults, Gb3 expression in glomeruli is strongly reduced to absent (41). Furthermore, endothelial cells have a high Gb3 content, and can be further stimulated by proinflammatory cytokines to express increased levels of Gb3 (42). Preclinical experiments in pigs have shown that the gastric mucosa of the fundus and antrum contains considerably less Gb3 than other internal organs such as kidney, colon, liver, and spleen (43).

Next, we analyzed n = 50 patients with gastric cancer for intratumoral Gb3 expression and distribution. We observed the majority of gastric carcinomas to express the glycosphingolipid Gb3 (36/50 cases, 72%). The fraction of Gb3-positive gastric cancers is similar to data reported for pancreatic adenocarcinoma, where increased Gb3 levels were found in 62% to 78% of tumors, respectively (5, 8). No Gb3 was detected in 14 patients (28%), as compared with normal tissue samples, where 45% of the samples (9/20) were devoid of Gb3 staining. Gb3 expression did not differ between patients with chronic gastritis or noninflamed mucosa. Of note, the heterogeneous pattern of intratumoral Gb3 expression resembled in size and extent to the Gb3 expressing gland-like structures in colon carcinoma observed previously (6). Expression was mainly confined to cancer cells in glandular structures in the intestinal-type lesions, but was also detected in tumor-associated blood vessels from cancers of all differentiation types. However, no significant differences between Gb3 expression were observable between intestinal or diffuse-type carcinoma (3).

We observed a significant negative association of Gb3 expression with increasing tumor TNM stage, as well as with the size of the primary lesion. This may indicate that Gb3-negative tumors

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**Figure 4.** Uptake kinetics intra cellular retrograde transport, after incubation of live St3051 cells with STxB-Cy3 (red), after fixation staining for golgin p97 (green) and nuclei (DAPI, blue). A, incubation on ice for 15 minutes inhibits active uptake, only plasma membrane staining is observable. B, after 15 minutes at 37°C, STxB is already accumulating within the cell, partially colocalizing with golgin p97 at perinuclear areas (arrows). C, after 60 minutes at 37°C, most of intracellular STxB has reached the Golgi (arrows). D, after 48-hour chase, STxB can still be detected in perinuclear areas around the Golgi (arrows). Enlargement, 1,000-fold; size bar, 10 μm.
have more aggressive properties. However, the link between Gb3 expression and pathologic parameters or prognosis is still largely unknown. In pancreatic, ovarian, and mammary carcinomas, a higher level of Gb3 expression was noticed in less differentiated tissue (5, 19, 25). In accordance with our findings on gastric cancer, Gb3-positive mammary carcinomas show lower malignant behavior, as compared with Gb3-negative tumors (25). There are only few reports on Gb3 expression in stomach cancer. However, it was previously shown that stroma fibroblasts in scirrhous carcinomas, a rare subgroup of diffuse-type cancer featuring important stromal components, express Gb3 (44).

Similar to the clinical samples, 8 of 10 gastric cancer cell lines expressed Gb3, as evidenced independently by flow cytometry and immunofluorescence microscopy. As intracellular uptake of STxB is a prerequisite for targeted therapy, we tested its uptake and intracellular routing in gastric cancer cells. Shiga toxin is taken up by clathrin-dependent (45) and clathrin-independent endocytosis (15), followed by intracellular transport to the endoplasmic reticulum, along the retrograde route (10). As the A-subunit of the holotoxin induces cell death (46), we used only nontoxic STxB as delivery tool. In accordance to earlier results for pancreatic or colorectal cancer cells (6), we observed a rapid and complete uptake of STxB in the gastric cancer cell line St3051. Within 15 minutes, STxB reached the Golgi apparatus. This shows for the first time that uptake of STxB in gastric cancer cells follows the retrograde trafficking route, avoiding the degrading environment of the lysosomes. Binding and intracellular uptake were maintained at acidic pH, even though this may not even be required for efficient tumor targeting within a gastric tumor mass beyond the acid-exposed surface.

In tissue sections of clinical samples, we frequently observed a striking heterogeneity of Gb3 expression. This might be explained by the genetic heterogeneity inherent to tumorigenesis, or by differences in tumor cell differentiation. In breast cancer cells, a functional implication of Gb3 expression for cancer stem cell properties is controversially discussed (47, 48). However, the physiologic or pathophysiologic regulation of Gb3 biosynthesis is not fully understood. In cell culture experiments, we observed a striking downregulation of Gb3 expression with increased culture periods, which was negatively correlated to increased cellular

![Figure 5](https://www.aacrjournals.org/MolCancerTher/article-pdf/15/5/1015/953692/15020551.pdf)

**Figure 5.** Cell proliferation assays show that the compound STxB–SN38 has highly increased and receptor-specific efficacy in gastric cancer cells, compared with the clinically used drug irinotecan. A, effects of irinotecan are comparable in Gb3-positive (St3051) or Gb3-negative (AGS) cell lines. B, STxB–SN38 inhibits cell growth efficiently in St3051 cells, whereas Gb3-negative AGS cells show only minor effects. C, quantitation of half-maximal inhibition (n = 3 independent assays, in triplicates). The efficiency of STxB–SN38 is over 100-fold increased as compared with irinotecan, and specific for Gb3-positive cells.
senescence, as indicated by altered cell morphology and senescence-associated β-galactosidase activity (49). The term senescence has originally been coined for primary cell lines, but was also observed to be induced by oncogenic stress in cancer cells. Depletion of glucose or serum components could be excluded as reason for the downregulated biosynthesis of Gb₃, as only complete replacement of the cell culture medium induced Gb₃ biosynthesis in long-term cultured cells, but not addition of glucose or fresh serum. However, further nutrient limitations or accumulation of catabolic end products in the culture medium might participate to the observed transient loss of proliferative capacity. Cellular senescence is thought to suppress tumorigenesis, defined as stable loss of proliferative capacity. Senescence can be induced by oncogenic stress, DNA damage, oxidative stress, and exposure to cytotoxic therapies. In accordance to in vitro results, staining of parallel tissue sections of gastric cancer indicated that Gb₃ was absent in senescent tissue areas that stained positive for senescence-associated β-galactosidase. Therefore, we propose cellular senescence, induced by oncogenic stress or hypoxia, to potentially inhibit Gb₃ expression in the tumor context.

As the majority of gastric carcinoma expressed Gb₃, and gastric cancer cells showed uptake of STxB along the retrograde route, we investigated the use of functionalized STxB covalently coupled via a disulfide-linker to SN38, the active metabolite of the cytotoxic drug irinotecan. Hence, binding of STxB–SN38 on the target cell results in a fast uptake of the STxB-drug conjugate, and in release of the drug, a topoisomerase type I inhibitor, within the target cells. As expected, our data show that SN38, whose hydrophobicity and toxicity limit its therapeutic efficacy, is orders of magnitude more effective than the clinically applied drug irinotecan (50). Moreover, the STxB–SN38 conjugate has the advantage of high receptor specificity, demonstrated by the fact that Gb₃-negative AGS cells show no detectable toxicity at the dosages used in our study, even though they are clearly sensitive to the type I topoisomerase inhibitors. Thus, a targeted therapy approach based on STxB–SN38 holds the potential to be far more efficient in tumor killing, with reduced toxic side effects. Several potential limitations need to be considered: Gb₃ was often expressed only in parts of the tumors, which may lead to acquired therapy resistance by escaping tumor cells. However, our data indicate that Gb₃-negative cells are frequently senescent, potentially reducing the risk of selective escape. Moreover, Gb₃ expression was decreased in more advanced tumor stages, which have the most urgent need for improved therapies. However, Gb₃ expression was not fully absent from more advanced tumors. In fact, two of three advanced-stage cancers were Gb₃-positive, indicating a need for patient stratification for Gb₃ expression. Lastly, some cells in normal gastric mucosa were also positive for Gb₃. Therefore, targeted therapy may affect noncancer cells as well, which could lead to transient tissue damage in the gastric mucosa that might be compensated by self-renewal of gastric epithelia. Importantly, Gb₃-positive cells in normal gastric mucosa were exclusively nondividing. Thus, by using a compound that affects proliferating cells, such as SN38, only cells that are (a) fast dividing and (b) Gb₃-positive would be targeted. In contrast, Gb₃-positive but nondividing cells in gastric and renal epithelium would be spared. Thus, a STxB-based targeted therapy may be feasible for patients with Gb₃-expressing gastric carcinoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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