Design and Optimization of 20-O-Linked Camptothecin Glycoconjugates as Anticancer Agents

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To improve the biological profile of 20(S)-camptothecin, a novel class of 20-O-linked camptothecin glycoconjugates has been designed for preferential cellular uptake into tumor cells by an active transport mechanism. Such conjugates have been optimized for enhanced solubility, stabilization of the camptothecin lactone ring, sufficient hydrolytic and proteolytic stability, and for an overall improvement in tumor selectivity. The constitution of the peptide spacer has a major impact on stability and biological activity of the conjugates both in vitro and in vivo. Glycoconjugates **17**−**22** with valine residues at the linkage position to camptothecin are sufficiently stable and show good antitumor activity in vitro against HT29 and other tumor cell lines. Fluorescence microscopy and flow cytometry experiments indicate that glycoconjugates such as **19** are taken up into lysosomal compartments of the tumor cell line HT29 by an active transport mechanism. The steric configuration of the particular amino acid residues linked to the camptothecin moiety has a major impact on the in vivo activity of the corresponding glycoconjugates in the breast cancer xenograft MX-1 model. Inhibiting tumor growth by >96%, the glycoconjugates **19** and **21** show the best activity in this particular model and have been investigated more extensively. The glycoconjugate **19** compares favorably to topotecan **4** and glycoconjugate **21** with respect to toxicity against hematopoietic stem cells and hepatocytes. Based on its profile, **19** has been selected for clinical trials.

Introduction

Camptothecin **1** is a pentacyclic alkaloid isolated by Wall et al. in the early 1960s from the Chinese tree *Camptotheca acuminata*.[1] It showed impressive activity against leukemias and a variety of solid tumors. Clinical trials were begun in 1970 with the more soluble sodium salt **2**. Despite some early evidence for activity, toxicities such as myelosuppression, vomiting, and diarrhea were severe, and clinical trials were suspended.[2] Nevertheless, due to its unique activity camptothecin still represents an important lead structure for extensive research programs directed towards novel anticancer compounds.

Important milestones in this field have been the elucidation of the mechanism of action in the mid 1980s and the approval and clinical success of the first representatives of the camptothecin class of compounds, irinotecan (**3**) (CPT-11; Yakult Honsha)[3] and topotecan (**4**) (SmithKline Beecham),[4] another decade later.

The camptothecins act by binding to the topoisomerase I-DNA complex, leading to an accumulation of DNA strand breaks upon replication, ultimately causing cell death.[5] The closed lactone E-ring is essential for activity based on the established mechanism of action.[6] A major issue of the camptothecin class of compounds is the opening of the lactone E-ring and the formation of an equilibrium between the ring closed lactone form and the open carboxylate form (Scheme 1) which is pH- and also species-dependent. Particularly, human serum albumin preferentially binds to the carboxylate form of camptothecin derivatives, shifting the equilibrium in favor of the carboxylate.[7] The lactone form is more stable in mouse serum, leading to the marked efficacy of these compounds in nude mouse xenografts,[8] which, however, might not be predictive for the same level of clinical activity for these compounds.

A number of research programs focused on improved analogues are ongoing. So far, several camptothecin derivatives such as 9-nitrocamptothecin (RFS-2000), exatecan (DX-8951), lurtotecan (NX-211), BNP-1350, diflomotecan (BN80915), and CKD-602 have entered clinical trials.[9]

Furthermore, a number of prodrugs and delivery systems have been developed. 20-O-Alkyl esters of camptothecin[10] and simple amino acid prodrugs[11] have been prepared to increase lactone ring stability and water solubility of camptothecins. Polymeric delivery systems such as poly(ethylene glycol) (PEG) conjugates[12] and N-2-hydroxypropylmethacrylamide polymer (HPMA) conjugates[13] have been developed to improve the pharmacokinetic profile of camptothecins.

In previous studies we optimized modified fucoside residues for preferential receptor-mediated uptake into tumor cells compared to liver cells utilizing neoglycoconjugates of bovine serum albumin (BSA).[14] In these model systems it has been shown that a chemical modification of fucoside residues at the 3-hydroxy group

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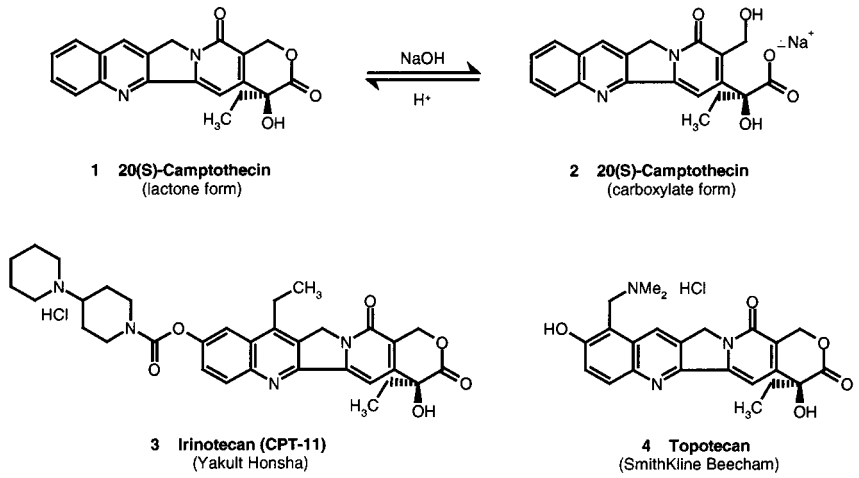
Scheme 1. Structures of 20(S)-Camptothecin, Irinotecan, and Topotecan



1 20(S)-Camptothecin (lactone form)

2 20(S)-Camptothecin (carboxylate form)

3 Irinotecan (CPT-11) (Yakult Honsha)

4 Topotecan (SmithKline Beecham)

led to a decreased uptake of the respective neoglycoconjugates of BSA into liver cells. In contrast, the efficient uptake of such BSA neoglycoconjugates by SW480 tumor cells was not negatively affected by modifications of the 3-hydroxy group. As preferred carbohydrate residues mediating a differential uptake into tumor cells vs liver cells 3-O-methyl-fucoside and 3-O-carboxymethyl-fucoside residues have been identified.[14] Our next goal was to improve the tumor selectivity and solubility of camptothecin by the synthesis of glycoconjugates designed for preferential cellular uptake into tumor cells by an active transport mechanism. Therefore, previously optimized fucoside derivatives such as p-aminophenyl 3-O-methyl-β-L-fucosides have been attached to the 20-hydroxy group of the camptothecin molecule via ester linked dipeptide spacers to obtain a novel class of low molecular weight glycoconjugates with improved water solubility and with increased stability of the lactone ring. With respect to the overall profile of such low molecular weight camptothecin glycoconjugates the optimization of the spacer group turned out to be of particular importance. Cellular uptake is not solely triggered by the carbohydrate residue, and, furthermore, the spacer group has a major impact on the cleavability of the conjugates which directly contributes to the cytotoxic activity. Such glycoconjugates should be sufficiently stable in culture medium and in serum and at the same time unfold their cytotoxic activity preferably by release of the toxophore inside the target cell after cellular uptake.

We have reported previously that a bulky side chain of the amino acid linked to camptothecin via an ester bond is crucial for hydrolytic stability of such 20-O-linked glycoconjugates.[15]

Here we describe the optimization of those camptothecin glycoconjugates according to criteria such as efficacy, stability, solubility, and reduced toxicity against hematopoietic stem cells and hepatocytes. The lead compound **19** (BAY 38-3441) has been selected for clinical trials. Investigations toward cellular uptake of **19** will also be described.

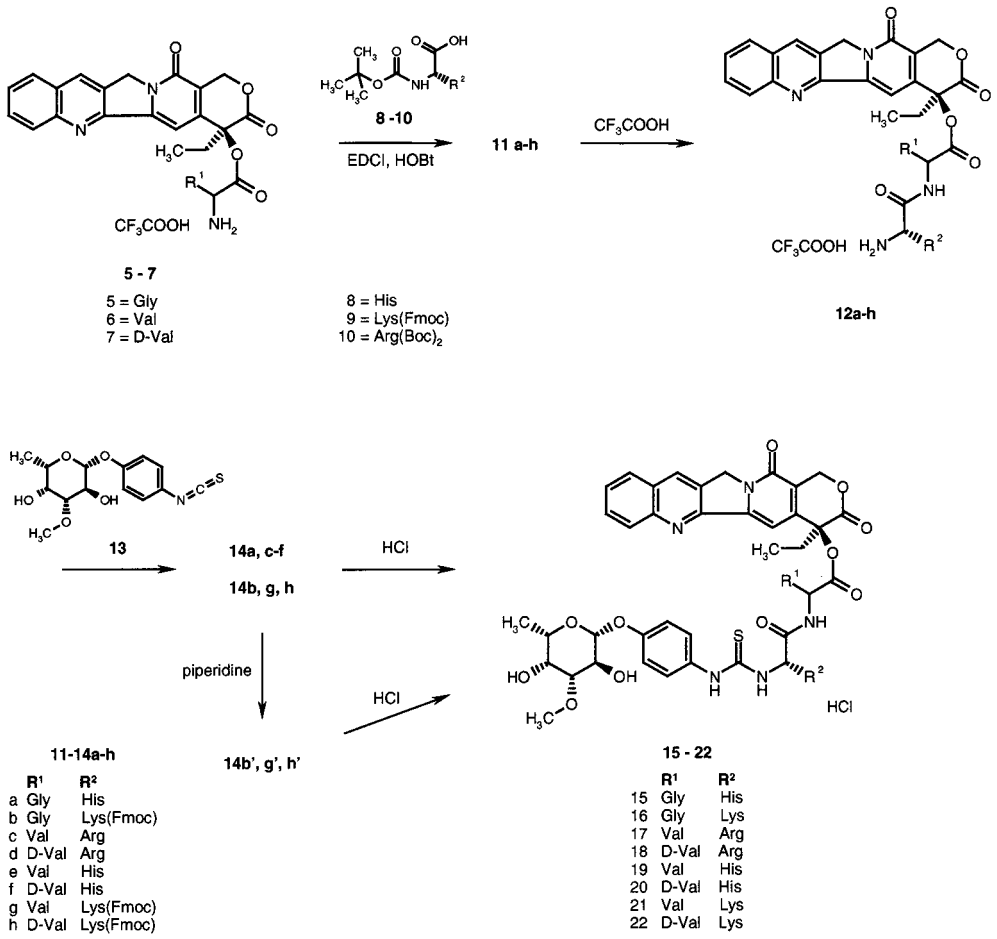
Results and Discussion

Chemistry. For the challenging acylation of the tertiary and unreactive 20-hydroxy group, particularly with bulky amino acid residues, we have developed an efficient method utilizing urethane-protected N-carboxy anhydrides[16] (UNCAs). The camptothecin amino acid conjugates **5**−**7** have been obtained by acylation of the 20-hydroxy group with UNCAs and subsequent deprotection of the terminal amino group as described previously.[15] Attachment of appropriately protected residues of basic amino acids **8**−**10** in the presence of *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI) and hydroxybenzotriazole (HOBt) yields the protected dipeptide conjugates **11a**−**h** (Scheme 2). In the case of the attachment of the histidine moiety **8**, no protection of the side chain functionality is required, whereas in the cases of lysine **9** fluorenyl-9-methoxycarbonyl (Fmoc) and arginine **10** bis-*tert*-butoxycarbonyl (Boc), protection of the side chain is required. The *tert*-butoxycarbonyl (Boc)-protecting group at the amino terminus is removed with trifluoroacetic acid, affording the dipeptide conjugates **12b, g, h** carrying lysine with a Fmoc-protected side chain at the N-terminus and the fully deprotected dipeptide conjugates carrying free histidine (**12a, e, f**) and arginine residues (**12c, d**) at the N-terminus, respectively.

The carbohydrate residues should be attached to the α-amino terminus of the dipeptide conjugates to enhance proteolytic stability of the glycoconjugates, whereas the basic side chains should contribute to water solubility. p-Aminophenyl 3-O-methyl-β-L-fucopyranoside was one of the preferred carbohydrate residues with promise for tumor targeting.[14] It is transformed into the isothiocyanate **13** ready for linkage to the dipeptide conjugates **12a**−**h**. The coupling reactions proceed smoothly in the presence of *N*-ethyl diisopropylamine. It is worth mentioning that in the case of dipeptide conjugates **12a** and **12c**−**f** with terminal arginine and histidine residues, respectively, the coupling reactions proceed with high regioselectivity at the α-amino group to yield the glycoconjugates **14a** and **14c**−**f** without any side chain protection required. In the case of the partially protected lysine-containing glycoconjugates **14b, g, h**, after the carbohydrate attachment the Fmoc group is removed with piperidine in dimethyl formamide. Finally, the glycoconjugates are treated with 1 equiv of 0.1 N aqueous hydrochloric acid to provide the water soluble glycoconjugate hydrochlorides **15**−**22**.

Solubility, Stability, and Antitumoral Activity of Camptothecin Glycoconjugates 15−**22**. As hy-

Scheme 2. Synthesis of Glycoconjugates of 20(S)-Camptothecin



drochlorides, the exemplified glycoconjugates **15**−**22** exhibit good water solubility [∼5 mg/mL at pH 4−5]. Furthermore, due to the α-acylation the lactone ring of the camptothecin moiety is markedly stabilized in these glycoconjugates. Even under drastic conditions such as addition of 2 equiv of aqueous sodium hydroxide, which leads to a quantitative ring opening in the case of topotecan **4**, the glycoconjugates **19** and **21** remain intact with less than 2% lactone ring opening.

According to our design criteria, camptothecin glycoconjugates should have reasonable hydrolytic and proteolytic stability in the culture medium of tumor cell lines. In this case the observed cytotoxic activity should mainly be due to a cellular uptake of glycoconjugates rather than an efficient release of the toxophore outside the cell. Therefore, in parallel to measuring cytotoxic activity against tumor cell lines (Table 1, column 5−7) using an MTT growth inhibition assay, the stability of the conjugates in the culture medium of HT29 tumor cells was assessed by HPLC analysis (Table 1, column 4).

The side chain of the amino acid residue linked to camptothecin has a significant impact on both stability and cytotoxic activity of the glycoconjugates. Glycoconjugates **15** and **16** with a glycine residue linked to camptothecin are readily cleaved in the culture medium with an almost quantitative camptothecin release within 24 h (Table 1, column 4). Therefore, the cytotoxic activity observed with the conjugates **15** and **16** is most probably due to the amount of camptothecin cleaved in the

Table 1. Stability and in Vitro Activity of Camptothecin Glycoconjugates

			stability in culture medium of HT29 after 24 h % CPT/	activity against cancer cell lines [IC_{50}/nM]		
compd	R^1	R^2	% conjugate	HT29	SW480	B16F10
15	Gly	His	95/0	10	15	30
16	Gly	Lys	68/23	10	30	50
17	Val	Arg	6/88	100	170	400
18	D-Val	Arg	6/89	100	110	400
19	Val	His	7/84	60	100	250
20	D-Val	His	5/86	160	230	600
21	Val	Lys	3/91	150	170	700
22	D-Val	Lys	4/86	120	150	600

culture medium. The cytotoxic activity of camptothecin **1** in these assays is in the same range (IC_{50} values are 5, 10, and 20 nM against HT29, SW480, and B16F10, respectively). Topotecan **4** in these systems displayed cytotoxic activity with IC_{50}s of 15, 20, and 150 nM.

In contrast, a bulky side chain R^1 of the amino acid residue linked to the hydroxy group of camptothecin significantly increases the stability of respective glycoconjugates in the supernatant of HT29 tumor cells (Table 1, column 4, conjugates **17**−**22**). At the same time these conjugates exhibit good antitumoral activity in vitro against HT29 and other tumor cell lines, which cannot be fully explained by the amount of camptothecin released extracellularly (Table 1, column 5−7). In a cellular assay the addition of 10 mol% of free camptothecin to glycoconjugate **19** does not significantly alter its cytotoxic activity against HT29 in vitro (Figure 1).

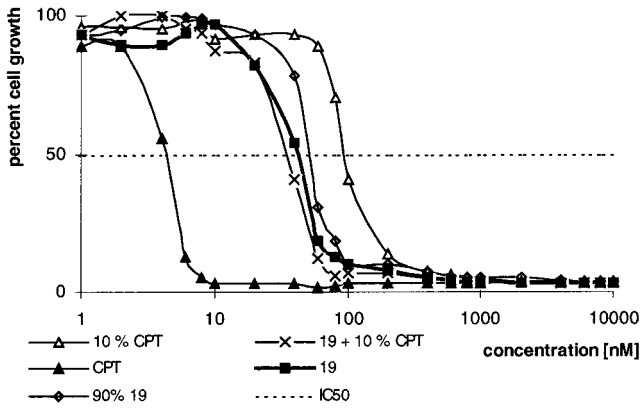


Figure 1. Effect of the addition of 10% camptothecin on the cytotoxic activity of glycoconjugate **19** against HT29 cell cultures.

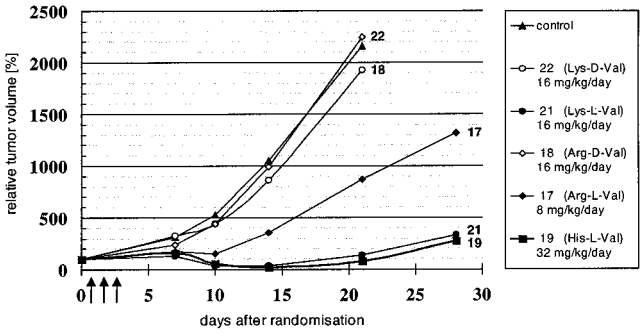


Figure 2. In vivo activity of glycoconjugates in the MX-1 breast cancer model with daily i.v. administration for 3 days at their MTD.

The steric configuration of the valine residue and the particular constitution of the side chain of the basic amino acid residue R^2 only have a minor impact on stability and cytotoxic activity against tumor cell lines in vitro.

Glycoconjugates with sufficient stability have been investigated in vivo against human tumor xenografts, such as the breast cancer MX-1, grown subcutaneously in mice. Glycoconjugates have been administered intravenously for the first 3 days after randomization at dose ranges between 8 and 32 mg/kg/day. The activities of the compounds at their respective maximum tolerated doses (MTDs) are exemplified in Figure 2.

The antitumor activities of compounds **17**−**22** against tumor cell lines in vitro are in the same range (Table 1). In contrast, the in vivo activities of these compounds in the MX-1 breast cancer model are significantly different. Particularly, a strong dependency on the configuration of the valine residue is observed when comparing the pairs of diastereoisomers **17**/**18** and **21**/**22**. The glycoconjugates **19** and **21** show the highest activity in this model and effected tumor remissions with optimal T/C values of 1.8 and 3.4 at their MTDs of 32 and 16 mg/kg/day, respectively. Topotecan in the same model effected tumor remissions with a T/C of 12.7 at an approximate MTD of 2.5 mg/kg/day with a 3-day therapy schedule. Therefore, further biological evaluations have been focused on the glycoconjugates **19** and **21** in comparison to topotecan.

Evaluation of Hematopoietic Activity of Conjugates 19 and 21. To assess the myelotoxicity of the most active compounds **19** and **21** in comparison to topotecan (**4**), C57BL/6 mice have been treated intraperitoneally daily for 3 days. Subsequently, 4 and 24 h after the last treatment hemopoietic stem cells have been removed from the femur and the ability for colony formation in vitro has been determined for stem cells isolated from the treated groups compared to the control groups. The counts of colony forming units (CFU-C) per femur is shown in Figure 3 for the untreated control group and for the groups treated with equitoxic doses of **19** and **21** and the reference compound **4**. Equitoxic doses have been estimated from a broader panel of pharmacology models.

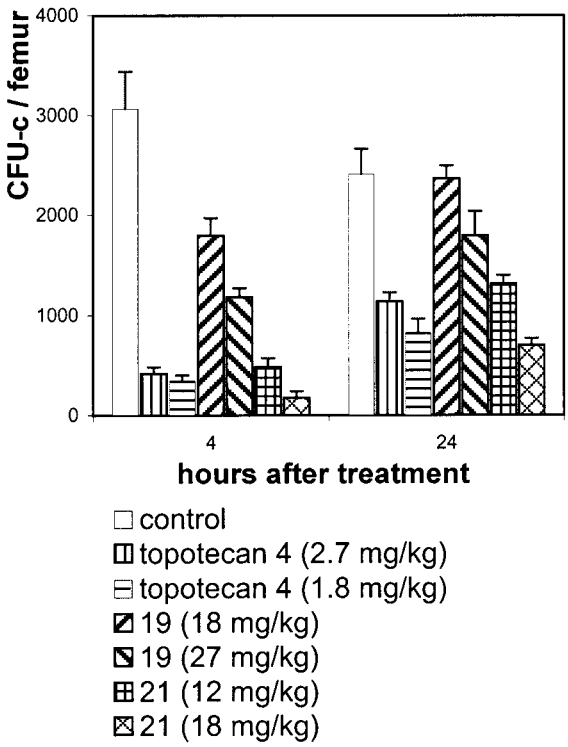


Figure 3. Hemopoietic activity in mice. Measurement of colony forming units counts (CFU-c) of progenitor stem cells per femur, 4 and 24 h after 3 days of treatment at the respective MTDs of topotecan **4**, **19**, and **21**.

The glycoconjugate **19** exhibits the lowest myelotoxicity in this model. Four hours after the third treatment with 18 and 27 mg/kg/day, respectively, the count of colony forming units is only moderately decreased compared to the untreated control group. The effect is reversible, and mice appeared completely recovered 24 h after the third treatment. In contrast, glycoconjugate **21** and topotecan **4** exhibit significantly higher myelotoxicity in this model.

Cytotoxicity on Primary Hepatocytes from Different Species in Vitro. Liver damage might be a possible limiting toxicity for camptothecin derivatives, particularly for glycoconjugates. An in vitro model using primary hepatocytes from rat, dog, and man cultivated in a "sandwich model" was used for the determination of the hepatotoxic potential. As endpoints, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) levels in the cellular supernatant have been determined, and the results obtained with compounds **19**, **21**, and **4** are exemplified in Table 2.

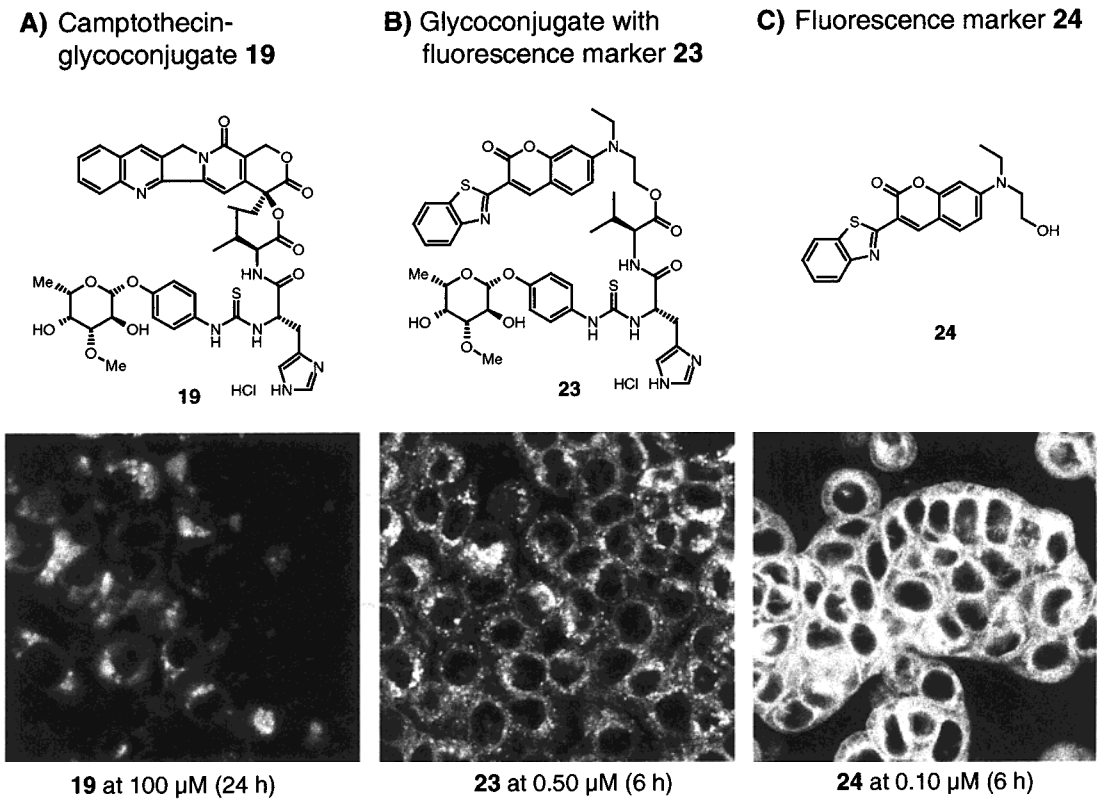


Figure 4. Uptake of glycoconjugates into the cellular compartments of HT29 cells determined by confocal laser scan microscopy.

Table 2. No Effect Concentrations (NOEC) with Respect to Elevated Levels of ALT,[a] AST,[b] and LDH[c] in the Supernatant of Cultivated Hepatocytes

	rat NOEC [μM]			dog NOEC[μM]			human NOEC [μM]		
compd	ALT	AST	LDH	ALT	AST	LDH	ALT	AST	LDH
19	10	10	10	10	10	10	30	30	30
21	30	30	30	10	30	30	30	30	30
4	0.1	0.1	1	1	0.1	1	0.1	1	10

[a] ALT: alanine aminotransferase. [b] AST: aspartate aminotransferase. [c] LDH: lactate dehydrogenase.

The glycoconjugates **19** and **21** exhibit a markedly decreased toxicity in vitro against cultivated hepatocytes compared to topotecan.

Studies on Cellular Uptake of Glycoconjugates. To investigate the mode of cellular uptake of glycoconjugates into the tumor cell line HT29, confocal laser scan fluorescence microscopy and fluorescence activated cell sorting (FACS) technologies have been employed. Due to the inadequate fluorescence of the camptothecin molecule, high concentrations (100 μM) and long incubation times (24 h) with camptothecin glycoconjugates such as **19** are required to effect detectable cellular staining (Figure 4, column A). After 24 h incubation of HT29 cells with 100 μM of glycoconjugate **19**, a cellular staining consisting predominantly of lysosomal compartments is observed.

To verify these results in the concentration range of the IC_{50} values of the glycoconjugate **19**, a model system was employed, where the poor fluorophor camptothecin was substituted by the fluorescence dye **24**, to obtain the highly fluorescent glycoconjugate **23**. The fluorophore **24** was chosen to closely reassemble the molecular wheight, size of the aromatic ring system, and polarity of camptothecin. The glycoconjugate **23** was synthesized in an analogous manner as described for camptothecin conjugates in Scheme 2.

Incubation of HT29 cells for 6 h with the nonconjugated fluorescence marker **24** effects an efficient cellular staining at concentrations as low as 0.1 μM. Confocal laser scan microscopy reveals a diffuse staining of the whole cell, only sparing the nucleus (Figure 4, column C). In contrast, the glycoconjugate **23** displays cellular staining at 0.5 μM, which is predominantly localized in the lysosomal compartments, the same pattern as observed for the camptothecin glycoconjugate **19** at 100 μM.

To further investigate the mode of cellular uptake, FACS studies have been performed with the fluorescence marker **24** and its respective glycoconjugate **23** at 4 °C and at 37 °C (Figure 5). As indicated by cellular staining and in consistency with an unspecific, passive membrane penetration, the cellular uptake of the fluorescence marker **24** does not show any temperature dependency. In contrast, the cellular uptake of the glycoconjugate **23** is markedly higher at 37 °C compared to 4 °C, which is almost at control level. This temperature dependency of cellular staining indicates an active transport of **23** into the cell. Taken together, these results support a cellular uptake of the camptothecin glycoconjugate **19** and its fluorescent analogue **23** into lysosomal compartments of HT29 tumor cells by an active transport mechanism.

Conclusions

To improve the biological profile of the potent anticancer compound camptothecin, glycoconjugates have been synthesized. A 3-O-methyl-fucoside residue, which has previously been shown to mediate a preferential uptake of neoglycoconjugates of bovine serum albumin

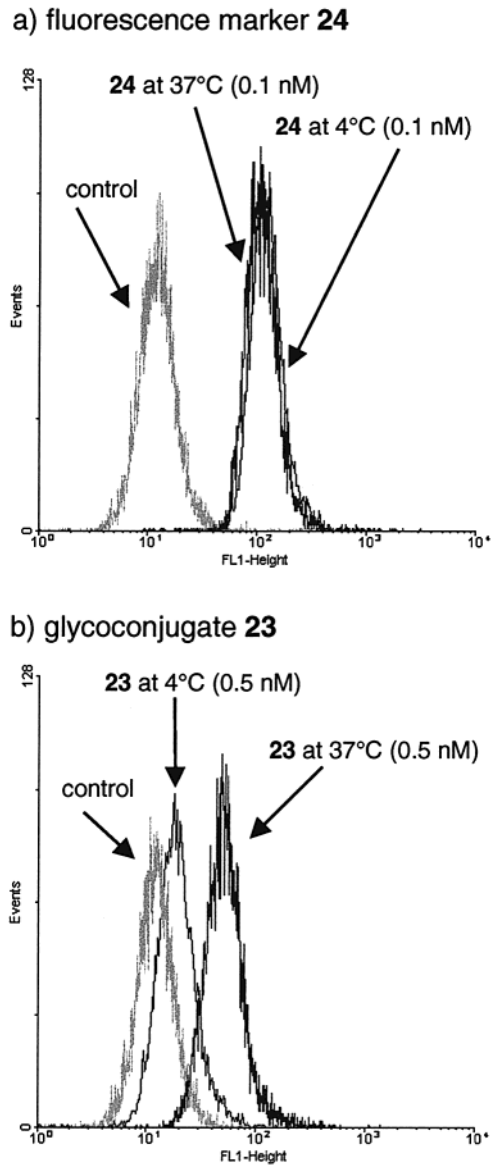


Figure 5. Temperature dependency of cellular uptake determined by FACS studies.

into SW480 tumor cells vs liver cells,[14] has been attached to the 20-hydroxy group of camptothecin via dipeptide spacers.

Such 20-O-linked camptothecin glycoconjugates have been optimized for enhanced stability of the lactone ring and for sufficient hydrolytic and proteolytic stability. The constitution of the peptide spacer has a major impact on stability and biological activity of the conjugates both in vitro and in vivo. A bulky side chain R^2 of the amino acid residue linked to the hydroxy group of camptothecin significantly increases the stability in the supernatant of HT29 tumor cells. Glycoconjugates **17**−**22**, with valine residues at the linkage position to camptothecin, are sufficiently stable and show good antitumor activity in vitro against HT29 and other tumor cell lines, which cannot be fully explained by the extracellular camptothecin release. The configuration of this particular α-amino acid residue linked to the camptothecin moiety does not have a significant impact on stability and cytotoxic activity in vitro. In contrast, its stereochemistry has a major impact on the in vivo activity of the corresponding glycoconjugates in the breast cancer xenograft MX-1 model. The glycoconjugates **19** and **21** showed the best activity in this particular model and have been investigated more extensively.

The glycoconjugate **19** shows an excellent activity in vivo against the MX-1 xenograft, inhibiting tumor growth by >96%. This activity has been confirmed in a variety of tumor models.[17] Furthermore, it shows a favorable profile compared to topotecan **4** and **21** with respect to toxicity against hematopoietic stem cells and hepatocytes. Fluorescence microscopic and flow cytometry experiments suggest that it is taken up into lysosomal compartments of the tumor cell line HT29 by an active transport mechanism. From this novel class of anticancer agents, the glycoconjugate **19** (BAY 38-3441) has been selected for clinical development.

Experimental Section

General Methods. Melting points were determined in open capillaries on a Büchi melting point apparatus and are uncorrected. Flash chromatography was carried out on *E. Merck* silica gel, 230−400 mesh. TLC analysis was carried out on *E. Merck* silica gel plates $60F_{254}$. NMR spectra were recorded at 400 MHz with equipment from *Bruker*. Chemical shifts and coupling constants are given in ppm and Hz, respectively. FAB-MS was performed on a *Finnigan* MAT 900 double focusing sector instrument. HPLC analysis was performed on a *Waters* Alliance 2690 instrument with UV detection at 257 nM or 356 nM; column: *E. Merck* LiChrospher 100, RP-18 (5 μm) 250 × 4 mm.

Chemistry. Amino acid derivatives **8**−**10** have been purchased from Bachem. Amino acid conjugates of camptothecin **5**−**7** and p-isocyanatophenyl-3-O-methyl-β-L-fucopyranoside **13** were synthesized as described earlier.[14,15]

Preparation of Dipeptide Conjugates of 20(S)-Camptothecin 12a−h with Either Free or Fmoc-Protected Side Chain Functionality (General Procedure). N^{α}-(*tert*-Butoxycarbonyl)-protected amino acid derivatives **8**−**10** (0.047 mol) are dissolved in 800 mL of anhydrous DMF and cooled to 0 °C. 1-Hydroxybenzotriazole (HOBt) (0.07 mol) and 0.056 mol of *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) are added, and the mixture is stirred for 30 min at 0 °C. Subsequently, 0.039 mol of the trifluoroacetate of the respective 20-hydroxy-linked amino acid conjugate of camptothecin **5**−**7** and finally 24.3 mL *N*-ethyl diisopropylamine are added. The mixture is stirred for 16 h at ambient temperature. The solvent is evaporated at 25 °C. After addition of 1.5 L of water the residue is stirred for 15 min and solidifies. The precipitate is filtered and washed with water. It is dissolved in 800 mL of dichloromethane, and the remaining water is removed in a separation funnel. Alternatively, 1.5 L of dichloromethane is added to the aqueous suspension, and the product mixture is distributed between the two layers. The organic layer is concentrated to 150 mL. This solution is dropped to 2 L of diethyl ether at 0 °C with stirring. The precipitate is filtered, washed with diethyl ether, and dried in vacuo. If required, an additional precipitation from dichloromethane/methanol with diethyl ether or purification by flash chromatography using acetonitrile as eluent is also possible.

The resulting protected intermediates from series **11 a**−**h** are dissolved in 600 mL of dichloromethane, and the solution is cooled to 5 °C. Two hundred milliliters of trifluoroacetic acid is added during stirring. The reaction mixture is allowed to warm to ambient temperature and is stirred for additional 2 h. The solvent and trifluoroacetic acid are evaporated in vacuo at 25 °C bath temperature. Diethyl ether is added to the residue, and the mixture is stirred for 10 min. The precipitate is filtered, washed with diethyl ether, and dried in vacuo overnight.

20(S)-20-O-[Histidylglycyl]camptothecin Bistrifluoracetate (12a). The activated histidine derivative Boc-His(Boc)-

OSu was utilized in the coupling reaction instead of EDCI/HOBT. Yield: 68% (over two steps); ^{1}H NMR (400 MHz, CD_3OD/CD_2Cl_2) δ 4.18, 4.45 (2 *d*, J = 18 Hz, 2H, CH_2 Gly), 4.19 (*t*, J = 7 Hz, 1H, α-CH His) 7.22 (*s*, 1H, imidazole) 7.40 (*s*, 1H, CPT D-ring), 7.74(*dd*, J = 7 Hz, 1H, CPT A-ring), 7.90 (*dd*, J = 7 Hz, 1H, CPT A-ring), 8.08 (*d*, J = 8 Hz, 1H, CPT A-ring),), 8.18 (*d*, J = 8 Hz, 1H, CPT A-ring), 8.4 (*s*, 1H, imidazole), 8.6 (*s*, 1H, CPT B-ring).

20(S)-20-O-[N$^\epsilon$-(Fluorenyl-9-methoxycarbonyl)lysyl-glycyl]camptothecin Trifluoracetate (12b). Boc-Lys-(Fmoc)-OH was used as starting material. Yield: 73% (over two steps); TLC: R_f = 0.56 (acetonitrile/water/glacial acetic acid 5/1/0.2).

20(S)-20-O-[Arginylvalyl]camptothecin Bistrifluoracetate (12c). Boc-Arg(Boc)$_2$-OH was used as starting material. The product of the coupling reaction was purified by flash chromatography using ethyl acetate as eluent. Yield: 56% (over two steps). TLC: R_f = 0.22 (acetonitrile/water/glacial acetic acid 10/2.5/1.2).

20(S)-20-O-[Arginyl-D-valyl]camptothecin Bistrifluoracetate (12d). Boc-Arg(Boc)$_2$-OH was used as starting material. The product of the coupling reaction was purified by flash chromatography using ethyl acetate as eluent. Yield: 56% (over two steps); TLC: R_f = 0.1 (acetonitrile/water/glacial acetic acid 5/1/0.2); ^{1}H NMR (400 MHz, CD_3OD/CD_2Cl_2) δ 3.18 (*t*, J = 7 Hz, 2H, N−CH_2 Arg), 3.88 (*t*, J = 6 Hz, 1H, α-CH Arg), 4.72 (*d*, J = 5 Hz, 1H, α-CH Val), 7.41 (*s*, 1H, CPT D-ring), 7.73 (*dd*, J = 7 Hz, 1H, CPT A-ring), 7.89 (*dd*, J = 7 Hz, 1H, CPT A-ring), 8.06 (*d*, J = 8 Hz, 1H, CPT A-ring), 8.2 (*d*, J = 8 Hz, 1H, CPT A-ring), 8.6 (*s*, 1H, CPT B-ring).

20(S)-20-O-[Histidylvalyl]camptothecin Bistrifluoracetate (12e). Boc-His-OH was used as starting material. Yield: 86% (over two steps); TLC: R_f = 0.2 (acetonitrile/water/glacial acetic acid 5/1/0.2); ^{1}H NMR (400 MHz, CD_3OD/CD_2Cl_2) δ 3.28 and 3.42 (2*dd*, J = 7 Hz, 15 Hz; 2H, N−CH_2 His), 4.68 (*d*, J = 5 Hz, 1H, α-CH Val), 7.20 (*s*, 1H, imidazole), 7.32 (*s*, 1H, CPT D-ring), 7.72 (*dd*, J = 7 Hz, 1H, CPT A-ring), 7.86 (*dd*, J = 7 Hz, 1H, CPT A-ring), 8.04 (*d*, J = 8 Hz, 1H, CPT A-ring), 8.16 (*d*, J = 8 Hz, 1H, CPT A-ring), 8.45 (*s*, 1H, imidazole), 8.56 (*s*, 1H, CPT B-ring).

20(S)-20-O-[Histidyl-D-valyl]camptothecin Bistrifluoracetate (12f). Boc-His-OH was used as starting material. The product of the coupling reaction was purified by flash chromatography using acetonitrile/water 20/1 as eluent. Yield: 79% (over two steps); TLC: R_f = 0.18 (acetonitrile/water/glacial acetic acid 5/1/0.2).

20(S)-20-O-[N$^\epsilon$-(Fluorenyl-9-methoxycarbonyl)lysyl-valyl]camptothecin Trifluoracetate (12 g). Boc-Lys(Fmoc)-OH was used as starting material. Yield: 86% (over two steps); TLC: R_f = 0.54 (acetonitrile/water/glacial acetic acid 5/1/0.2); ^{1}H NMR (400 MHz, CD_3OD/CD_2Cl_2 1:1) δ 3.13 (*t*, J = 5 Hz, 2H, N−CH_2 Lys), 4.15 (*t*, J = 7 Hz, 1H, CH Fmoc), 4.81 (*d*, J = 7 Hz, 2H, CH_2 Fmoc), 7.70 (*dd*, J = 7 Hz, 1H, CPT A-ring), 7.86 (*dd*, J = 7 Hz, 1H, CPT A-ring), 8.02 (*d*, J = 8 Hz, 1H, CPT A-ring), 8.18 (*d*, J = 8 Hz, 1H, CPT A-ring), 8.51 (*s*, 1H, CPT B-ring).

20(S)-20-O-[N$^\epsilon$-(Fluorenyl-9-methoxycarbonyl)lysyl-D-valyl]camptothecin Trifluoracetate (12h). Boc-Lys(Fmoc)-OH was used as starting material. Yield: 55% (over two steps); TLC: R_f = 0.60 (acetonitrile/water/glacial acetic acid 5/1/0.2).

p-Isothiocyanatophenyl 3-O-Methyl-β-L-fucopyranoside (13). p-Aminophenyl 3-O-methyl-β-L-fucopyranoside (9.43 g, 0.035 mol) is dissolved in 0.9 L of dioxane/water 1:1 (v/v). Thiophosgene (3.7 mL, 0.048 mol) is added, and the mixture is stirred for 10 min at ambient temperature. *N*-Ethyl diisopropylamine (35 mL, 0.2 mol) is added, and the mixture is stirred for an additional 15 min. Dioxane is evaporated in vacuo at a bath temperature of maximum 25 °C. The aqueous layer is extracted twice with 200 mL of dichloromethane. The combined dichloromethane layers are washed twice with 200 mL of water, dried upon sodium sulfate, and evaporated. The residue is stirred with 200 mL of diethyl ether. After 10 min 200 mL of petrolether is added, and the mixture is stirred for additional 10 min. The precipitate is filtered, washed with petrolether, and dried in vacuo. 8.9 g (82%) are obtained; TLC (acetonitrile/water 10:1): R_f = 0.7.

Preparation of Glycoconjugate Hydrochlorides of 20(S)-Camptothecin 14a−h (General Procedure). Camptothecin dipeptide conjugates **12a−h** (0.027 mol) are dissolved in 1 L of anhydrous DMF and cooled to 0 °C. *N*-Ethyl diisopropylamine (18.9 mL, 0.11 mol) and 9.4 g (0.03 mol) of p-isothiocyanato-phenyl 3-O-methyl-β-L-fucopyranoside **13** are added. After stirring for 2 h the reaction mixture is allowed to warm to room temperature and stirring is continued for 16 h. After evaporating the solvent in vacuo at 25 °C, 1.5 L of water is added to the residue, and the mixture is stirred for 30 min. During this step the product solidifies. The precipitate is filtered, washed with water, and dried in vacuo. If required, the crude product is purified by flash chromatography (acetonitrile/water 30:1). The corresponding fractions are collected, concentrated, and dissolved in 300 mL of dichloromethane. Methanol is added dropwise until complete solution occurs. This solution is added to 1.5 L of diethyl ether and stirred for 30 min. The glycoconjugates **14a−h** precipitate and are filtered, washed with diethyl ether, and dried in vacuo. The fully deprotected glycoconjugates **14a** and **14c−f** are ready for the transformation into corresponding hydrochlorides. From **14b, g, h** prior to this the Fmoc group is removed.

For removal of the Fmoc-group, 0.021 mol of the Fmoc-protected intermediates **14b, g, h** is dissolved in 700 mL of DMF, the solution is cooled to 0 °C, and 36 mL of piperidine is added. The reaction mixture is allowed to warm to room temperature, and stirring is continued for 1 h. The solvent is evaporated, and the residue is dissolved in 200 mL of dichloromethane and precipitated with 600 mL of diethyl ether. The precipitate is collected by filtration, dissolved in 400 mL of methanol, and stirred for 1 h. Four hundred milliliters of diethyl ether is added, and stirring is continued for 30 min. The precipitating glycoconjugates **14b′, g′, h′** are filtered, washed with diethyl ether, and then dried in vacuo.

Subsequently, 0.016 mol of the fully deprotected conjugates **14a**, **14c−f,** and **14b′, g′, h′** are suspended in 1.4 L of distilled water. One equiv of a 0.1 M aqueous hydrochloric acid is added in small portions. The mixture is ultrasonified until complete solution. It is lyophilized, and the glycoconjugate hydrochlorides are obtained as white to yellowish solids.

20(S)-20-O-{N$^\alpha$-[4-(3-O-Methyl-β-L-fucopyranosyloxy)-phenylaminothiocarbonyl]histidylglycyl}camptothecin Hydrochloride (15). Yield: 77% (over two steps); TLC: R_f = 0.33 (acetonitrile/water/glacial acetic acid 5/1/0.2); ^{1}H NMR (400 MHz, CD_3OD/CD_2Cl_2) δ 1.03 (*t*, J = 7 Hz, 3H, CH_3 CPT), 1.31 (*d*, J = 6 Hz, 3H, CH_3 Fuc), 3.1 (*dd*, J = 6 Hz, J = 15 Hz, 1H, N−CH_2 His), 3.23 (*dd*, J = 3 Hz, J = 10 Hz, 1H, 3-CH Fuc), 3.4 (*dd*, J = 6 Hz, J = 15 Hz, 1H, N−CH_2 His), 3.5 (*s*, 3H, OCH_3 Fuc), 3.72 (dq, J = 1 Hz, J = 5 Hz, 1H, 5-CH Fuc), 3.80 (dd, J = 9 Hz, J = 8 Hz, 1H, 2-CH Fuc), 3.9 (d, J = 3 Hz, 1H, 4-CH Fuc), 4.82 (*d*, J = 8 Hz, 1H, 1-CH Fuc), 7.0 (*d*, J = 9 Hz, 2H, oxy-phenylamino-), 7.2 (*s*, 1H, imidazole), 7.18 (*d*, J = 9 Hz, 2H, oxy-phenylamino-), 7.41 (*s*, 1H, CPT D-ring), 7.73 (*dd*, J = 7 Hz, 1H, CPT A-ring), 7.89 (*dd*, J = 7 Hz, 1H, CPT A-ring), 8.05 (*d*, J = 8 Hz, 1H, CPT A-ring), 8.18 (*d*, J = 8 Hz, 1H, CPT A-ring), 8.54 (*s*, 1H, CPT B-ring), 8.6 (*s*, 1H, imidazole); MS (FAB) *m/e* 854 (M + H)$^+$. Anal. ($C_{42}H_{43}N_7O_{11}S \times HCl \times 4H_2O$) C, H, N, Cl.

20(S)-20-O-{N$^\alpha$-[4-(3-O-Methyl-β-L-fucopyranosyloxy)-phenylaminothiocarbonyl]lysylglycyl}camptothecin Hydrochloride (16). Yield: 67% (over three steps); TLC: R_f = 0.25 (acetonitrile/water/glacial acetic acid 5/1/0.2); ^{1}H NMR (400 MHz, CD_3OD/CD_2Cl_2) δ 1.01 (*t*, J = 7 Hz, 3H, CH_3 CPT), 1.31 (*d*, J = 6 Hz, 3H, CH_3 Fuc), 2.86 (*t*, J = 7 Hz, 2H, N−CH_2 Lys), 3.23 (*dd*, J = 3 Hz, J = 9 Hz, 1H, 3-CH Fuc), 3.5 (*s*, 3H, OCH_3 Fuc), 3.73 (dq, J = 1 Hz, J = 5 Hz, 1H, 5-CH Fuc), 3.8 (dd, J = 9 Hz, J = 8 Hz, 1H, 2-CH Fuc),), 3.9 (d, J = 3 Hz, 1H, 4-CH Fuc), 4.82 (*d*, J = 8 Hz, 1H, 1-CH Fuc), 7.02 (*d*, J = 9 Hz, 2H, oxy-phenylamino-), 7.23 (*d*, J = 9 Hz, 2H, oxy-phenylamino-), 7.41 (*s*, 1H, CPT D-ring), 7.72 (*dd*, J = 7 Hz, 1H, CPT A-ring), 7.89 (*dd*, J = 7 Hz, 1H, CPT A-ring), 8.06 (*d*, J = 8 Hz, 1H, CPT A-ring), 8.2 (*d*, J = 8 Hz, 1H, CPT

A-ring), 8.6 (*s*, 1H, CPT B-ring); MS (ESI) *m/e* 845 $(M + H)^+$. Anal. ($C_{42}H_{48}N_6O_{11}S \times HCl \times 4H_2O$) C, H, N, Cl.

20(S)-20-O-{N$^\alpha$-[4-(3-O-Methyl-β-L-fucopyranosyloxy)-phenylaminothiocarbonyl]arginylvalyl}camptothecin Hydrochloride (17). Yield: 83%; TLC: $R_f = 0.3$ (acetonitrile/water/glacial acetic acid 5/1/0.2); ^{1}H NMR (400 MHz; CD_3OD/CD_2Cl_2) δ 0.95 (*t*, $J = 7$ Hz, 3H, CH_3 CPT), 1.32 (*d*, $J = 6$ Hz, 3H, CH_3 Fuc), 3.23 (*dd*, $J = 3$ Hz, $J = 10$ Hz, 1H, 3-CH Fuc), 3.51 (*s*, 3H, OCH_3 Fuc), 3.74 (dq, $J = 1$ Hz, $J = 5$ Hz, 1H, 5-CH Fuc), 3.81 (dd, $J = 9$ Hz, $J = 8$ Hz, 1H, 2-CH Fuc), 3.9 (d, $J = 3$ Hz, 1H, 4-CH Fuc), 4.83 (*d*, $J = 8$ Hz, 1H, 1-CH Fuc), 7.4 (*d*, $J = 9$ Hz, 2H, oxy-phenylamino-), 7.26 (*d*, $J = 9$ Hz, 2H, oxy-phenylamino-), 7.37 (*s*, 1H, CPT D-ring), 7.72 (*dd*, $J = 7$ Hz, 1H, CPT A-ring), 7.86 (*dd*, $J = 7$ Hz, 1H, CPT A-ring), 8.06 (*d*, $J = 8$ Hz, 1H, CPT A-ring), 8.18 (*d*, $J = 8$ Hz, 1H, CPT A-ring), 8.56 (s, 1H, CPT B-ring); MS (FAB) *m/e* 915 $(M + H)^+$. Anal. ($C_{45}H_{54}N_8O_{11}S \times HCl \times 5H_2O$) C, H, N, Cl.

20(S)-20-O-{N$^\alpha$-[4-(3-O-Methyl-β-L-fucopyranosyloxy)-phenylaminothiocarbonyl]arginyl-D-valyl}camptothecin Hydrochloride (18). Yield: 77%; TLC: $R_f = 0.27$ (acetonitrile/water/glacial acetic acid 5/1/0.2); ^{1}H NMR (400 MHz; CD_3OD/CD_2Cl_2) δ 1.01 (*t*, $J = 7$ Hz, 3H, CH_3 CPT), 1.32 (*d*, $J = 6$ Hz, 3H, CH_3 Fuc), 3.24 (*dd*, $J = 3$ Hz, $J = 10$ Hz, 1H, 3-CH Fuc), 3.50 (*s*, 3H, OCH_3 Fuc), 3.72 (dq, $J = 1$ Hz, $J = 5$ Hz, 1H, 5-CH Fuc), 3.8 (dd, $J = 9$ Hz, $J = 8$ Hz, 1H, 2-CH Fuc), 3.9 (d, $J = 3$ Hz, 1H, 4-CH Fuc), 4.82 (*d*, $J = 8$ Hz, 1H, 1-CH Fuc), 6.9 (*d*, $J = 9$ Hz, 2H, oxy-phenylamino-), 7.13 (*d*, $J = 9$ Hz, 2H, oxy-phenylamino-), 7.4 (*s*, 1H, CPT D-ring), 7.68 (*dd*, $J = 7$ Hz, 1H, CPT A-ring), 7.84 (*dd*, $J = 7$ Hz, 1H, CPT A-ring), 8.0 (*d*, $J = 8$ Hz, 1H, CPT A-ring), 8.19 (*d*, $J = 8$ Hz, 1H, CPT A-ring), 8.52 (*s*, 1H, CPT B-ring). Anal. ($C_{45}H_{54}N_8O_{11}S \times HCl \times 6H_2O$) C, H, N, Cl.

20(S)-20-O-{N$^\alpha$-[4-(3-O-Methyl-β-L-fucopyranosyloxy)-phenylaminothiocarbonyl]histidylvalyl}camptothecin Hydrochloride (19). Yield: 78%; mp 180 °C (dec); TLC: $R_f = 0.27$ (dichloromethane/methanol/ammonia 17% 15/2/0.2); $[\alpha]^{22}_D$ −41.5° (*c* 0.2 DMF); ^{1}H NMR (400 MHz; CD_3OD/CD_2Cl_2) δ 1.0 (*t*, $J = 7$ Hz, 3H, CH_3 CPT), 0.92 and 1.08 (*2d*, $J = 7$ Hz, 6H, CH_3 Val), 1.33 (*d*, $J = 6$ Hz, 3H, CH_3 Fuc), 3.24 (*dd*, $J = 3$ Hz, $J = 10$ Hz, 1H, 3-CH Fuc), 3.23 and 3.42 (*2dd*, $J = 6$ Hz, $J = 15$ Hz, 2H, CH_2 His), 3.50 (*s*, 3H, OCH_3 Fuc), 3.75 (dq, $J = 1$ Hz, $J = 6$ Hz, 1H, 5-CH Fuc), 3.8 (dd, $J = 9$ Hz, $J = 8$ Hz, 1H, 2-CH Fuc), 3.91 (d, $J = 3$ Hz, 1H, 4-CH Fuc), 4.85 (*d*, $J = 8$ Hz, 1H, 1-CH Fuc), 7.05 (*d*, $J = 9$ Hz, 2H, oxy-phenylamino-), 7.2 (*s*, 1H, imidazole), 7.21 (*d*, $J = 9$ Hz, 2H, oxy-phenylamino-), 7.36 (*s*, 1H, CPT D-ring), 7.72 (*dd*, $J = 7$ Hz, 1H, CPT A-ring), 7.86 (*dd*, $J = 7$ Hz, 1H, CPT A-ring), 8.05 (*d*, $J = 8$ Hz, 1H, CPT A-ring), 8.14 (*d*, $J = 8$ Hz, 1H, CPT A-ring), 8.52 (*s*, 1H, CPT B-ring), 8.6 (*s*, 1H, imidazole); MS (FAB) *m/e* 896 $(M + H)^+$. Anal. ($C_{45}H_{49}N_7O_{11}S \times HCl \times 4H_2O$) C, H, N, Cl.

20(S)-20-O-{N$^\alpha$-[4-(3-O-Methyl-β-L-fucopyranosyloxy)-phenylaminothiocarbonyl]histidyl-D-valyl}camptothecin Hydrochloride (20). Yield: 75%; TLC: $R_f = 0.36$ (acetonitrile/water/glacial acetic acid 5/1/0.2); ^{1}H NMR (400 MHz; CD_3OD/CD_2Cl_2) δ 1.1 (*t*, $J = 7$ Hz, 3H, CH_3 CPT), 1.05 and 1.06 (*2d*, $J = 7$ Hz, 6H, CH_3 Val), 1.31 (*d*, $J = 6$ Hz, 3H, CH_3 Fuc), 3.24 (*dd*, $J = 3$ Hz, $J = 10$ Hz, 1H, 3-CH Fuc), 3.1 and 3.32 (*2dd*, $J = 7$ Hz, $J = 15$ Hz, 2H, CH_2 His), 3.51 (*s*, 3H, OCH_3 Fuc), 3.72 (dq, $J = 1$ Hz, $J = 6$ Hz, 1H, 5-CH Fuc), 3.81 (dd, $J = 9$ Hz, $J = 8$ Hz, 1H, 2-CH Fuc), 3.9 (d, $J = 3$ Hz, 1H, 4-CH Fuc), 4.8 (*d*, $J = 8$ Hz, 1H, 1-CH Fuc), 6.88 (*d*, $J = 9$ Hz, 2H, oxy-phenylamino), 7.01 (*d*, $J = 9$ Hz, 2H, oxy-phenylamino-), 7.2 (*s*, 1H, imidazole), 7.41 (*s*, 1H, CPT D-ring), 7.71 (*dd*, $J = 7$ Hz, 1H, CPT A-ring), 7.84 (*dd*, $J = 7$ Hz, 1H, CPT A-ring), 8.03 (*d*, $J = 8$ Hz, 1H, CPT A-ring), 8.18 (*d*, $J = 8$ Hz, 1H, CPT A-ring), 8.55 (*s*, 1H, CPT B-ring), 8.61 (*s*, 1H, imidazole). Anal. ($C_{45}H_{49}N_7O_{11}S \times HCl \times 5H_2O$) C, H, N.

20(S)-20-O-{N$^\alpha$-[4-(3-O-Methyl-β-L-fucopyranosyloxy)-phenylaminothiocarbonyl]lysylvalyl}camptothecin Hydrochloride (21). Yield: 63%; mp 200 °C (dec); TLC: $R_f = 0.36$ (acetonitrile/water/glacial acetic acid 5/1/0.2); $[\alpha]^{22}_D$ −40.6° (*c* 0.17 DMF); ^{1}H NMR (400 MHz; CD_3OD/CD_2Cl_2) δ 0.93 (*t*, $J = 7$ Hz, 3H, CH_3 CPT), 0.9 and 1.0 (*2d*, $J = 7$ Hz, 6H, CH_3 Val), 1.18 (*d*, $J = 6$ Hz, 3H, CH_3 Fuc), 3.27 (*dd*, $J = 3$ Hz, $J = 10$ Hz, 1H, 3-CH Fuc), 2.83 (*t*, $J = 7$ Hz, 2H, N−CH_2 Lys), 3.4 (*s*, 3H, OCH_3 Fuc), 3.66 (dd, $J = 10$ Hz, $J = 8$ Hz, 1H, 2-CH Fuc), 3.74 (dq, $J = 1$ Hz, $J = 6$ Hz, 1H, 5-CH Fuc), 3.91 (d, $J = 3$ Hz, 1H, 4-CH Fuc), 4.55 (*d*, $J = 5$ Hz, 1H, α-CH Val), 4.83 (*d*, $J = 8$ Hz, 1H, 1-CH Fuc), 7.02 (*d*, $J = 9$ Hz, 2H, oxy-phenylamino-), 7.21 (*d*, $J = 9$ Hz, 2H, oxy-phenylamino-), 7.3 (*s*, 1H, CPT D-ring), 7.70 (*dd*, $J = 7$ Hz, 1H, CPT A-ring), 7.87 (*dd*, $J = 7$ Hz, 1H, CPT A-ring), 8.03 (*d*, $J = 8$ Hz, 1H, CPT A-ring), 8.12 (*d*, $J = 8$ Hz, 1H, CPT A-ring), 8.58 (*s*, 1H, CPT B-ring); MS (FAB) *m/e* 887 $(M + H)^+$. Anal. ($C_{45}H_{54}N_6O_{11}S \times HCl \times 3H_2O$) C, H, N, Cl.

20(S)-20-O-{N$^\alpha$-[4-(3-O-Methyl-β-L-fucopyranosyloxy)-phenylaminothiocarbonyl]-lysyl-D-valyl}camptothecin Hydrochloride (22). Yield: 53%; TLC: $R_f = 0.30$ (acetonitrile/water/glacial acetic acid 5/1/0.2); $[\alpha]^{22}_D$ −74.8° (*c* 0.12 DMF); ^{1}H NMR (400 MHz; CD_3OD/CD_2Cl_2) δ 1.0 (t, $J = 7$ Hz, 3H, CH_3 CPT), 1.06 (d, $J = 7$ Hz, 6H, CH_3 Val), 1.3 (d, $J = 6$ Hz, 3H, CH_3 Fuc), 2.86 (t, $J = 7$ Hz, 2H, N−CH_2 Lys), 3.24 (dd, $J = 3$ Hz, $J = 10$ Hz, 1H, 3-CH Fuc), 3.5 (s, 3H, OCH_3 Fuc), 3.72 (dq, $J = 1$ Hz, $J = 6$ Hz, 1H, 5-CH Fuc), 3.8 (dd, $J = 10$ Hz, $J = 8$ Hz, 1H, 2-CH Fuc), 3.90 (d, $J = 3$ Hz, 1H, 4-CH Fuc), 4.81 (d, $J = 8$ Hz, 1H, 1-CH Fuc), 6.9 (d, $J = 9$ Hz, 2H, oxy-phenylamino-), 7.08 (d, $J = 9$ Hz, 2H, oxy-phenylamino-), 7.4 (s, 1H, CPT D-ring), 7.70 (dd, $J = 7$ Hz, 1H, CPT A-ring), 7.86 (dd, $J = 7$ Hz, 1H, CPT A-ring), 8.02 (d, $J = 8$ Hz, 1H, CPT A-ring), 8.2 (d, $J = 8$ Hz, 1H, CPT A-ring), 8.56 (s, 1H, CPT B-ring). Anal. ($C_{45}H_{54}N_6O_{11}S \times HCl \times 4H_2O$) C, H, N, Cl.

Determination of Glycoconjugate Stability by HPLC Analysis. Lactone Ring Stability. Topotecan **4** and glycoconjugates **19** and **21** were dissolved in acetonitrile/water 1:1 at concentrations of 1 mg/10 mL. Subsequently 2 equiv of 1 N sodium hydroxide was added. After 1 h, lactone ring opening was quantified by HPLC analysis (UV detetection at 257 nm).

Camptothecin Release in the Supernatant of HT29. HT29 tumor cells (200 000 cells/mL) were grown in RPMI 1640 medium supplemented with 10% FCS for 24 h. Aqueous solutions of the glycoconjugates **15**−**22** in concentrations of 10 μM were added and incubated for another 6 and 24 h, respectively. Fifty microliters of the cellular supernatant was injected into HPLC using an RP18 (5 μm) column with 70% $HClO_4$/water (0.4% v/v) as eluent A and acetonitrile as eluent B (UV detection at 365 nm). Both glycoconjugates and released camptothecin were quantified by UV detection at 356 nm.

Biology. MTT Assay for Determination of Cytotoxic Activity Against Tumor Cell Lines. The human colon cancer cell lines SW480 and HT29 and the murine melanoma cell line B16F10 were grown in RPMI 1640 medium supplemented with 10% FCS. Confluent cells were trypsinized and resuspended in RPMI at 50 000 cells/mL (SW480 and HT29) and 20 000 cells/mL (B16F10). One hundred microliters of these cell suspensions was transferred to each well of a 96 well microtiter plate and incubated for 1 day. Subsequently, 100 μL of RPMI and 1 μL of a solution of camptothecin derivatives either in DMSO or water were added. Growth inhibition was assessed 6 days after incubation using the MTT assay. Twenty microliters of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) of a starting concentration of 5 mg per mL of H_2O was added and incubated at 37 °C for 5 h. Subsequently, the medium was removed, 100 μL of 2-propanol per well was added, and 30 min later an additional 100 μL of H_2O was added. The extinction was measured at 595 nm using an ELISA-Multiplate Reader.

Determination of the in Vivo Activity in the Breast Cancer Model MX-1. Six to eight week old athymic nude mice (NMRI nu/nu strain) grown in the breeding facilities of the Drug Development Laboratory Oncotest GmbH, Freiburg, Germany were used. Human tumors established in serial passage in nude mice were implanted subcutaneously in both flanks of female animals. Treatment was started as soon as the tumors reached a diameter of 5−7 mm depending on the doubling time. Mice were randomly assigned to treatment

groups and control groups (5 mice per group bearing 8−10 evaluable tumors). Tumor size was measured twice weekly by two-dimensional measurement with calipers. Volumes were calculated according to the formula $a \times b^2/2$ with a and b representing two perpendicular tumor diameters. Relative tumor volume values were calculated for each single tumor by dividing the tumor size day × by the tumor size day 0 at the day of randomization. Median RTV values were used for further evaluation. Tumor volume inhibition (volume of test group/control group T/C) was the main end point. Furthermore, body weight changes and mortality were assessed as parameters for toxicity. Glycoconjugates were dissolved in water (c = 1−3 mg/mL) and were administered daily for the first 3 days intravenously.

Determination of Hemopoietic Activity (Colony Formation of Progenitor Cells). C57bl/6 mice (n = 3) were treated intraperitoneally once a day for 3 consecutive days with the glycoconjugates **19** or **21** in comparison to topotecan **4** at different doses in the range of the respective MTDs calculated from the nude mice models. At 4 and 24 h after the last treatment, bone marrow cells were isolated from both femurs and cultured (2×10^5/culture) on semisolid, CSF-supplemented medium. After 7 days incubation (37 °C, 5.5% CO_2) colony formation (number of colonies with >50 cells/colony), indicating proliferation and differentiation of myeloid progenitor stem cells, was determined.

Determination of Cytotoxicity Against Hepatocytes. Liver was first perfused with P-I (perfusion buffer I, consisting of ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA, 0.5 mM) and HEPES (10 mM) in Hank's Balanced Salt Solution (HBSS, Gibco, without Ca^{2+} and Mg^{2+})) at a flow rate of 20−30 mL/(min × cannula) for 20 min and subsequently with P-II (perfusion buffer II, consisting of HEPES (10 mM, pH 7.4) in HBSS, $CaCl_2$ (5.0 mM), and 1 mg/mL collagenase type IV). Cells were suspended in HBSS and centrifuged at 80 g for 3 min. After washing, cells were counted in a hemocytometer after staining with trypan blue. Viability was estimated to be about 80−90%.

Hepatocytes were cultured in collagen sandwich gels according to described methods.[18,19] Briefly, 6-well plates (Greiner, Solingen) were coated with 1.0 mL per well of rat tail tendon collagen dissolved in William's Medium E.[20] Hepatocytes (2×10^6) were seeded into each well and allowed to attach overnight. After removal of the unattached cells, the cells were coated with 1 mL of the same collagen solution which was previously used to prepare the lower part of the sandwich. Culture medium (2.0 mL) was added (supplemented with insulin [0.16 U/mL], glucagon [0.014 μg/mL], prednisolone [9.6 μg/mL], glutamine [1%], penicillin/streptomycin [200 U/mL], and 10% fetal calf serum) and renewed daily. After 48 h of adaptation cells were incubated with the test compounds at the indicated concentrations for up to 7 days.

Stock solutions of test compounds in DMSO (3 mM) were further diluted in culture medium to the desired concentration. The primary hepatocytes were treated for 1−7 days, and the cell medium containing the test compound was changed every day.

The cytotoxic effects of the compound on the cells were determined by measuring release of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) in the cell supernatants.

In addition, the mitochondrial dehydrogenase activity was measured in an MTTassay.

Confocal Laser Scan Fluorescence Microscopy. HT29 cells were grown for 48 h on coverslips and then incubated with test compounds for 6 and 24 h at temperatures of 37 °C. The camptothecin glycoconjugate **19** was tested at 100 μM, whereas the fluorescence dye conjugate **23** was tested at 0.5 μM and the fluorescence dye **24** at 0.1 μM. The confocal images were generated using an inverted LSM 410 UV, Zeiss, Germany.

Flow Cytometry. HT29 cells were incubated with 0.1 μM of the glycoconjugates for 6 h at either 37 °C or 4 °C. Cells were isolated with accutase, washed twice with PBS, and resuspended in PBS at 2×10^6 cells/mL. Flow cytometric measurements were performed using a FACStar^{Plus}, Becton Dickinson, Ca., U.S.A. The cells were excited at 488 nm using an argon ion laser. The emitted fluorescence was measured using a 530 BP30 band-passfilter (Standard green fluorescence). Viability staining was done by using propidiumiodide dye (Molecular Probes, NL).

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