Tumor-Specific Targeting of Pancreatic Cancer with Shiga Toxin B-Subunit

Matthias Maak, Ulrich Nitsche, Larissa Keller, Petra Wolf, Marianne San, Marine Thiebaud, Robert Rosenberg, Rupert Langer, Jörg Kleeff, Helmut Friess, Ludger Johannes, and Klaus-Peter Janssen

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 globotriaosylceramide (Gb3 or CD77) is strongly increased in colorectal adenocarcinoma and their metastases. Here, we report an upregulation of Gb3 in pancreatic adenocarcinoma (21 of 27 cases) as compared with matched normal tissue (n = 27). The mean expression was highly significantly increased from 30 ± 16 ng Gb3/mg tissue in normal pancreas to 61 ± 41 ng Gb3/mg tissue (mean ± SD, P = 0.0006), as evidenced by thin layer chromatography. Upregulation 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 with irinotecan. Moreover, this effect was effectively blocked by competing incubation with nonlabeled STxB, showing the specificity of the targeting. Thus, STxB constitutes a promising new tool for specific targeting of pancreatic cancer. Mol Cancer Ther; 10(10); 1918–28. ©2011 AACR.
adenocarcinoma by lipid extraction and thin layer chromatography (TLC), as well as by immunofluorescence 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 7-ethyl-10-hydroxycamptothecin (SN38), 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). Anticancer cytotoxicity was reported, even though no obvious survival benefit was reported so far. The STxB-SN38 compound has been shown previously to possess highly increased cytotoxicity as compared with irinotecan when tested on a colorectal cancer cell line (15). Moreover, the STxB-SN38 compound showed high receptor specificity, as 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 uses of STxB on pancreatic carcinoma. Taken together, we report a significantly increased expression of Gb3 in pancreatic cancer, as compared with 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 about 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, 3 patients died because of unrelated causes and 17 died because of cancer progression or disease recurrence. Tumors were classified according to the International Union Against Cancer, as summarized in Table 1 (21). Histology-guided sample selection was conducted 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 as follows: anti-golgin p97 (Molecular Probes); anti-STxB (22); anti-CD31 (BD Biosciences Pharmingen); DAPI, Ki67, and staurosporine (from Sigma-Aldrich); and calnexin and antivimentin (both from Santa Cruz Biotechnology). Secondary antibodies coupled to fluorophores were purchased from Jackson Immunoresearch and cell culture reagents from Invitrogen. Irinotecan was obtained from the Pharmacy of the Klinikum rechts der Isar. SN38 was coupled to STxB as described (15).

**Indirect immunofluorescence**

Immunostaining was conducted as described before (6). Briefly, after paraformaldehyde fixation, cell lines on coverslips were permeabilized with 0.1% Triton X-100, blocked with PBS containing 2% bovine serum albumin, 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) was carried out according to the supplier’s instructions. For staining of endogenous Gb3, tissue cryosections were fixed with 3% paraformaldehyde for 20 minutes and incubated with STxB-Cy3 for 30 minutes at a final concentration of 10 μg/mL in PBS containing 0.2% bovine serum albumin. For counterstaining against the nuclear antigen Ki67, 0.1% Triton X-100 was used on cryosections. For image acquisition, epifluorescence or confocal microscopes (Zeiss) were used. Images were processed using Adobe Photoshop Software CS3 and colocalization was tested with ImageJ version 1.42q (NIH).

**Quantification of Gb3 expression by TLC**

Gb3 expression was quantified as previously described (refs. 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 hydroalcoholic 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 hour 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 TLC plates (Merck) and separated with chloroform/methanol/water (65:25:4). Dried plates were soaked in 0.1% polyisobutylmethacrylate in hexane, floated for 1 hour in blocking solution, followed by incubation with STxB (20 nmol/L), primary polyclonal antiserum against STxB, and secondary horseradish peroxidase–coupled antirabbit antibodies. Reactive bands were revealed with enhanced chemiluminescence (Amer- sham Pharmacia Biotech).

**Cell culture and STxB uptake assays**

Pancreatic (DanG, MiaPaCa2, and BxPC3) and color- ecal (DLD1 and HT29) cancer cell lines were kept in Dulbecco’s Modified Eagle’s Media (DMEM) with fetal calf serum (FCS; 10% for BxPC3, 7% for all other cells), 1% Pen/Strep, and 1% glucose. 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 described (see above). Cell lines were authenticated by the German cell line repository (DSMZ).

**Cytotoxicity assays**

Cell growth analysis was conducted using the Cell Proliferation Kit II (XTT) from Roche Diagnostics, the amount of dye corresponding to the number of metabolically active cells. The cell lines were split into 6-well plates (at 1 × 10⁶ cells per well) and grown for 3 days. Treatment with irinotecan or STxB-SN38 was carried out for 6 hours in cell medium. Cells were washed with DMEM and substituted with fresh medium for an additional 48 hours. Cells were then seeded onto 96-well plates (in triplicates at 0.5 × 10³ cells per well and 1 × 10³ cells per well) in medium containing 0.5% FCS. After 12 to 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) at 450 to 500 nm. Mean values of 5 independent analyses are shown. A competition assay was conducted with 5 μmol/L STxB-SN38 or 50 μmol/L unlabeled STxB or 50 μmol/L of unlabeled STxB 5 minutes before treatment with 5 μmol/L STxB-SN38.

**Statistical analysis**

Statistical analyses were conducted using SPSS (version 16.0), Graph Pad Prism 5, and GraphPad InStat3 (Graph Pad Software). Data are presented as mean ± SD or where specified as median (range). The comparison of Gb3 expression in pancreatic adenocarcinoma and corresponding nondiseased pancreas was done using paired Student’s 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. The Mann–Whitney U tests were used for evaluating the correlation of Gb3 expression with histopathologic 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 overexpressed in human colorectal cancer (5). Here, we have analyzed surgically resected pancreatic adenocarcinoma and corresponding nondiseased pancreas by lipid extraction and TLC. Gb3 expression was detectable in all tumors tested (n = 28; Table 1). Average median expression was 54.9 ng Gb3/mg tissue as compared with 29.0 ng Gb3/mg tissue in normal pancreas (n = 27). The mean difference of 31.2 ng Gb3/mg tissue was highly significant (95% 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 with the surrounding normal pancreas (Fig. 1A, Supplementary Fig. S1). In 48% (13 of 27) of the samples, the Gb3 level was higher than the calculated median expression of 54.9 ng Gb3/mg tissue. In addition, established pancreatic cancer cell lines were tested and all showed Gb3 expression (Fig. 1B). However, Gb3 expression in cell lines was lower than in primary patient samples. Thus, Gb3 was clearly upregulated 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 histopathologic or survival data. In fact, the HR for the level of Gb3 expression and survival time was 1.005. The correlation with histopathologic 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 SII). 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 SIII). 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 upregulated intratumoral Gb3 levels. Previous work showed 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 TLC. Despite different sensitivities, both detection methods were in good accordance. In normal pancreas, Gb3 expression was mainly detected in endothelia (Fig. 2A–C). In tumors with low Gb3 expression, stromal components like blood vessels were the major source of Gb3 expression (Fig. 2D–F). However, in tumors with high Gb3 expression, duct-like cancer cells were strongly stained with fluorescently labeled STxB (Fig. 2G–L). Tumor-associated endothelial cells were only weakly stained for STxB (Fig. 2F and J). No association of tumor cell growth with Gb3 expression was detected in carcinoma with Gb3 overexpression (Supplementary Table SIII). However, in carcinoma with low Gb3 expression, the few cells that were positive for Gb3 were negative for the proliferation marker Ki67 (Supplementary Table SIII).
Intracellular uptake of STxB in pancreatic cancer cells

Next, we investigated whether 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 colocalized with the Golgi marker Golgin p97 or with the endoplasmic reticulum marker calnexin (Fig. 3, Supplementary Figs. S2 and 3), indicating that STxB followed the retrograde route to the Golgi apparatus. Two days after the initial uptake, STxB was still detectable in punctate structures, colocalizing with the Golgi in DanG and MiaPaCa2 cells. However, no obvious colocalization of STxB could be detected with Golgi structures or endoplasmic reticulum in BxPC3 cells after 48 hours (Supplementary 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 with 56% for HT29 colon cancer cells (positive control), and 0% of DLD1 cells (negative control; not shown).
Supplementary Fig. S3 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

Because 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 with 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 (Supplementary Table SII and Supplementary Fig. S4). The inhibitory concentration 50% (IC_{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 (Supplementary Fig. S5). Irinotecan had a cytotoxic effect on DLD1 cells that lack the receptor Gb3, 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 nonspecific 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 Gb3 receptor.
62% of pancreatic carcinoma (Gb3 levels, based on wet weight of the tissue samples developed mass spectrometry method (13). We used a way, avoiding degradation in the lysosomes (8–10).

Consistent with recent data, we detected increased Gb3 levels in 78% of pancreatic carcinoma compared with nondiseased pancreas (n = 27 cases; ref. 13). Distler and colleagues reported overexpression of Gb3 in 62% of pancreatic carcinoma (n = 21) based on a newly developed mass spectrometry method (13). We used a TLC 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 nondiseased pancreas was 0.5 ng/mg of tissue, as opposed to 55 ng Gb3/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 clinicopathologic 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 data (P = 0.21). Furthermore, our analysis provided no evidence for a correlation of Gb3 expression with clinical or pathologic 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 pathophysiologic role of upregulated Gb3 expression in tumors is still unclear (29, 30).

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 that is frequently found in pancreatic cancer may lead to an underestimation of the actual Gb3 levels. Interestingly, not all tumor cells within a given sample may lead to an underestimation of the actual Gb3 levels. 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; refs. 5, 6). The binding kinetics of STxB to the receptor Gb3 are complex. The pentameric STxB has 3 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 5 days (5, 7, 22, 23).

Consistent with recent data, we detected increased levels of Gb3 in 78% of pancreatic carcinoma compared with nondiseased pancreas (n = 21 of 27 cases; ref. 13). Distler and colleagues reported overexpression of Gb3 in 62% of pancreatic carcinoma (n = 21) based on a newly developed mass spectrometry method (13). We used a TLC 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 nondiseased pancreas was 29 ng/mg of tissue, as opposed to 55 ng Gb3/mg tumor tissue. However, the data reported...
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. Colocalization 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 time points. Therefore, targeted chemotherapy mediated by STxB may be feasible on pancreatic cancer. As proof of principle, we used 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 (15, 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 (40, 41). In vitro, the cytotoxicity was successfully shown 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 that 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 the 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; refs. 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 showed cytotoxicity of STxB-SN38 on HT29 cells (not shown). To validate the receptor specificity, tests were conducted 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 with irinotecan, in accordance with earlier findings on colorectal cancer cells (ref. 15; Fig. 4). However, there was no positive correlation between the Gb3 expression levels in the cell lines and sensitivity toward STxB-SN38. This may indicate that additional parameters, such as regulation of apoptotic pathways, may determine the cellular sensitivity to the cytotoxic compound. Moreover, a competition assay with a 10-fold molar excess of unlabeled STxB in competition with STxB-SN38 showed 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.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


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