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Synthesis, Hydrolytic Activation and Cytotoxicity of Etoposide Prodrugs

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Abstract—Two 4′-propylcarbonoxy derivatives (**2**,**3**) of etoposide (**1**), a topoisomerase II inhibitor, were synthesized and evaluated as potential prodrugs for anticancer therapy. Their activation via hydrolysis mechanisms was determined as a function of pH in buffer solutions, in human serum and in the presence of carboxyl ester hydrolase. Cytotoxicity was determined on various tumor cell lines and compared to the parent compound. On cell lines exhibiting resistance to etoposide we observed an enhanced cytotoxicity of the prodrugs of up to three orders of magnitude.

Etoposide (**1**) is a widely used, highly effective anticancer drug against a broad spectrum of tumors including pediatric cancers such as acute lymphatic lymphomas, rhabdomyosarcomas and neuroblastomas, as well as in most common adult cancers.[1,2] It is also used in bone marrow transplantation conditioning regimens. However, the therapeutic use of etoposide is limited by toxicity involving mainly myelosuppression.[3] A major problem for the use of this drug (and other topoisomerase inhibitors) is the development of multidrug resistance.[4,5] Efforts are presently made to synthesize new derivatives of the natural podophyllotoxin to overcome some of these shortcomings.[6–8] An alternative approach to new analogues of therapeutic agents is the synthesis of prodrugs. In previous work[9–12] we synthesized prodrugs of various antitumor agents to improve their bio-availability, phamacokinetics and aqueous solubility. For paclitaxel[11,12] we established that prodrugs incorporating hydrolytically cleavable moieties are effective in lowering the systemic toxicity of the drug in animal models and most importantly also in patients.[13] Furthermore these prodrugs of paclitaxel revealed a dramatically improved pharmacokinetics and could be formulated for intravenous delivery with a minimum of toxic organic vehicles. Based on these findings the hydrolytic activation approach was applied to etoposide, an important drug in pediatrics and adult oncology.

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Synthesis

Compounds **2** and **3** were prepared as follows. Treatment of a solution of **1** (Sigma, Germany) in dichloromethane, with one equivalent of soketal chloroformate at −70 °C followed by the addition of one equivalent of pyridine and reacting for 6 h, lead to **2**. The workup consisted of extraction with brine, drying over magnesium sulfate, centrifugation and concentrating the organic layer to one fifth of the reaction volume. The product was isolated by HPLC purification (C_{18} reverse-phase column with 50/50 vol% acetonitrile–water), followed by lyophilization of the eluate (yield 83.5%, purity 99.2%). Compound **3** was prepared from a THF solution of **2** by the addition of 2 N HCl and reacting for 2 h at ambient temperature. Workup consisted of washing with brine, drying over magnesium sulfate, reducing the volume to 25% of the reaction volume and purification via HPLC under the conditions given for **2**. The yield of purified product was 85.5% and the purity by HPLC was greater than 99%.

The structures of **2** and **3** were established by ^{1}H NMR, ^{13}C NMR and MS data.[14,15]

Solution stability

The chemical stability of solutions of **2** and **3** were determined by dissolving the compounds in 50/50 vol% chromophore–ethanol (1 mg/10 μL solvent mixture)

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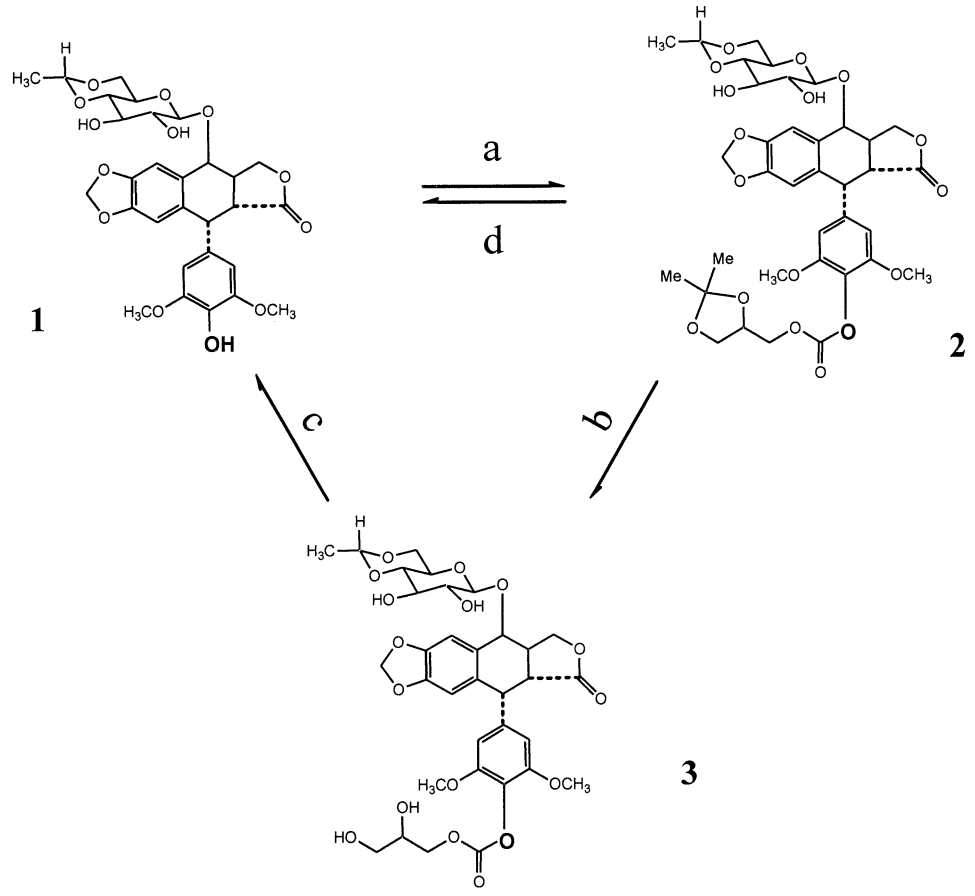

Scheme 1. (a) Soketal* chloroformate, CH_2Cl_2; (b) THF, 2 N HCl, (c) PBS, pH > 8.0; or carboxylesterase, pH 7.3; or serum (d) carboxyl esterase, pH 7.3; or serum (*2,2-dimethyl-4-hydroxymethyl-1,3-dioxolan).

and adding 90 μL of 1× PBS, pH 7.3 to obtain concentrations of about 14 mM. Solutions were also prepared in 50 vol% acetonitrile–water. These solutions were stored under ambient laboratory conditions with average temperatures of about 22 °C. Samples, 2 μL, were periodically removed and analyzed by HPLC over a period of 3 weeks.

Hydrolysis studies

Hydrolysis rates were determined by dissolving the compounds in acetonitrile–aqueous buffer solutions (20/80 vol%) at different pH, in human serum or with carboxylic ester hydrolase (CEH) solutions (1 μM, from pig liver, Sigma Co., Germany). In addition, human serum was fractionated via HPLC size exclusion chromatography (G200), resulting in four peaks (ranging in M_r cutoffs between 350,000 and 10,000) and fractions containing these peaks were used in hydrolysis experiments. All experiments were conducted at 37 °C at drug concentrations of about 6 mM. Serum samples were pre-separated using Sep-Pac C18 cartridges. Samples were worked up by removing 10 μL aliquots, adding 10 μL of acetonitrile, centrifuging at 14,000 rpm and using 10 μL of the clear supernatant for HPLC analysis. The data were quantified from peak integrated areas of the HPLC chromatograms.

Cytotoxicity

Anti-proliferative activities of **1**–**3** were determined using the XTT assay.[16] Briefly, 10,000 tumor cells/well were added to 96-well plates and incubated under standard tissue culture conditions (37 °C, 5% CO_2) for 24 h before addition of drug at concentrations ranging from 100 μM to 100 fM by direct addition from stock solutions in DMSO. After 72 h, XTT (Aldrich Chemical Co., Germany) was added to a final concentration of 250 μg/well. The plates were then incubated for an additional 4 h and the OD determined at 450 nm using a microplate reader. The cell viability–drug dilution profiles were obtained from sigma plots, and drug concentrations which inhibited growth by 50% were calculated from duplicate runs (IC_{50}).

Protein binding

Serum affinity binding experiments were conducted by dissolving the prodrug in a minimum of methanol and adding human serum to a final concentration of about 100 μM. Samples were ultrafiltered using the microcon YM-10 (Amicon Corp.) membrane centrifugation system and analyzed by HPLC. The values obtained for **2** and **3** were 97±4% and 35±7%, respectively.

Results and Discussion

The reaction scheme for the synthesis of prodrugs **2** and **3** and their activation to the parent compound **1** is shown in Scheme. 1. One-step reactions resulted in these compounds in high yield (>80%) and both compounds were easily obtained in high purity (99%) by conventional HPLC methods. Both prodrugs show, surprisingly, exceptional solution stabilities for several weeks in formulations (surfactant–ethanol, 1% saline combinations) and under conditions encountered in clinical applications (intravenous delivery). Under acidic conditions (1% HCl) compound **2** converts to **3** within 2 h with some degradation (about 10%) of the glycoside moiety.

In preliminary experiments at neutral pH in PBS buffered solutions, the prodrugs were very stable, but in the presence of plasma or serum both **2** and **3** hydrolyzed to **1**, with the elimination of soketal and glycerine, respectively. When serum was fractionated by size exclusion chromatography (G-200 column) it was found that all the conversion to the parent drug occurred in a fraction of molecular weights between 80 and 40 kD, which was within the molecular weight range of carboxyl esterases.

The kinetics of prodrug hydrolysis were then determined at 37 °C in PBS buffer at various pH, in human serum and in buffer solutions containing porcine liver esterase.

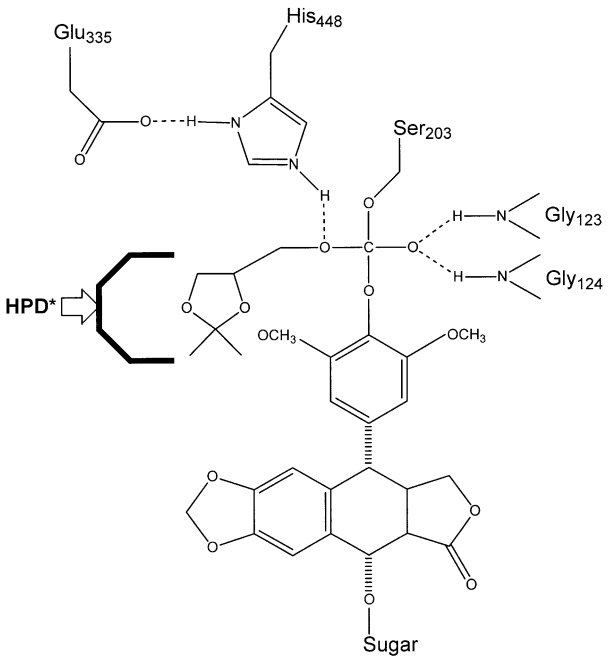

Figure 1. Transition state complex of ProVP-16 I and carboxyl esterase. *HPD, hydrophobic positioning domain. See ref 17 for anion hole.

Table 1. Kinetic parameters for hydrolysis of etoposide prodrugs

Compd	Media (37 °C ± 0.5)	pH (±0.1)	k_{obs} (10^{-3} min^{-1})[a]	$t_{1/2}$ (min)
Prodrug I	PBS buffer	5.0–10.0	No conversion	—
2		11.8	8.333 ± 0.50	83.16
	Human serum esterase[b]	7.3	0.923 ± 0.046	750.8
		7.3	48.89 ± 3.62	14.17
Prodrug II	PBS buffer	5.0–7.3	No conversion	—
3		8.0	3.201 ± 0.428	216.49
		8.8	7.142 ± 0.351	97.03
		10.5	100.01 ± 12.72	6.93
	Human serum esterase	7.3	12.36 ± 0.965	56.07
		7.3	1.348 ± 0.081	514.10

[a]Mean ±SD.
[b]Porcine liver carboxyl ester hydrolase.

Table 2. Cytotoxicity of etoposide and prodrugs (XTT assay)

Cell line	Type	MDR-1	IC_{50} (μM)[a]		
			Etoposide	Prodrug I	Prodrug II
			1	**2**	**3**
Molt 3	T-cell leukemia, parental	–	0.065	0.010	0.035
MOVP-3	VP16 resistant Molt 3	++	7.55	0.012	0.050
VCR100	T-lymphoblastoide VC res.	+	15.5	3.2	7.6
HT-29	Colon carcinoma	–	10[b]	0.0035	0.0057
SW480	Colon adenocarcinoma res.	+	50[c]	0.11[c]	0.11[c]
A2780	Ovarian carcinoma parental	–	65	0.09	0.58
ADR5000	Doxo resistant A2780	++	100	6.5	38

[a]Average of duplicate determinations.
[b]Only 35% of cells were affected.
[c]~50% cells affected by drug.

In all experiments conversion–time curves exhibited first-order kinetics from which linear log conversion–time plots were obtained. The rate constants, k_{obs}, and half-lives, $t_{1/2}$, were calculated from the slopes of these plots. The data are summarized in Table 1.

In PBS buffer, **2** is surprisingly stable over a broad range of pH from 5 to 10. Only above pH 11, this prodrug hydrolyzes to give etoposide at relatively fast rates with a half life of 83 min at pH 11.8. This contrasts with reaction characteristics of **3** which undergoes base-catalyzed hydrolysis above pH 8 at rates which rapidly increase with increasing pH. We attribute the unusual hydrolytic stability of **2** to the hydrophobic nature of the entire southern region of this molecule and, to a lesser extent, also to steric hindrance from the two *ortho* methoxy groups surrounding the carbonate moiety. For the esterase-catalyzed conversion, the rate constant for **2** is nearly 40 times that of **3**. This increase in substrate specificity of **2** over **3** for the enzyme may be due to the presence of a hydrophobic positioning domain next to the oxyanion hole within the catalytic region, with a high affinity for the isopropylideneglyceryl side chain, as shown in Figure 1. In human serum, both compounds exhibit prodrug behavior. Interestingly, the activation rate of **3** in human serum is 14 times faster than that of **2**. Based on the dramatically increased affinity of **2** to serum proteins, we suggest that a reduction of free **2** in human serum (due to binding competition of various proteins components) may contribute to its lower hydrolysis rate. The relatively large differences in HPLC elution times (C_{18} column) of 7.1 min for **3** compared to 18.5 min for **2** (lipophilic index) support the above observations. In any event, the kinetic data presented here clearly show the significance of specific structural motives (other than those directly involved in the catalytic process, i.e., sp^2 carbonyl) in determining the transformation of the prodrug to the parent compound.

Next, we tested the cytotoxicity of **1**–**3** on a number of parental and drug-resistant tumor cell lines (Table 2). Both **2** and **3** exhibit no initial cytotoxicity compared to **1**. Their IC_{50} values after 72 h in cell culture (the drug concentration of 50% cell viability) were significantly below the parent drug, etoposide. With cell lines which strongly expressed the MDR-1 gene (data not shown), we observed significant differences of up to three orders of magnitude. The nearly identical IC_{50} values for the prodrugs between the parental cell line Molt-3 and its etoposide-resistant clone (MOVP-3) are direct evidence that these compounds completely circumvent drug resistance mechanisms.

Detailed preclinical investigations are presently being done and will be reported shortly.

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14. 4′-(2″,3″-Isopropylideneglyceryl carbonoxy)-etoposide: ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.01 (s, 1H, H_5), 6.54 (s, 1H, H_8), 6.28 (s, 2H, $H_{2'}$, $H_{6'}$), 6.02 (s,1H, O-CH-O), 5.23 (d, 1H, $H_{1'}$), 4.94 (d, 1H, H_4), 4.72 (m, 2H, H_2,$H_{7''}$), 4.58 (t, 2H, H_1), 4.28 (d, 1H, H_{9a}), 4.20–4.02, (m, 3H, H_{9b},$H_{5''}$, $H_{6''}$), 3.60 (m, 3H, OCH_3), 3.32 (m, 1H, H_5''), 3.28 (d, 1H, H_2), 3.15 (d, 2H, $H_{3''}$,$H_{4''}$), 2.48 (s, 3H, CH_3), 1,26 (m, 3H, C(2″,3″),CH_3). ^{13}C NMR 174.58, 152.33, 150.69, 147.83, 146.35, 139.11, 131.96, 128.93, 127.01, 109.77, 108.97, 107.28,. 101.49, 101.36, 98.57, 80.10, 74.37, 72.94, 72.69, 71.71, 68.52, 67.32, 65.75, 64.98, 55.80, 43.18, 40.34, 40.05, 39.78, 39.50, 39.23, 38.95, 38.67, 26.36, 25.32, 20.31. UV max 248 nm (ε 1942, 271 nm (ε 1504). FAB LRMS *m*/*e* 746.85 $(M+H)+$ calc. for $C_{36}H_{42}O_{17}$ 746.70. HPLC elution time (C_{18} column, 50/50 CAN–H_2O, flow 0.5 mL/min), 18.46 min (etoposide 8.42 min).
15. 4′-(2″,3″-dihydroxypropyl carbonoxy)-etoposide: FAB LRMS *m*/*e* $(M+H)^+$ 706.91, calcd for $C_{33}H_{38}O_{17}$ 706.64. UV max 246 nm (ε 1704), 273 nm (ε 1355). HPLC elution time (C_{18} column, 50 vol% ACN–H_2O, flow 0.5 mL/min) 7.1 min.
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