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# Shiga Toxin-Mediated Retrograde Delivery of a Topoisomerase I Inhibitor Prodrug**

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Selectivity remains one of the principal challenges in cancer chemotherapy. With more-selective agents, it should be possible to minimize side effects and to use more efficient active-compound concentrations.[1] One way to achieve this goal is targeted delivery to cancer cells.[2,3] Such targeting strategies have already been described, and the use of monoclonal antibodies is by far the best-explored example,[4,5] with compounds such as Mylotarg having reached the market.[6]

Here, we present a new delivery strategy based on the nontoxic B-subunit of Shiga toxin, termed STxB. Shiga toxin is produced by intestinal pathogenic bacteria.[7] STxB associates with the catalytic A-subunit that modifies ribosomal RNA in the cytosol of target cells, thus leading to protein biosynthesis inhibition. To reach the cytosol from the extracellular space where the toxin is produced by the bacteria, STxB binds to the cellular toxin receptor, the glycolipid Gb3. By following a recently discovered pathway, termed the retrograde route (Figure 1), Shiga toxin is then transported via endosomes and the Golgi apparatus to the endoplasmic reticulum, from where the A-subunit is translocated across the membrane to the cytosol.[8,9]

The Shiga toxin receptor Gb3 is strongly expressed by certain human cancers,[9] including colorectal carcinoma.[10] In a mouse model of spontaneous intestinal adenocarcinomatosis, we have shown that STxB can reach Gb3-expressing tumors in vivo.[11] As a delivery tool, STxB exhibits characteristics that the protein has acquired as an intestinal pathogen in co-evolution with its hosts: stability at extreme pH and in the presence of proteases, capacity to cross tissue barriers and to distribute in the organism, and resistance against extra- and intracellular inactivation.[9]

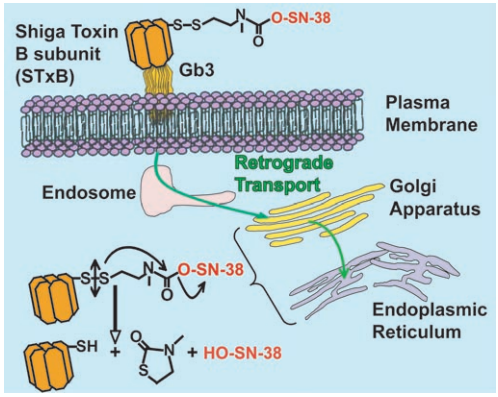
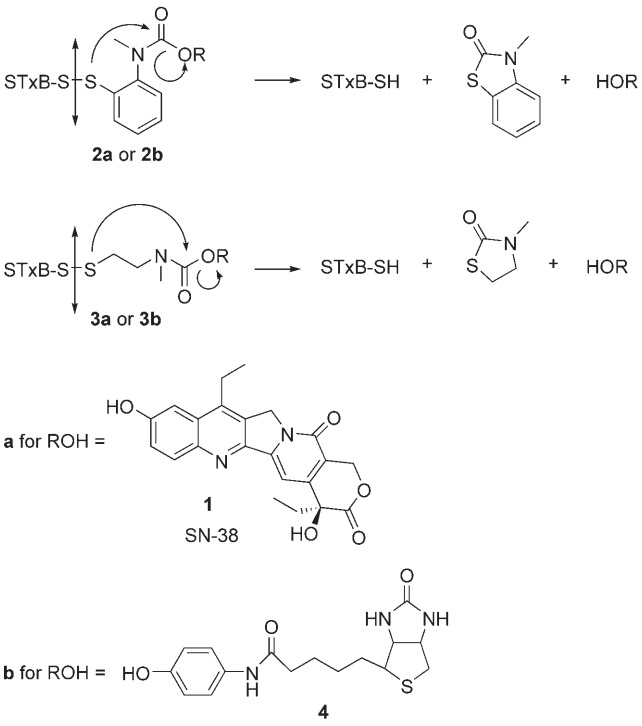



***Figure 1.*** Principle of retrograde delivery.

We designed a prodrug based on SN-38 (**1**; Scheme 1), the active principle of CPT11 (Campto), which is used for the treatment of colorectal carcinoma.[12] SN-38 belongs to the class of camptothecin derivatives that are cytotoxic by inhibition of topoisomerase I, and is one of the most efficient




***Scheme 1.*** Cleavage reactions of compounds **2** and **3**. The double-headed arrows indicate the bonds that are cleaved first.

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compounds in this family.[13] For coupling to the prodrug, an STxB variant with a thiol functionality, termed STxB-SH, was used that was specifically designed for site-directed chemical cross-linking.[14,15]

The phenolic position of SN-38 was chosen to build self-immolative spacers that include disulfide bonds. After cleavage of these bonds and release of a free thiol function, the free phenol must be released without any other external reactant. To this end, we envisioned two different spacers with variable stabilities in the biological system. One is based on an aromatic ring and the other on an aliphatic chain.

These two compounds (**2** and **3**) and their respective cleavage reactions are depicted in Scheme 1. Two variants were synthesized for each spacer arm: one (**a**) with SN-38 (**1**), and the other (**b**) with biotin derivative **4**. The latter model allowed circumvention of the fact that release of SN-38 from compounds **2a** and **3a** could not be monitored in vivo because of a lack of sensitivity. The biotin group was derivatized with a phenol spacer to obtain a similar susceptibility to cleavage as that with the phenol function of SN-38. The compounds were obtained according to Scheme 2 and the Supporting Information.

For the synthesis of compounds **3a** and **3b**, commercial amino alcohol **5** was first monoprotected as a *tert*-butoxycarbonyl (Boc) derivative. The hydroxy group was converted to bromide with $CBr_4$ and then substituted by a thioacetate. The thiol function of **8** was activated as a pyridine disulfide **9**. Liberation of the free amine was performed in acidic medium and the formed chlorhydrate **10** was kept as a salt because the free amine was unstable. Compound **10** was then reacted with phosgene and triethylamine to give the stable carbamoyl chloride **11**. The phenol (SN-38 or biotin derivative) was first coupled in the presence of a stoichiometric amount of 4-dimethylaminopyridine (DMAP) to this bifunctional intermediate **11**. Finally, STxB-SH was reacted with carbamate **12** under basic conditions (pH 9).

The substitution levels of the coupling products were determined as five SN-38 or biotin molecules per STxB pentamer, by using mass spectrometric analysis (see the Supporting Information) and fluorimetric dosage.

As a first step towards the biological evaluation of compounds **2** and **3**, the stability of the biotin versions was tested in different media. Compound **2b** turned out to be readily activated even in the absence of cells, thus precluding its use in vivo. In contrast, compound **3b** was completely stable over extended periods of up to 48 h at 37 °C in all media, including pure fetal calf serum. Prodrug **3a** was also stable in pure fetal calf serum, as shown by fluorimetric measurements (see the Supporting Information).

Compound **3** was chosen for an in-depth characterization on HT-29 colorectal carcinoma cells. ELISA analysis with **3b** demonstrated that cleavage became detectable in the 6–24-h time interval, and was essentially complete at 48 h (Figure 2).

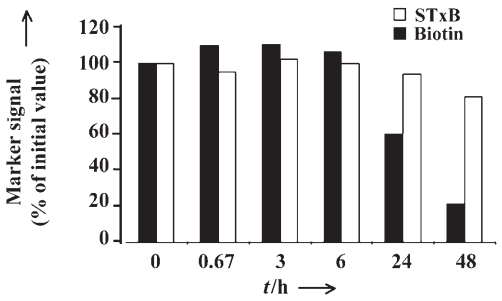
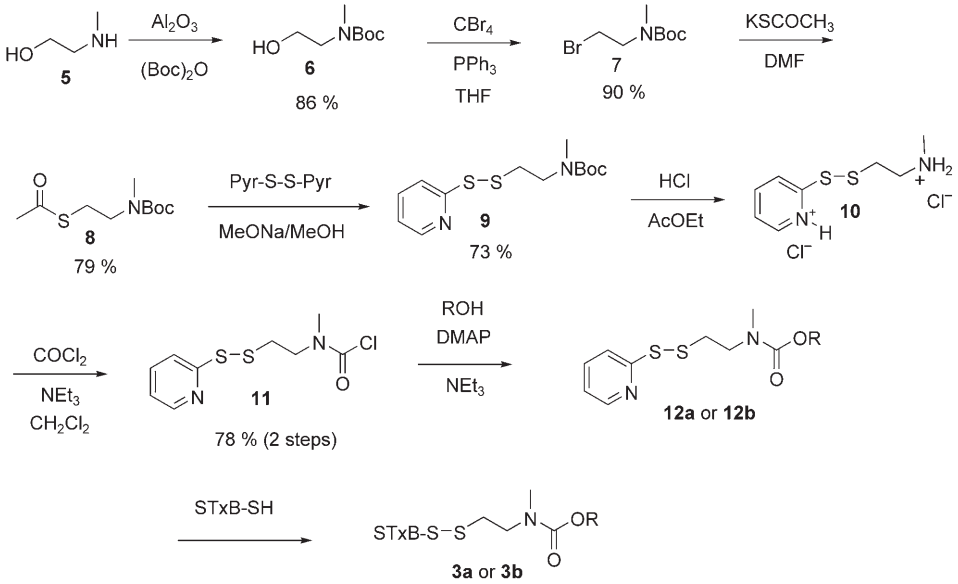



***Figure 2.*** ELISA analysis of activation of **3b** on HT-29 cells. Compound **3b** (1 μM) was incubated with HT-29 cells on ice. After washing, the cells were shifted to 37 °C for the indicated times, lysed, and the lysates were analyzed by ELISA for the indicated markers. Means of two determinations are shown. $t$ = incubation time.

The same results were obtained on HeLa cells. To demonstrate that cleavage occurred intracellularly, we used immunofluorescence analysis (Figure 3). Consistent with the ELISA data, no cleavage could be detected after short times of internalization (45 min), in which STxB (red) and biotin (green) co-localized with the Golgi marker Rab6 (blue). After 48 h, STxB (red) could still be detected in the Golgi region (blue). However, the biotin signal was largely gone, which strongly suggests that the reduction of the disulfide bond occurred within membranes of the biosynthetic/secretory pathway.

Having established that biotin model compound **3b** is activated in HT-29 cells, we sought to determine the cytotoxic effect of the corresponding prodrug **3a**. As shown in Figure 4, an $IC_{50}$ value of 300 nM (in SN-38 equivalents) was observed on Gb3-expressing HT-29 cells. To establish specif-




***Scheme 2.*** Synthesis of compounds **3a** and **3b**.

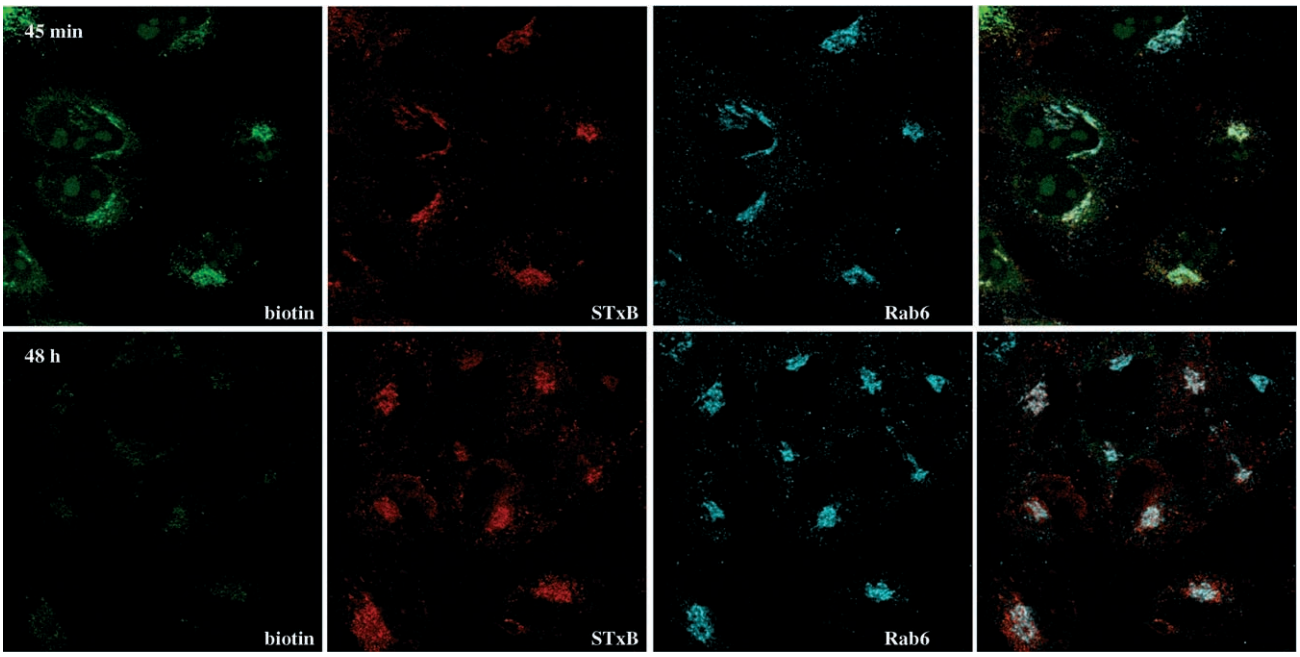



***Figure 3.*** Activation of **3b** after retrograde trafficking to the biosynthetic/secretory pathway. Compound **3b** (1 μM) was incubated with HT-29 cells on ice. After washing, the cells were shifted to 37 °C for the indicated times, fixed, and stained for the indicated markers. Top: 45 min of uptake; bottom: 48 h of uptake. Biotin: green; STxB: red; Rab6 (Golgi): blue.

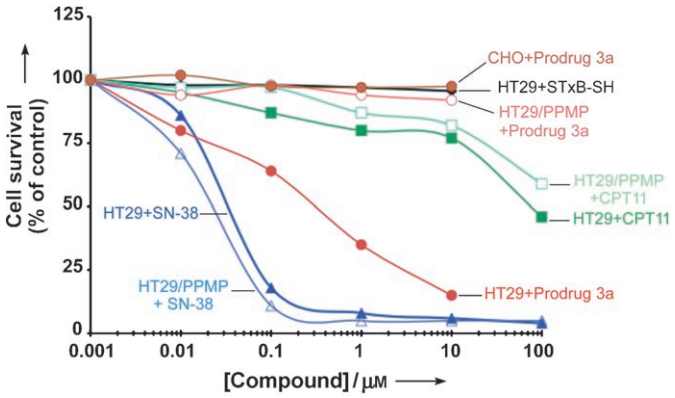



***Figure 4.*** Cytotoxic activity of compounds on HT-29 cells. The indicated compounds were incubated for 6 h at 37 °C with HT-29 (with or without PPMP) or CHO cells. After washing, incubation was continued for 7 days at 37 °C, followed by live-cell counting by using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

icity, two Gb3 negative-control situations were tested: HT-29 cells that were treated with the glycosylceramide synthase inhibitor 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP),[16] or spontaneously Gb3-negative Chinese hamster ovary (CHO) cells. In both cases, the cells were not sensitive at all to incubation with prodrug **3a**, which was used in the same concentration range for all experiments with **3a** (Figure 4). Furthermore, we showed that nonderivatized STxB-SH had no measurable cytotoxicity on Gb3-expressing HT-29 cells under our experimental conditions (Figure 4).

Importantly, neither nonvectorized SN-38 ($IC_{50}$: 30 nM) nor its prodrug CPT-11 used in clinics ($IC_{50}$: 70 μM) had a cytotoxic effect on HT-29 cells that were dependent on Gb3 expression (Figure 4), thus further establishing the selectivity of compound **3a** for Gb3-expressing tumor cells.

In summary, we have identified a novel tumor-delivery approach based on retrograde prodrug targeting to membranes of the biosynthetic/secretory pathway, by using STxB. The disulfide linkage of prodrug **3a** is slowly released, most likely in the endoplasmic reticulum whose function in cellular redox homeostasis is well-recognized.[17] This slow release should sustain the continued presence of the active principle in dividing tumor cells, with prodrug being otherwise rapidly cleared from the circulation. Retrograde delivery will also place the site of drug release close to the nucleus, where the molecular target of hydrophobic SN-38 resides. In the light of high Gb3 expression levels in tumors (more than $10^7$ binding sites per cancer cell[18]) and the preferential retrograde transport in tumor cells, when compared to nontumoral Gb3-expressing cells,[18,19] we expect that retrograde delivery will make a major contribution to improve the selectivity of cancer chemotherapy.

## Experimental Section

The synthesis and characterization of all new compounds and the conditions of the biological experiments are described in the Supporting Information.

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[1] T. M. Allen, *Nat. Rev. Cancer* **2002**, *2*, 750.
[2] B. Druker, *Cancer Cell* **2002**, *1*, 31.
[3] V. Torchilin, *Eur. J. Pharm. Sci.* **2000**, *11*, S81.
[4] M. C. Garnett, *Adv. Drug Delivery Rev.* **2001**, *53*, 171.
[5] C. Monneret, J. C. Florent, *Bull. Cancer* **2000**, *87*, 829.
[6] I. D. Bernstein, *Leukemia* **2000**, *14*, 474.
[7] A. O'Brien, V. Tesh, A. Donohue-Rolfe, M. Jackson, S. Olsnes, K. Sandvig, A. Lindberg, G. Keusch, *Curr. Top. Microbiol. Immunol.* **1992**, *180*, 65.
[8] K. Sandvig, B. van Deurs, *Gene Ther.* **2005**, *12*, 865.
[9] L. Johannes, D. Decaudin, *Gene Ther.* **2005**, *12*, 1360.
[10] O. Kovbasnjuk, R. Mourtazina, B. Baibakov, T. Wang, C. Elowsky, M. Choti, A. Kane, M. Donowitz, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 19087.
[11] K.-P. Janssen, D. Vignjevic, R. Boisgard, T. Falguières, G. Bousquet, D. Decaudin, F. Dollé, D. Louvard, B. Tavitian, S. Robine, L. Johannes, *Cancer Res.* **2006**, *66*, 7230.
[12] E. Van Cutsem, D. Cunningham, W. W. Ten Bokkel Huinink, C. J. Punt, C. G. Alexopoulos, L. Dirix, M. Symann, G. H. Blijham, P. Cholet, G. Fillet, C. Van Groeningen, J. M. Vannetzel, F. Levi, G. Panagos, C. Unger, J. Wils, C. Cote, C. Blanc, P. Herait, H. Bleiberg, *Eur. J. Cancer* **1999**, *35*, 54.
[13] B. Gatto, G. Capranico, M. Palumbo, *Curr. Pharm. Des.* **1999**, *5*, 195.
[14] L. Johannes, B. Goud, W. H. Fridman, E. Tartour, Patent WO 02/060937, **2002**.
[15] M. Amessou, V. Popoff, B. Yelamos, A. Saint-Pol, L. Johannes, *Current Protocols in Cell Biology* (Eds.: J. Bonifacino et al.), Wiley, Hoboken, **2006**, chap. 15.10.
[16] A. Abe, J. Inokuchi, M. Jimbo, H. Shimeno, A. Nagamatsu, J. A. Shayman, G. S. Shukla, N. S. Radin, *J. Biochem.* **1992**, *111*, 191.
[17] A. Görlach, P. Klappa, T. Kietzmann, *Antioxid. Redox Signaling* **2006**, *8*, 1391.
[18] T. Falguières, F. Mallard, C. L. Baron, D. Hanau, C. Lingwood, B. Goud, J. Salamero, L. Johannes, *Mol. Biol. Cell* **2001**, *12*, 2453.
[19] M. Warnier, W. Römer, J. Geelen, J. Lesieur, M. Amessou, L. van den Heuvel, L. A. Monnens, L. Johannes, *Kidney Int.* **2006**, *70*, 2085.

  