J|A|C|S
COMMUNICATIONS

Published on Web 06/07/2002

A Versatile Prodrug Approach for Liposomal Core-Loading of Water-Insoluble Camptothecin Anticancer Drugs

Xinli Liu, Bert C. Lynn, Junhong Zhang, Lin Song, David Bom, Wu Du, Dennis P. Curran, and Thomas G. Burke*

Departments of Chemistry and Pharmaceutical Sciences, and The Experimental Therapeutics Program, Markey Cancer Center, University of Kentucky, Lexington, Kentucky 40506, Tigen Pharmaceuticals, Inc., 235 Bolivar Street, Lexington, Kentucky 40508, and Department of Chemistry, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

Received January 16, 2002

Camptothecin analogues are anticancer agents that act on topoisomerase I.[1] Topotecan and CPT-11 have received FDA approval, and several other derivatives are now in pre-clinical and clinical development. In patients, camptothecins are known to undergo widespread biodistribution to both tumor and nontumor sites, with the latter frequently resulting in notable toxicities to healthy tissues (e.g. bone marrow, gastrointestinal mucosa).[2] Attenuation of such toxicities and tumor-targeting are proven benefits of liposomal core-loaded drug formulations.[3] To date industrial production of core-loaded liposomal camptothecin formulations has been limited to the water-soluble camptothecins topotecan, CPT-11, GG-211 (lurtotecan), and CDK-602. These agents contain basic amines, and they are loaded into the core of pre-made small unilamellar vesicles by well-established chemical gradient methods.[4] Although liposomal core-loaded formulations of water-insoluble camptothecins such as 7-ethyl-10-hydroxy-camptothecin (SN-38) and 7-*tert*-butyldimethylsilyl-10-hydroxy-camptothecin (DB-67) are of significant clinical interest,[5] these agents lack amino groups and do not load with use of gradient driven processes. Ester prodrug development is a well-established approach to derivatizing such agents containing a hydroxyl group,[6] and here we describe a prodrug approach that allows water-insoluble camptothecin prodrugs to load into the liposomal lumen. Novel mechanistic insight into the pH-mediated prodrug hydrolysis and the influence of ester functionality is provided.

The procedure involves conversion of an active camptothecin analogue to a 20-OR ω-aminoalkanoanic ester prodrug,[7] in which R = $CO[CH_2]_nNH_2$ and $n = 1-3$. The basic amino group of the prodrug serves three roles. First, at pH ranges of 3−5, it enhances aqueous solubility.[8] Second, it enhances responsiveness to a transmembrane ammonium sulfate gradient across the liposomal bilayer, thereby facilitating active loading of the agent into the liposomal aqueous core. Third, at a physiological pH of 7 or above (the pH to be encountered following drug release at the tumor site), the nucleophilicity of the amine manifests itself and cyclization to the C-21 carbonyl carbon occurs. This cyclization triggers a rapid and convenient nonenzymatic[9] decomposition process that releases active camptothecin. Accordingly, this novel liposomal approach may allow for the tumor-targeting of water-insoluble camptothecins.

In contrast to 10-OR-CPT-20-OH compounds such as CPT-11, which are known to exhibit hydrolysis-resistant carbamate bonds that require enzymatic cleavage,[10] reversed-phase high-performance liquid chromatographic (RP-HPLC) studies of the stabilities of several 10-OH-CPT-20-OR and CPT-20-OR glycinate ester prodrugs (where R = $COCH_2NH_2$) revealed extensive chemical (i.e. nonenzymatic) decomposition in every case.[11] Prodrug decomposition was observed in phosphate buffered saline (PBS) at pH 7.4, as well as in human plasma and blood.

The presence of the amine functionality was found to be essential for the prodrug decomposition. Whereas camptothecin-20(*S*)-glycinate **1** [1 μM, PBS (pH 7.4), 37 °C] underwent extensive decomposition (~90%) within 3 h, its corresponding aliphatic ester analogue camptothecin-20(*S*)-acetate (differing solely by the replacement of the amino group with a proton) demonstrated excellent stability in the above fluids for days with negligible evidence of hydrolysis. Amine-containing esters of camptothecin with longer alkyl functionalities (R = $COCH_2CH_2NH_2$, R = $COCH_2CH_2CH_2NH_2$) also reacted under similar conditions, albeit at a slower rate (half-lives of 26.4 and 36.7 h, respectively) than their glycinate counterparts.

To better understand the mechanism of prodrug decomposition, RP-HPLC coupled with electrospray ionization-tandem mass spectrometry (ESI-MS/MS) and ^{13}C, ^{15}N, and 2D NMR experiments were used to study the decomposition reaction of camptothecin-20(*S*)-glycinate. Camptothecin-20(*S*)-glycinate **1** decomposed to produce several products[12] (see proposed Scheme 1): the closed-ring lactone form of camptothecin **5**, the ring-opened carboxylate form of camptothecin **6**, and two novel decomposition products **3** and **4** generated following the formation of an unusual six-membered morpholine 2,5-dione ring **2** (or lactam intermediate). The lactam intermediate arose by intramolecular nucleophilic attack of the amino group on the lactone E-ring carbonyl carbon of camptothecin and is in fast equilibrium with structures **3** (which we refer to as the ortho lactone) and **4**. Ortho lactone **3** arose by a second intramolecular reaction within the lactam intermediate and **3** exhibited the same mass as camptothecin-20(*S*)-glycinate ester but with a strikingly different fragmentation pattern. **4** arose by a competitive intermolecular reaction to the lactam intermediate and reacted to release both **5** and **6**.[13]

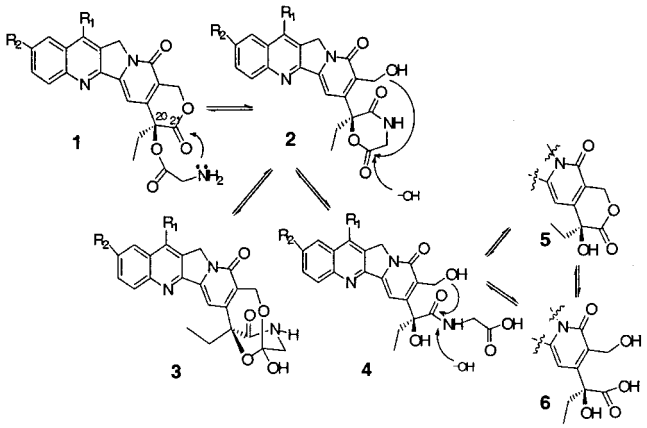
Likewise, glycinate ester prodrug analogues of other camptothecins (1 μM, in PBS, pH 7.4), including DB-67 and SN-38, underwent extensive decomposition (~90%) within 3 h. HPLC data for each analogue studied was consistent with the generation of the novel decomposition products, along with the lactone and carboxylate forms of the parent drug.[12] ESI-MS/MS analysis indicated that the proposed ortho lactone **3** generated for DB-67-(20)*S*-glycinate displayed the same mass as the glycinate ester prodrug but with a different fragmentation pattern.[12] As in the case for the camptothecin prodrug, the novel degradation products were generated from the proposed lactam intermediate.

We suggest that the generation of these lactam intermediates is central to the rapid decomposition of this class of prodrugs. Initiation of drug release by prodrug hydrolysis at physiological pH is a pre-

* Address correspondence to this author at the University of Kentucky. E-mail: tgburke@uky.edu.

10.1021/ja0256212 CCC: $22.00

Scheme 1 [a]



[a] Glycinate ester of CPT (R_1 = H, R_2 = H), of SN-38 (R_1 = CH_2CH_3, R_2 = OH), and of DB-67 (R_1 = $Si(CH_3)_2C(CH_3)_3$, R_2 = OH)

dictable chemical process with less likelihood of interpatient variability compared to enzyme-mediated drug release. For this reason we prioritized our subsequent efforts on the liposomal core-loading of 20-OR prodrugs over the 10-OR prodrug counterparts. At low pH values in the 3−5 range we found that 20-OR prodrugs (where R = $CO[CH_2]_nNH_2$ and n = 1−3) displayed markedly improved stability, thereby facilitating liposomal loading methodologies conducted at low pH values. For example, the amounts of intact camptothecin-20(*S*)-glycinate (1 μM in PBS, 37 °C) remaining after 2 h at pH values of 7.4, 6.0, 5.0, and 3.0 were 18%, 68%, 95%, and 99%, respectively.

Remote "active" loading of prodrug into premade small unilamellar vesicles, with diameters of 100 nm, was carried out by using transmembrane ammonium sulfate gradients. Prodrug was added to a liposomal suspension where initially $[(NH_4)_2SO_4]_{CORE} \gg [(NH_4)_2SO_4]_{EXTERNAL}$; loading of the prodrug occurred as a result of base exchange (initiated by NH_3 gas molecules departing the liposome).[14] Whereas underivatized camptothecin and DB-67 localize predominantly in the bilayer compartment of the liposome,[3b] their 20-OR prodrugs, where R = $CO[CH_2]_nNH_2$ and n = 1−3, loaded with high efficiency (≈60 to 90%) into the core of liposomes at clinically relevant drug-to-lipid ratios (between 1:4 to 1:20). More importantly, these core-loaded liposomal formulations of camptothecin 4-aminobutanoate ester and DB-67 4-aminobutanoate ester exhibited markedly improved stabilities in whole blood relative to their free forms (Figure 1). Whereas the decomposition of free prodrug in both cases was extensive, liposomal entrapment prevented the degradation process from occurring, providing indirect evidence that the prodrug was effectively retained within the liposome for periods up to 40 h. These time periods are known to be sufficient for successful tumor-targeting to be achieved.[3]

In conclusion, we have demonstrated the utility of a convenient ester prodrug strategy for the liposomal core-loading of water-insoluble camptothecins. Our prodrug approach opens the door for liposomal loading and tumor-targeting of a much expanded selection of camptothecins. If premature drug leakage from the particle can be reduced and tumor-targeting optimized by using agents such as SN-38 and DB-67 and related 7-silyl-containing camptothecins (silatecans), their high intrinsic potencies and persistent lactone ring stabilities suggest they may be of significant value for targeted delivery.

Acknowledgment. This research was supported by NCI grants CA63653, CA83362, and CA75751.

Supporting Information Available: Table of structure of camptothecin analogues, experimental procedures for LC/MS; 2D NMR spectrum; and synthesis of new compounds as well as liposome active loading methods (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

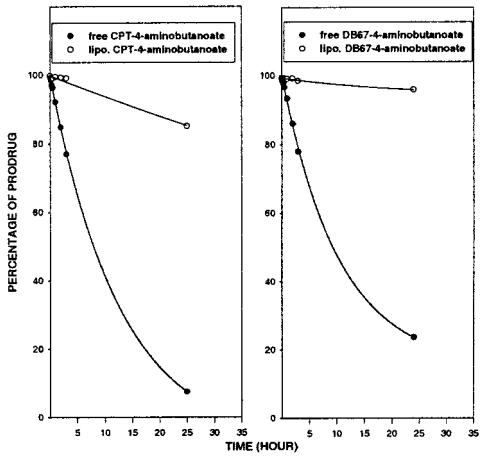


Figure 1. (Left panel) Free vs core-loaded CPT-4-aminobutanoate in whole blood. (Right panel) Free vs core-loaded DB-67-4-aminobutanoate in whole blood (pH 7.4, 37 °C).

References

(1) For a review of the campothecin biochemistry and drug development see: Liehr, J., Giovanella, B. C., Verschraegen, C. F., Eds. The Camptothecins: Unfolding Their Anticancer Potential. *Ann. N.Y. Acad. Sci.* **2000**, *922*, 1−363.

(2) (a) Arun, B.; Frenkel, E. P. *Expert. Opin. Pharmacother.* **1989**, *246*, 1046−1048. (b) Ratain M. J. *J. Clin. Oncol.* **2002,** *20*, 7−8.

(3) Reviews: (a) Barenholz, Y. *Curr. Opin. Colloid Interface Sci.* **2001**, *6*, 66−77. (b) Burke, T.G.; Bom, D. *Ann. N.Y. Acad. Sci*. **2000**, *922*, 36−45

(4) (a) Tardi, P.; Choice, E.; Masin, D.; Redelmeier, T.; Bally, M.; Madden, T. D. *Cancer Res.* **2000**, *60* (13), 3389−93. (b) Sadzuka, Y.; Hirotsu, S.; Hirota, S. *Jpn. J. Cancer Res.* **1999**, *90* (2), 226−32.

(5) These agents display potent antitopoisomerase I activities and improved inherent stabilities of the key 20(*S*)-α-hydroxy-δ-lactone pharmacophore in blood and tissues.[3b]

(6) Denny, W. A. *Eur. J. Med. Chem.* **2001** Aug, *36* (7−8), 577−95.

(7) (a) Wall, M. E.; Wani, M. C.; Nicholas, A. W.; Manikumar, G.; Tele, C.; Moore, L.; Truesdale, A.; Leitner, P.; Besterman, J. M. *J. Med. Chem.* **1993**, *36*, 2689−2700. (b) See Supporting Information for our novel and modified semisynthetic procedures of 10-OH-CPT-20-OR and CPT-20-OR glycinate ester prodrugs, respectively.

(8) (a) Vishnuvajjala, B. R.; Garzon-Aburbeh, A. U.S. Patent 4943579, 1990. (b) Wadkins, R. M.; Potter, P. M.; Vladu, B.; Marty, J.; Mangold, G.; Weitman, S.; Manikumar, G.; Wani, M. C.; Wall, M. E.; Van Hoff, D. D. *Cancer Res.* **1999**, *59*, 3424−3428.

(9) Wadkins et al.[8b] previously employed HPLC and noted that 20(*S*)-glycinate esters of camptothecins hydrolyzed in phosphate buffer (pH 7.5) in the absence of enzymes to release active drug. HPLC peaks were assigned to the parent drug and the ionized and un-ionized forms of the glycinate (both lactone and hydroxy acid forms). No discussion of the hydrolysis mechanism or evidence for the presence of a lactam intermediate and ortho lactone was presented.[8b]

(10) Kawato, Y.; Aonuma, M.; Hirota, Y.; Kuga, H.; Sato, K. *Cancer Res.* **1991**, *51* (16), 4187−91.

(11) CPT, SN-38, and DB-67 glycinate esters were studied in this report.

(12) (a) The observed mass values for the protonated molecular (MH^+) ions of CPT glycinate were as follows: **1** (406), **3** (406), **4** (424), **5** (349), **6** (367). (b) CPT glycinate fragmentation pattern [*m*/*z* (%)]: 406 (25), 331 (100), 303(30). Ortho lactone fragmentation pattern [*m*/*z* (%)]: 406 (100), 388 (40), 303 (20). (c) DB-67 glycinate fragmentation pattern [*m*/*z* (%)]: 536 (100), 461 (50), 433 (20). Ortho lactone fragmentation pattern [*m*/*z* (%)]: 536 (100), 518 (10).

(13) A manuscript fully elucidating the hydrolysis mechanism is in preparation. Evidence for proposed degradation products **3** and **4** includes the following: (1) glycine carbonyl ^{13}C isotopic labeling NMR experiment in PBS showing that this carbon exists as a tetrahedral carbon as in **3**; (2) glycine ^{15}N isotopic labeling NMR experiments showing that **3** and **4** have amide bonds; (3) molecular mass of **3** and **4** are consistent with ref 12a; (4) see Supporting Information for the 2D NMR spectrum of the *N*-methyl analogue of **3** (this analogue is isolatable due to longer half-life).

(14) (a) Maurer-Spurej, E.; Wong, K. F.; Maurer, N.; Fenske, D. B.; Cullis, P. R. *Biochim. Biophys. Acta* **1999**, *1416*, 1−10. (b) See Supporting Information for liposomal active loading.

JA0256212