

# Dendrimer-Encapsulated Camptothecins: Increased Solubility, Cellular Uptake, and Cellular Retention Affords Enhanced Anticancer Activity *In vitro*

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## Abstract

**A biocompatible polyester dendrimer composed of the natural metabolites, glycerol and succinic acid, is described for the encapsulation of the antitumor camptothecins, 10-hydroxycamptothecin and 7-butyl-10-aminocamptothecin. The cytotoxicity of the dendrimer-drug complex toward four different human cancer cell lines [human breast adenocarcinoma (MCF-7), colorectal adenocarcinoma (HT-29), non-small cell lung carcinoma (NCI-H460), and glioblastoma (SF-268)] is also reported, and low nmol/L IC<sub>50</sub> values are measured. Cellular uptake and efflux measurements in MCF-7 cells show an increase of 16-fold for cellular uptake and an increase in drug retention within the cell when using the dendrimer vehicle.** (Cancer Res 2006; 66(24): 11913-21)

## Introduction

In developed countries, cancer represents the second leading cause of death, exceeded only by heart disease. Despite the litany of improvements made in cancer diagnosis, treatment, and prognosis since President Nixon first declared a "War on Cancer" in 1971, ~1,500 people die from cancer every day in the U.S., with the four deadliest cancers being lung, colon, breast, and pancreatic (1). Today, there are a multitude of research efforts aimed at improving patient survival, including, among many others, the search for new natural products, the synthesis of chemotherapeutic agents, the development of improved anticancer drug delivery methods, and the understanding of how cancers originate, grow, and metastasize.

Natural products have been a very successful source of new antineoplastic agents, contributing to >60% of the anticancer pharmaceuticals in use today (2). Of these, two analogues of camptothecin, an alkaloid isolated from *Camptotheca acuminata* reported in 1966 (3), are increasingly in clinical use (4–7). Camptothecins have had their greatest utility in treating primary and metastatic colon carcinoma, platinum-refractory ovarian cancer, and small cell lung carcinoma. Of the \$9 billion annual market for cancer chemotherapeutics in 2004, the Food and Drug Administration (FDA)-approved camptothecins, topotecan and irinotecan (Fig. 1), accounted for >\$1 billion, indicative of their growing use by tens of thousands of individuals (4).

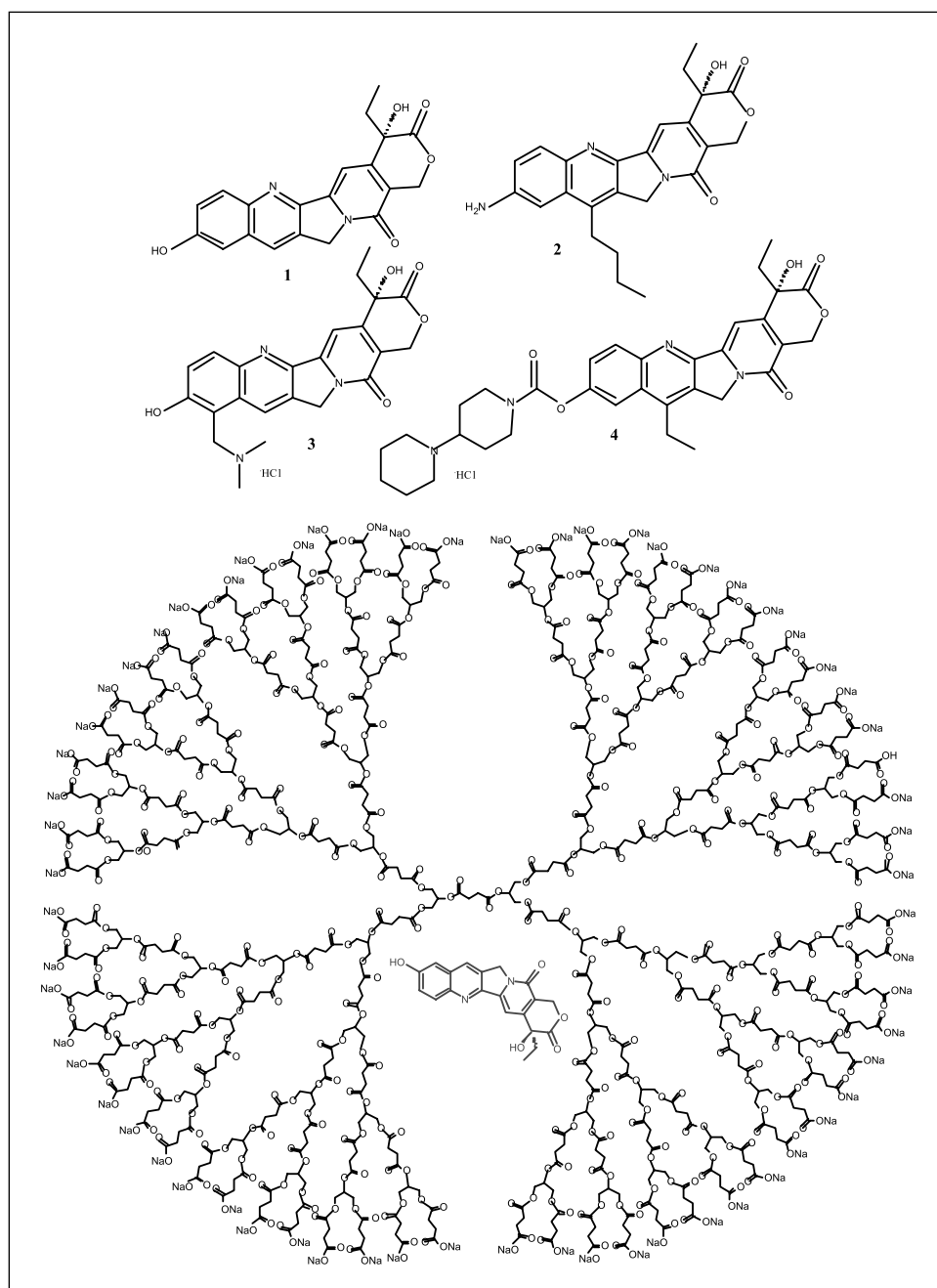
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doi:10.1158/0008-5472.CAN-06-2066

Like many natural products, one of the major anticancer benefits of camptothecin research has been the elucidation of its unique and novel mode of action, which is the poisoning of DNA topoisomerase I during its catalytic cycle of DNA relaxation (8–10). As a pharmaceutical candidate, however, camptothecin did not have optimal properties, particularly with respect to aqueous solubility, and failed in clinical trials conducted in the 1970s (4). Besides the two aforementioned water-soluble analogues that are currently approved by the FDA, there were at least a dozen other analogues of camptothecin in clinical trials as of 2003 (11). Many of these new analogues attempt to circumvent the poor water solubility of camptothecins while retaining anticancer potency and, importantly, minimizing its side effects (12). Although irinotecan and topotecan continue to find growing use in clinical oncology, major side effects caused by the water-solubilizing functionalities include severe (grade 3) to life-threatening (grade 4) diarrhea and myelosuppression, respectively, which are the primary dose-limiting toxicities associated with these treatments (13, 14). Because of these inadequacies, there is significant incentive to develop alternative prodrugs or drug delivery systems for camptothecins that will reduce side effects and enhance potency.

As mentioned above, camptothecins, like a large number of highly active pharmaceuticals, lack appreciable water solubility. Therefore, these drugs are either not used clinically, delivered in large volumes of aqueous solution, delivered in conjunction with surfactants (e.g., Cremophor EL), chemically derivatized to afford soluble prodrugs, or linked to large water-soluble polymers (e.g., PEGylation), all of which may result in side effects or reduced anticancer efficacy (15, 16).

Approaches to delivering unaltered natural products using polymeric carriers is of widespread interest (17, 18). Recently, dendritic polymers (19–25) have been explored for the encapsulation of hydrophobic compounds and for the delivery of anticancer drugs. Dendrimers are globular, highly branched macromolecules possessing a well-defined core, an interior region, and a large number of end groups. The physical characteristics of dendrimers, including their monodispersity, water solubility, encapsulation ability, and large number of functionalizable peripheral groups, make these macromolecules ideal candidates for evaluation as drug delivery vehicles. Several previous reviews have covered the early work of drug delivery with dendrimers (25, 26). Currently, there are three methods for using dendrimers in drug delivery: (a) the drug is covalently attached to the periphery of the dendrimer to form dendrimer prodrugs, (b) the drug is coordinated to the outer functional groups via ionic interactions, or (c) the dendrimer acts as a unimolecular micelle by



**Figure 1.** *Top*, chemical structures of (1) 10-hydroxycamptothecin (10HCPT), (2) 7-butyl-10-aminocamptothecin (BACPT), (3) topotecan (Hycamtin), and (4) irinotecan (Camptosar). *Bottom*, chemical structure of the [G4.5]-PGLSA-COONa dendrimer with encapsulated 10HCPT (1). Not drawn to scale.

encapsulating a pharmaceutical through the formation of a dendrimer-drug (i.e., host-guest) supramolecular assembly.

The latter approach is of interest for multiple reasons and provides an opportunity to encapsulate pharmacologically active compounds and to study the supramolecular assemblies formed in these systems. For example, rose bengal (27) and acetylsalicylic acid (28) have been noncovalently encapsulated within poly(propylene imine) and poly(amidoamine) dendrimers. In the case of rose bengal, the internalized dye molecules were confined within the dendrimer as a consequence of steric congestion at the dendrimer periphery (27). Pyrene was encapsulated within both poly(propylene imine) dendrimers and unimolecular micelles based on PEGylated Fréchet-type dendrimers (29, 30). Additionally, fluorescent dyes such as phenol blue (31) and 4-(dicyanomethylene)-2-methyl-6-(4-

dimethylaminostyryl)-4H-pyran (32) have been encapsulated. In a further example, poly(amidoamine) dendrimers have been used to enhance the delivery of ibuprofen to A549 lung epithelial cells (33).

We are synthesizing and evaluating dendrimers composed of natural metabolites such as glycerol and succinic acid for medical uses (34–39). We have found that crosslinkable derivatives of these dendrimers are useful for the repair of ophthalmic wounds and articular cartilage (35, 37, 40–42). Recently, we described the encapsulation characteristics of a poly(glycerol-succinic acid) (PGLSA) dendrimer and the use of this macromolecule as a potential vehicle for drug delivery (43). In this report, we expand on the potential use of these polyester dendrimers for the delivery of potent and poorly water-soluble anticancer drugs, like the camptothecins. These PGLSA dendrimers are attractive for cancer

therapy because: (a) the chemical composition, physical structure, and molecular weight can be precisely controlled; (b) the dendrimer structure permits diverse and extensive site-specific functionalization; (c) the container-like properties enable the entrapment and delivery of poorly water-soluble anticancer agents; and (d) the potential for enhanced uptake of the dendrimer in specific cell lines or reduced drug efflux from the cells. Herein, we report the encapsulation of 10-hydroxycamptothecin (10HCPT) and 7-butyl-10-aminocamptothecin (BACPT) within PGLSA dendrimers and the cytotoxicity of these supramolecular assemblies towards human breast adenocarcinoma (MCF-7), colorectal adenocarcinoma (HT-29), non-small cell lung carcinoma (NCI-H460), and glioblastoma (SF-268) cell lines. We also report the effect of encapsulating 10HCPT within PGLSA dendrimers on drug uptake and efflux rates when exposed to MCF-7 cells.

## Materials and Methods

### Chemicals and Instrumentation

All solvents were dried and freshly distilled prior to use (pyridine with CaH and THF with Na). All chemicals were purchased from Aldrich or Acros at the highest purity grade available and used without further purification. All reactions were done under nitrogen atmosphere at room temperature unless specified otherwise. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian INOVA spectrometer (operating at 400 and 100.6 MHz for  $^1\text{H}$  and  $^{13}\text{C}$  NMR, respectively). Fourier transform IR (FTIR) spectra were recorded on a Nicolet Smart MIRacle Avatar 360 using a zinc selenide crystal. MALDI-TOF mass spectra were obtained using a PerSpective Biosystems Voyager-DE Biospectrometry Workstation operating in the positive ion mode using 2-(4-hydroxyphenylazo)-benzoic acid. UV-Vis spectra were recorded on a Hewlett Packard 8453 spectrophotometer. Fluorescence spectra were acquired on a Photon Technology International QM-4/2005 spectrofluorimeter. cLogP values were calculated using the ACD/LogP DB, version 9.09 (Advanced Chemistry Development, Inc., Toronto, Ontario, Canada).

### Synthesis

10HCPT was isolated from *C. acuminata* (44) and BACPT was synthesized as described recently (45). The synthesis of the hydroxyl-terminated generation four-PGLSA dendrimer, [G4]-PGLSA-OH, was carried out as described in a previous publication (34). The synthesis of the carboxylated derivative of the [G4]-PGLSA-OH, which was used in this study, is described below.

**Synthesis of [G4.5]-PGLSA-COONa.** [G4]-PGLSA-OH (0.140 g, 0.0131 mmol) was dissolved in pyridine (10 mL) and stirred while succinic anhydride (0.167 g, 1.68 mmol) was added. The reaction mixture was stirred for 16 hours before the pyridine was removed under reduced pressure. The contents were partially dissolved in  $\text{CH}_2\text{Cl}_2$  (15 mL), 0.1 N HCl (15 mL) was added, and then the mixture was stirred for an additional 15 minutes. After stirring, the organic and aqueous phases separated, and a layer was formed between the two phases. Although avoiding the interface, most of the aqueous and organic phases were removed. This washing procedure, using 15 mL of  $\text{CH}_2\text{Cl}_2$  and 0.1 N of HCl, was repeated twice. Any remaining organic or aqueous phase was removed first by rotoevaporation followed by lyophilization to yield 0.191 g of a highly viscous liquid (85% yield). To dissolve the dendrimer in water, deionized water (10 mL) and brine (0.5 mL) were added to the solution, and 0.05 N of NaOH was added drop-wise to the stirring solution until the pH remained at 7.0. The dendrimer was purified via dialysis with 7,000 molecular weight cutoff dialysis tubing for 24 hours in deionized water. The water was then removed via lyophilization to obtain a white solid.  $^1\text{H-NMR}$  ( $\text{D}_2\text{O}$ ):  $\delta$  2.32 (m, 130,  $-\text{CH}_2-\text{CH}_2-$ ), 2.46 (m, 133,  $-\text{CH}_2-\text{CH}_2-\text{CH}_2-$ ), 2.58 (m, 228,  $-\text{CH}_2-\text{CH}_2-$ ) 4.13 to 4.21 (m, 240,  $-\text{CH}_2-\text{CH}-\text{CH}_2-$ ), 5.18 (m, 62,  $-\text{CH}_2-\text{CH}-\text{CH}_2-$ ).  $^{13}\text{C NMR}$  ( $\text{D}_2\text{O}$ ):  $\delta$  180.72 (COOH), 175.37 (COOH), 173.52 (COOR), 70.14 (CH), 69.76 (CH), 62.80 (CH<sub>2</sub>), 34.31 (CH<sub>2</sub>), 32.10 (CH<sub>2</sub>), 30.72 (CH<sub>2</sub>), (CH<sub>2</sub>), 29.01 (CH<sub>2</sub>). FTIR:  $\nu$  ( $\text{cm}^{-1}$ ) 3,368 (OH), 2,964 (aliphatic C-H stretch), 1,732 (C = O), 1,567 (asymmetric COO<sup>-</sup> stretch), 1,409 (symmetric COO<sup>-</sup>

stretch), 1,149 (C-O stretch). MALDI mass spectrometry 17,168  $m/z$  ( $\text{M} + \text{Na}$ )<sup>+</sup>, 8602  $m/z$  ( $\text{M} + \text{Na}$ )<sup>2+</sup> [theory, 17,120.0  $m/z$  ( $\text{M}^+$ )].

### Encapsulation Procedure

The encapsulation procedure requires both the dendrimer and hydrophobic compound to be soluble in a volatile organic solvent that is miscible with water.

**10HCPT encapsulated within [G4.5]-PGLSA-COONa.** For molar calculations, we assumed a molecular weight of 17,823 for the dendrimer, corresponding to a half-protonated/half-sodium salt carboxylic acid-terminated dendrimer. Twenty-five milligrams ( $1.4 \times 10^{-6}$  mol) of the [G4.5]-PGLSA-COONa dendrimer was dissolved in 2.0 mL of  $\text{CH}_3\text{OH}$ . A solution of 10HCPT (0.5 mg/ $1.4 \times 10^{-6}$  mol) in 1.0 mL of  $\text{CH}_3\text{OH}$  was added to the dendrimer solution and stirred for 10 minutes. Next, 1.0 mL of water was added to the  $\text{CH}_3\text{OH}$  solution and stirred for 1 hour. The uncovered solution was then stirred overnight in the dark to allow the  $\text{CH}_3\text{OH}$  to slowly evaporate. The remaining  $\text{CH}_3\text{OH}$  was removed via rotary evaporation over several hours. A small amount of drug precipitated from the solution and was removed via centrifugation. The concentration of the encapsulated 10HCPT was measured via UV-Vis ( $\lambda_{\text{max}} = 382$  nm;  $\epsilon_{382} = 28,000$ ) and was found to be 240  $\mu\text{mol/L}$ . The encapsulated drug-dendrimer solution was then stored in the dark, at room temperature, until further use. All experiments were done within 24 hours of preparing the sample. A similar procedure was used for encapsulating BACPT and the concentration of encapsulated BACPT was determined to be 440  $\mu\text{mol/L}$  ( $\lambda_{\text{max}} = 339$  nm;  $\epsilon_{339} = 15,020$ ). Samples for NMR analysis were prepared using deuterated solvents.

### NMR Experiments

NMR data on the encapsulated species were recorded at 25°C in 5 mm NMR tubes using Varian Inova 500 and 600 MHz NMR spectrometers with 5 mm Varian probes. The 500 MHz  $^1\text{H-NMR}$  spectra of the drug, dendrimer, and drug-dendrimer complex were obtained with a spectral width of 5.5 kHz, a 77-degree pulse flip angle (5  $\mu\text{s}$ ), a 5.8 second acquisition time, 1 second relaxation delay, and digitized using 64,000 points to obtain a digital resolution of 0.17 Hz/pt. One-dimensional difference NOE spectra (NOEDS) for the dendrimer/BACPT sample were generated from a spectrum recorded with a 6.5-second selective on-resonance irradiation of a succinic acid methylene signal at an estimated power level of 0.1 mW and a 5.8-second acquisition period to build up the steady state NOE and a control spectrum irradiated off-resonance of the methylene signal. Suppression of the water signal was achieved with a separate 1.5-second long presaturation pulse at the water frequency incorporated within the 6.5-second irradiation period. To improve free induction decay (FID) subtraction, the dendrimer/BACPT sample was equilibrated in the magnet for 30 minutes before recording data. The NOEDS were obtained in an interleaved manner with eight scans accumulated for the on- or off-resonance FID and looping around 128 times to achieve a good signal-to-noise ratio with 1,024 scans per FID.

### Human Cancer Cell Panel

The cytotoxicity of 10HCPT, BACPT, and their corresponding dendrimer encapsulation formulations were evaluated in a human cancer cell panel using a procedure described previously (46). MCF-7 human breast carcinoma (Barbara A. Karmanos Cancer Center, Detroit, MI), NCI-H460 human large cell lung carcinoma (American Type Culture Collection, Manassas, VA), and SF-268 human astrocytoma (NCI Developmental Therapeutics Program, Frederick, MD) cell lines were all adapted and maintained in RPMI 1640 supplemented with fetal bovine serum (Life Technologies/Invitrogen, Carlsbad, CA) at 10% (v/v) and the antibiotics, penicillin G (100 units/mL) and streptomycin sulfate (100  $\mu\text{g/mL}$ ), in a humidified 5%  $\text{CO}_2$  atmosphere kept at 37°C. HT-29 human colorectal adenocarcinoma cells (American Type Culture Collection) were maintained in McCoy's 5A medium (Life Technologies/Invitrogen) under the same conditions described above. Strict attention was paid to using cells when in the logarithmic phase of cell growth. Cells were harvested from subconfluent cultures using trypsin/EDTA (0.05%/0.02%) and were suspended in medium. Cell viability was determined using a sulforhodamine B (SRB) protein binding assay.

Cell suspensions were first prepared at densities of 3,000 (MCF-7), 1,500 (NCI-H460), 10,000 (SF-268), or 4,000 (HT-29) cells per 50  $\mu\text{L}$  of medium for each well of 96-well culture dishes. The medium of each well was then replaced with 100  $\mu\text{L}$  of antibiotic-free medium containing various concentrations of the dendrimer, free camptothecin, or dendrimer-encapsulated camptothecin. Three replicates were tested for each concentration. For  $\text{IC}_{50}$  determinations, the formulations were diluted serially in half-log steps. Blank wells and wells with media but no cells were included for background correction because trichloroacetic acid (TCA)-precipitated proteins from serum alone result in some background SRB absorbance.

After a 3-day continuous exposure, cells were fixed by the addition of 25  $\mu\text{L}$  of cold 50% (w/v) TCA to the growth medium in each well at 4°C for 1 hour, then washed five times with water. The TCA-fixed cells were then stained for 30 minutes with 50  $\mu\text{L}$  of 0.4% (w/v) SRB in 1% (v/v) acetic acid followed by five rinses with 1% (v/v) acetic acid to remove unbound dye. The fixed, stained plates were air-dried and bound dye was then solubilized by incubation with 100  $\mu\text{L}$  of 10 mmol/L Tris base for at least 5 minutes. Absorbance was measured at 540 nm using a Tecan Ultra multiplate reader. Absorbance measured on wells containing cells that did not receive the drug represented 100% growth, and absorbance measured on wells containing no cells represented 0% growth. For  $\text{IC}_{50}$  calculations, survival data were evaluated by variable slope curve-fitting using Prism 4.0 software (GraphPad, San Diego, CA).

### Cell Uptake and Efflux Studies

The intracellular accumulation and retention of 10HCPT with and without dendrimer encapsulation was measured. MCF-7 cells were plated into 12-well plates at a density of  $3 \times 10^5$  cells/well and incubated overnight before cell uptake and efflux experiments. The medium from each well was replaced with 1 mL of medium containing either free 10HCPT or dendrimer-encapsulated 10HCPT at a drug concentration of 1, 5, or 8  $\mu\text{mol/L}$ . In the first set of trials, the cellular uptake of both free and dendrimer-encapsulated 10HCPT was measured. After 2, 10, or 24 hours of drug exposure, the medium was removed, and the attached cells were washed directly with PBS and lysed with 200  $\mu\text{L}$  of DNase I lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 5 mmol/L  $\text{MgCl}_2$ , 5% glycerol, 0.05 mg/mL DNase I, 0.25% (w/v) SDS, and 10 mmol/L DTT; ref. 47]. No floating cells were detectable in these studies, even after 24 hours of treatment with the highest drug concentration.

In the second set of experiments, the retention of both free and dendrimer-encapsulated 10HCPT was measured. MCF-7 cells were exposed to medium containing either 10HCPT or dendrimer-encapsulated 10HCPT at a concentration of 1, 5, or 8  $\mu\text{mol/L}$  for 24 hours. At the end of 24 hours of drug exposure, the medium was removed, cells washed with PBS, and then replaced with fresh medium devoid of 10HCPT or dendrimer-encapsulated 10HCPT. At 0.5, 8, or 32 hours after incubation in the drug-free media, the medium was removed and floating cells were recovered by centrifugation at  $1,000 \times g$  for 5 minutes. The cell monolayer was washed with PBS as above and the floating cell pellet was lysed together with the monolayer using the DNase I lysis buffer (47) described above.

In both sets of experiments, the concentrations of 10HCPT in the cell lysate samples were determined fluorometrically using a standard curve via excitation at 382 nm, and measuring the emission at 550 nm. Fluorescence spectroscopy was used instead of UV-Vis spectroscopy for better accuracy at low concentration.

## Results and Discussion

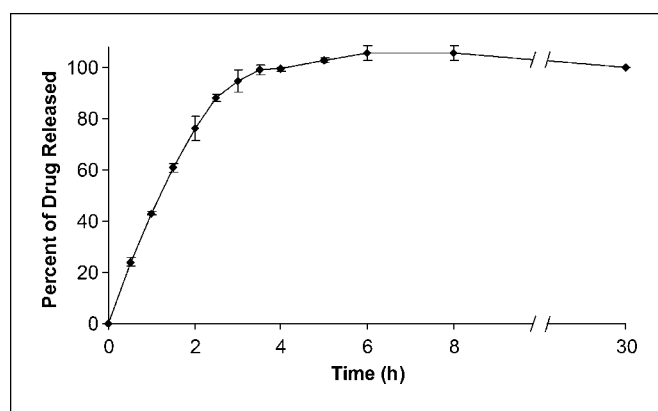
**Encapsulation studies.** The carboxylate-terminated fourth generation [G4.5]-PGLSA-COONa dendrimer (Fig. 1) was used in these encapsulation studies because it provided better encapsulation characteristics than the smaller generation dendrimers and the hydroxy-terminated dendrimers (43). The [G4.5]-PGLSA-COONa dendrimer was synthesized and possessed a molecular weight of 17,823 (half sodium salt). It was fully characterized by NMR, FTIR, and MALDI-TOF mass spectrometry.

10HCPT (1) and BACPT (2) were selected for encapsulation because: (a) both camptothecins possess poor aqueous solubility ( $<25 \mu\text{mol/L}$ ), (b) BACPT is more lipophilic than 10HCPT based on  $\text{cLogP}$  of 3.14 versus 1.63, respectively, (c) 10HCPT and BACPT exhibit different cell line cytotoxicity profiles, and (d) BACPT is more cytotoxic than 10HCPT.

The encapsulation procedure required both the dendrimer and the hydrophobic camptothecin to be soluble in a volatile organic solvent that is miscible with water. Briefly stated, a 1:1 molar ratio of the dendrimer and drug was dissolved in methanol and water, and then the methanol was removed to afford the dendrimer-encapsulated camptothecin. The concentration of the encapsulated 10HCPT was determined to be 240  $\mu\text{mol/L}$ , which represents an  $\sim 10$ -fold increase in the water solubility of 10HCPT ( $\approx 20 \mu\text{mol/L}$ ). The concentration of encapsulated BACPT was determined to be 440  $\mu\text{mol/L}$ , an  $\sim 10$ -fold increase in the water solubility of BACPT ( $\approx 40 \mu\text{mol/L}$ ).

To assess the drug release profile of the encapsulated 10HCPT/[G4.5]-PGLSA-COONa vehicle, we monitored the release of 10HCPT in PBS buffer at a pH of 7.4. Specifically, a solution of dendrimer-encapsulated 10HCPT was prepared using the encapsulation procedure described previously, and the drug concentration in the solution was determined to be 218  $\mu\text{mol/L}$ . Next, a 100  $\mu\text{L}$  aliquot of the dendrimer-encapsulated 10HCPT was added to 3 mL of PBS buffer and subsequently transferred into a dialysis cassette (0.5–3 mL, Slide-A-Lyser; Pierce, Rockford, IL) with a molecular weight cutoff of 3,500. The dialysis cassette was then placed into a beaker containing 100 mL of PBS buffer at 37°C. Next, 200  $\mu\text{L}$  aliquots of the buffer were removed from the beaker at different time points over the next 30 hours. The concentration of 10HCPT in the buffer aliquots at each time point was determined by fluorescence spectroscopy ( $\lambda_{\text{Ex}} = 382 \text{ nm}$  and  $\lambda_{\text{Em}} = 550 \text{ nm}$ ). A series of nine samples having known concentrations of 10HCPT were used to create a calibration curve from which the concentrations of the unknown samples were determined. This experiment was done in duplicate. The results are displayed in Fig. 2 and show that the drug is released relatively linearly over the first 2 hours and reaches full release at  $\sim 6$  hours. Based on the release profile, the dendrimer vehicle is likely to be more effectively used for intratumoral delivery instead of i.v.

**NMR characterization of dendrimer-encapsulated camptothecins.** To further characterize the supramolecular assembly formed between the dendrimer and the camptothecin analogues,



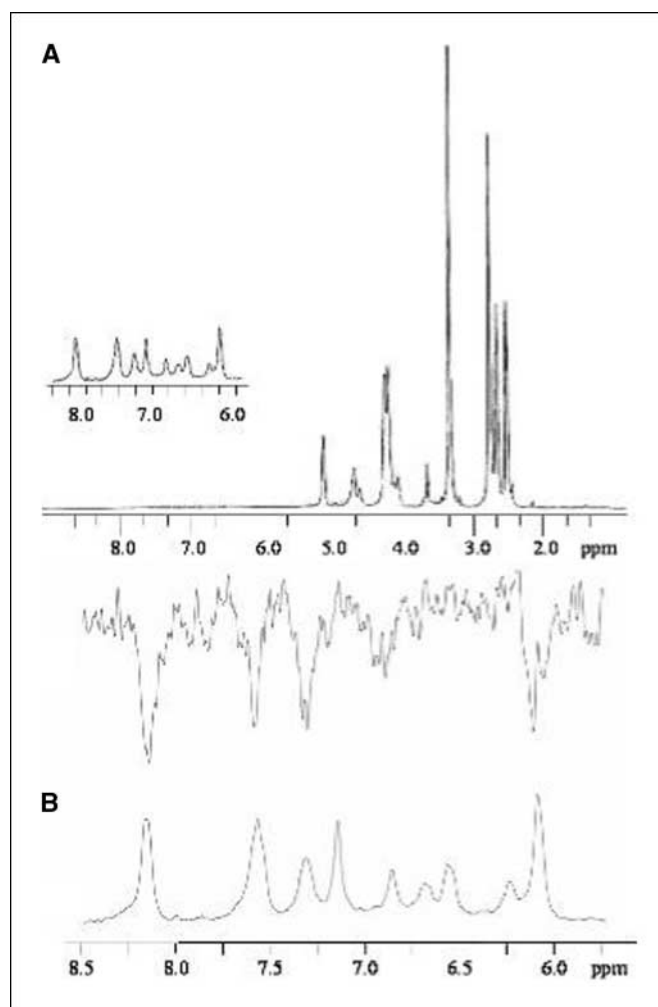
**Figure 2.** [G4.5]-PGLSA-COONa encapsulated 10HCPT release profile. Points, mean; bars, range ( $n = 2$ ).

we did a series of NMR experiments. In the  $^1\text{H}$ -NMR spectra, the aromatic protons of the encapsulated 10HCPT and BACPT are distinct and downfield from the dendrimer protons. Selective irradiation of the internal succinic acid protons of the dendrimer reveals NOE dipolar interactions to the aromatic proton resonances of the camptothecins. As shown in Fig. 3, the NOESY, with selective irradiation of the internal succinic acid protons (2.68 ppm), reveal NOE dipolar interactions in four of the nine proton resonances of BACPT. Similar results were observed and reported previously with the encapsulated 10HCPT (data not shown; ref. 43). These data suggest internalization of the camptothecins within the dendrimer structure and not localization at the periphery. These findings were supported by performing a similar experiment on the peripheral succinic acid protons (2.55 ppm), which when irradiated, did not generate selective NOEs to the aromatic protons of 10HCPT or BACPT.

**Cytotoxicity studies (human colorectal adenocarcinoma, breast adenocarcinoma, non-small cell lung carcinoma, and glioblastoma cells).** Cytotoxicity experiments were done using standard NCI assay methods, which involved a 3-day drug exposure, followed by cellular fixation and quantification of cell survival by staining with SRB, and monitoring the absorbance at 562 nm. Studies were initiated with the HT-29 human colorectal adenocarcinoma line, a p53-null line representing one of the most common indications for currently FDA-approved camptothecins. To do so, a series of vehicle controls and controls relative to the encapsulated camptothecins were generated, specifically: (a) 10HCPT dissolved in DMSO for both stock solution and subsequent dilutions—"DMSO"; (b) 10HCPT dissolved in DMSO with subsequent dilutions using distilled, deionized water—"DMSO/Water"; (c) 10HCPT dissolved in DMSO and then simply diluted in water already containing the dendrimer—"Simple mix"; and (d) 10HCPT encapsulated within the dendrimer using the solvent evaporation technique followed by vehicle dilution in aqueous solution—"Encapsulated." In addition, the dendrimer alone was tested in aqueous solution at a concentration of 0.34 mg/mL, which is higher than the drug delivery studies. The dendrimer alone was not cytotoxic ( $98.1 \pm 1.8\%$  survival, in quadruplicate in each of three experiments).

The results of a single representative experiment with 10HCPT in HT-29 cells are shown in Fig. 4. The  $\text{IC}_{50}$  of the dendrimer-10HCPT assembly was 32.3 nmol/L. This dendrimer-10HCPT assembly had a 3.5-fold increase in potency relative to DMSO-dissolved 10HCPT and a 4.1-fold increase in comparison to DMSO-dissolved 10HCPT stock with subsequent dilutions made in water. This experiment was repeated twice with another preparation of dendrimer-encapsulated drug, and the same activity trend of enhanced potency relative to DMSO preparations was observed. DMSO was used in these experiments to increase the solubility of 10HCPT, but this approach is not acceptable for clinical use.

To determine whether the increase in anticancer activity conferred by the dendrimer-encapsulated 10HCPT was observed with other cell lines, the 10HCPT dissolved in DMSO and the dendrimer-encapsulated 10HCPT formulations were compared in a diverse human cancer cell panel (Fig. 5). This cell line panel consists of the MCF-7 human breast adenocarcinoma, the NCI-H460 human large cell lung carcinoma, and the SF-268 human astrocytoma. Data are expressed as the  $\text{IC}_{50}$  values for each drug using a standard 3-day continuous exposure followed by fixation and SRB staining. Interestingly, the dendrimer-encapsulated 10HCPT exhibited an improved degree of potency relative to the DMSO-dissolved drug in each cell line. Specifically, the  $\text{IC}_{50}$  values



**Figure 3.** Top,  $^1\text{H}$ -NMR spectrum of BACPT encapsulated within [G4.5]-PGLSA-COONa ( $\text{D}_2\text{O}$ , ppm from TMS) in which the singlet and doublet in the aliphatic region (2–3 ppm) correspond to the interior and exterior succinic acid protons of the dendrimer, respectively (inset, expansion of aromatic region). Bottom, one-dimensional  $^1\text{H}$ -NOESY (A) between encapsulated BACPT (B) and interior succinic acid protons of [G4.5]-PGLSA-COONa.

for the DMSO dissolved 10HCPT compared with the dendrimer-encapsulated 10HCPT was reduced from 72.0 to 10.1 nmol/L for the MCF-7-treated cells, from 32.4 to 16.7 nmol/L for the NCI-H460-treated cells, and from 13.1 to 4.6 nmol/L for the SF-268-treated cells. Four important observations were noted: first, the use of the dendrimer lowered the  $\text{IC}_{50}$ s compared with the 10HCPT dissolved in DMSO (e.g., 2- to 7-fold); second, the magnitude of effect with the dendrimer carrier is not the same for each cell line; third, the greatest magnitude of enhanced potency was in the least sensitive cell line (MCF-7); and fourth, potentially clinically relevant,  $\text{IC}_{50}$ s (<20 nmol/L) were observed for three distinct cancer cell lines.

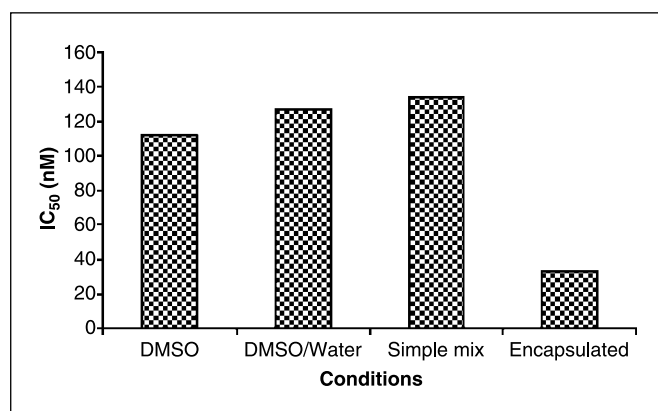
We next evaluated a more lipophilic camptothecin analogue, BACPT, which possesses an  $\sim 10$ -fold greater cytotoxic activity in most tumor cell lines as compared with 10HCPT (Fig. 1), possibly due to structural characteristics that enhance the interaction with DNA topoisomerase I (45). Due to its limited aqueous solubility BACPT, like 10HCPT, is a challenge in pharmaceutical formulation and subsequent use in the clinic. The dendrimer encapsulation process afforded an even greater aqueous solubility to the BACPT of 440  $\mu\text{mol/L}$  (relative to 10HCPT). This result suggests that the

dendrimer may be of significant utility to improving the aqueous solubility of other camptothecin analogues.

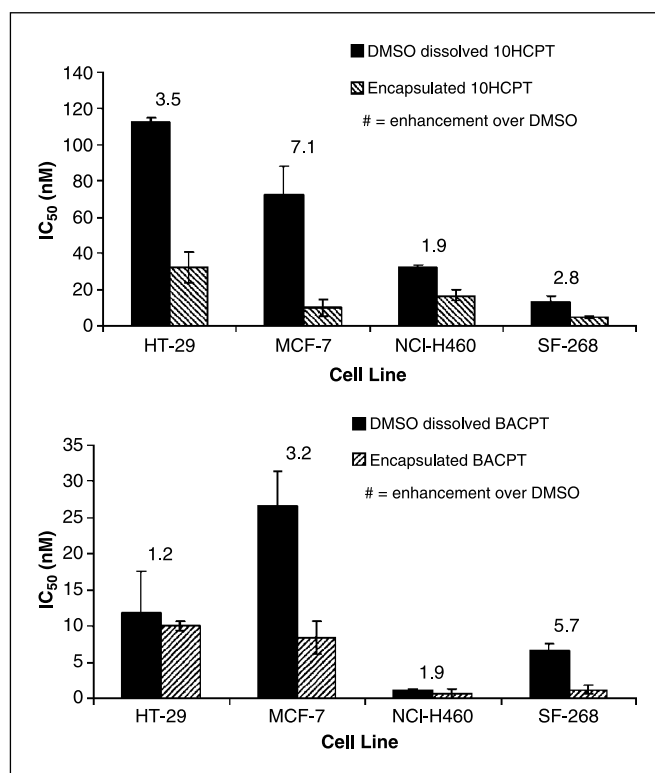
Surprisingly, unlike the case with 10HCPT, the dendrimer encapsulation afforded no additional increment of cytotoxicity toward HT-29 cells relative to DMSO-dissolved drug: 11.9 nmol/L in DMSO versus 10.0 nmol/L encapsulated in dendrimer (Fig. 5). However, like 10HCPT, it is notable that BAPCT is not used clinically, likely due to negligible aqueous solubility. Thus, encapsulation within the dendrimer formulation maintains an aqueous bioactivity similar to that in DMSO, thereby overcoming a major challenge in the potential clinical utility of this compound.

To determine whether the lack of additional enhancement of BAPCT activity conferred by the dendrimer was common to the other three cell lines investigated, the BAPCT dissolved in DMSO and dendrimer-encapsulated BAPCT preparations were compared in the human cancer cell panel as described earlier (Fig. 5). As before, these data are expressed as the  $IC_{50}$  values for each compound using a standard 3-day continuous exposure followed by fixation and SRB staining. The dendrimer-encapsulated BAPCT exhibited an improved degree of potency relative to the DMSO-dissolved agent in each of the other cell lines. Comparing the DMSO-dissolved BAPCT to the dendrimer-encapsulated BAPCT, the  $IC_{50}$  values were reduced from 26.7 to 8.3 nmol/L for the MCF-7-treated cells, from 1.2 to 0.6 nmol/L for the NCI-H460-treated cells, and from 6.6 to 1.2 nmol/L for the SF-268-treated cells. The greatest magnitude of enhanced potency was in the SF268 cell line (5.7-fold), and a 1- to 3-fold increase in potency was observed in the others. Importantly, low nanomolar to subnanomolar  $IC_{50}$ s were observed for the cell lines, with a 0.64 nmol/L  $IC_{50}$  for the NCI-H460 lung cancer cells.

**Influence of dendrimer on camptothecin cellular uptake and retention with MCF-7 breast adenocarcinoma cells.** Finally, the intracellular accumulation and retention of 10HCPT with and without dendrimer encapsulation was measured to help elucidate the potential mechanism underlying the dendrimer-mediated enhancement of cytotoxic potency. In the first set of trials, the cellular uptake of both free and dendrimer-encapsulated 10HCPT was measured. The MCF-7 cells were exposed to medium containing free 10HCPT or dendrimer-encapsulated 10HCPT at a drug concentration of 1, 5, or 8  $\mu$ mol/L. After 2, 10, or 24 hours of exposure, the medium was removed, and the cells were washed with buffer and lysed. The concentration of 10HCPT in the cell lysate samples was determined fluorometrically.



**Figure 4.** Columns,  $IC_{50}$  values (in nanomolar) of different 10HCPT formulations toward HT-29 cells.



**Figure 5.** Top, anticancer activity of dendrimer-encapsulated 10HCPT versus DMSO-dissolved 10HCPT expressed as  $IC_{50}$  values (in nanomolar). Bottom, anticancer activity of dendrimer-encapsulated BAPCT versus DMSO-dissolved BAPCT expressed as  $IC_{50}$  values (in nanomolar). Columns, mean; bars, SD ( $n = 3$ ).

The results displayed in Fig. 6, show that dendrimer-encapsulated 10HCPT was taken up by the cells at a significantly greater rate than the free 10HCPT at the same concentration. At the 2-hour mark, the dendrimer-encapsulated 10HCPT showed, on average, a 16-fold greater intracellular 10HCPT concentration than for free 10HCPT at the same exposure concentration. Even at the 10-hour mark, the encapsulated 10HCPT still showed approximately eight times the uptake of free 10HCPT at the same exposure concentration. Clearly, encapsulation of the 10HCPT within the dendrimer facilitated uptake.

A second set of experiments was done to measure the retention of both free and dendrimer-encapsulated 10HCPT. In these trials, MCF-7 cells were exposed to medium containing either free or dendrimer-encapsulated 10HCPT at a concentration of 1, 5, or 8  $\mu$ mol/L for 24 hours. At the end of the 24-hour exposure period, the medium was removed and replaced with fresh medium devoid of 10HCPT, free or encapsulated. After 0.5, 8, or 32 hours of exposure to the new media, the new medium was removed, and the cells were washed with buffer and lysed. The concentrations of 10HCPT in the cell lysate samples were determined fluorometrically as above. As shown in Fig. 6, even after 32 hours, the cells exposed to the dendrimer-encapsulated 10HCPT retained a higher amount of 10HCPT than those cells exposed to the same concentration of free drug. In fact, at 32 hours, the cells treated with the encapsulated 10HCPT retained ~50% of the amount of drug that they contained at 0.5 hours. For the cells treated with free 10HCPT, the cells retained only ~35% of the 10HCPT that they contained at the 0.5-hour mark. These data show that 10HCPT, when delivered as encapsulated in the dendrimer, is

both taken up by the cells at a much greater rate and is retained longer than the free 10HCPT. This may be a result of the reduced relative rate of drug efflux or simply enhanced relative retention. These results indicate that the increased cytotoxicity of the encapsulated 10HCPT toward the MCF-7 cells is likely due to higher intracellular levels of 10HCPT as a result of increased uptake and retention.

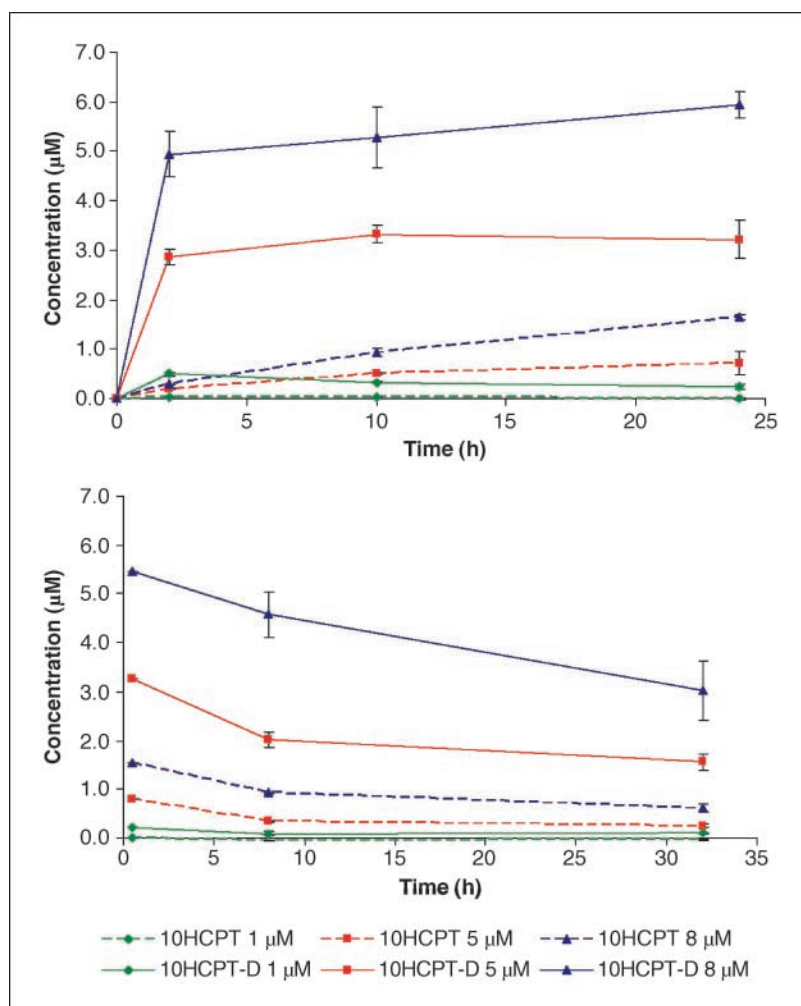
## Conclusions

The use of dendrimers as drug carriers by encapsulating hydrophobic drugs is a potential method for delivering highly active pharmaceutical compounds that may not be in clinical use due to their limited water solubility and resulting suboptimal pharmacokinetics. The dendrimers used in these studies are well-defined, single molecular weight, polyester macromolecules composed of the natural metabolites, succinic acid and glycerol. The encapsulation of two hydrophobic anticancer compounds, 10HCPT and BACPT, was carried out successfully using the solvent evaporation method. Encapsulation of the camptothecins within the dendrimer structure was confirmed by NMR spectroscopy.

The dendrimer-encapsulation procedure enhanced the potency of both 10HCPT and BACPT toward human cancer cell lines. The magnitude of the enhancement over the DMSO-dissolved

formulations was greatest for 10HCPT in MCF-7 cells and for BACPT in SF-268 cells, and the trends of the two systems are different. The most potent formulation was with dendrimer-encapsulated BACPT against lung cancer cells. These data are striking and of interest for six reasons. First, using the dendrimer vehicle affords significantly more camptothecin uptake than the DMSO formulation. Second, the differential rank order of enhanced potency leads us to hypothesize that using the dendrimer affords unique cell-specific uptake/retention characteristics. Third, and in support of this hypothesis, one can recall that the sensitivity of HT-29 cells to BACPT was not significantly enhanced by the dendrimer whereas sensitivity was enhanced in the other human cancer cell lines (breast, lung, central nervous system). Therefore, we are eager to submit these formulations for the NCI-60 cell line screening panel, as we anticipate that COMPARE analysis may reveal a non-camptothecin profile for each drug that might be suggestive of greater efficacy in organ systems in which camptothecins may not be under investigation currently for clinical applications. Fourth, the dendrimer increases aqueous solubility like DMSO, but the increased solubility alone does not account for the observed cytotoxicity profiles, as the DMSO and dendrimer preparations afforded different cytotoxicity profiles. Fifth, our initial studies afford formulations with low clinically relevant  $IC_{50}$ s for lung cancer cells. Sixth, the dendrimer provides a means to deliver the

**Figure 6.** *Top*, effect of dendrimer encapsulation on MCF-7 cellular uptake of 10HCPT. Cells were exposed to medium containing free (*dashed lines*) or encapsulated (*solid lines*) 10HCPT at 1, 5, or 8  $\mu\text{mol/L}$ , followed by removal of the medium, washing, and lysis of the cells at 2, 10, and 24 hours. The concentration of 10HCPT in the cell lysate samples was then determined fluorometrically. *Bottom*, effect of dendrimer encapsulation on retention of 10HCPT in MCF-7 cells. Cells were exposed to medium containing free (*dashed lines*) or encapsulated (*solid lines*) 10HCPT at 1, 5, or 8  $\mu\text{mol/L}$ , followed by removal of the medium, washing, and the addition of drug-free medium at 24 hours. After 0.5, 8, or 32 hours of exposure to the fresh media, the medium was removed, the cells washed and lysed together with floating cells pelleted from the media, and the concentration of 10HCPT in the cell lysate samples was then determined fluorometrically. Points, mean; bars, SD ( $n = 3$ ). 10HCPT, free drug; 10HCPT-D, dendrimer-encapsulated drug.



highly active BACPT in a small volume of aqueous solution—a current challenge that limits the preclinical development of this potent camptothecin analogue.

Through drug uptake and retention experiments with MCF-7 cells, it was observed that encapsulation of 10HCPT within the dendrimer resulted in greater intracellular drug concentration due both to increased uptake and improved retention, which collectively provides a rationale for the increased cytotoxicity observed for the drug-dendrimer complex. With this information in hand, we can hypothesize on the possible mechanism(s) for the increased uptake and resulting cytotoxicity of the camptothecins when using the dendrimer carrier. The dendrimer could be acting as (a) a depot for the camptothecin which releases the camptothecin into the aqueous solution over time for subsequent uptake by the cells—but this proposal is not consistent with the data observed between cell lines and the DMSO controls; (b) a vehicle that transports the camptothecin to the cell membrane for subsequent passive absorption of the camptothecin or the camptothecin-dendrimer complex via the intracellular junctions—although this transport route is generally accepted for smaller materials of <1.5 nm (48); or (c) a vehicle and its cargo that is taken up by the endocytosis pathway. The last proposal is more likely and would be consistent with reports on anionic carboxylated PAMAM and hydroxyl terminated PAMAM dendrimers which are proposed to enter the cell through fluid-phase endocytosis (49, 50). Cell trafficking studies with labeled dendrimer with and without the camptothecin are planned to elucidate the role of the dendrimer in the delivery of camptothecins.

In summary, the delivery of hydrophobic molecules, such as the camptothecins, can be accomplished using these biodegradable, biocompatible dendrimers that increase aqueous solubility by approximately an order of magnitude without the introduction of additional functional groups or other compounds to the formulation that may present unwanted effects. Thus, these results may provide a reason to reinvestigate once promising anticancer compounds, which may not have been developed because these compounds could not be formulated for preclinical evaluation, let alone clinical use. Moreover, the dendrimer enhances both the uptake and retention of these compounds within cancer cells, a finding that was not anticipated at the onset of these studies. This also opens up new avenues of research into the further development of drug-dendrimer complexes specific for a cancer and/or targeted organ system. These encouraging results provide further impetus to design, synthesize, and evaluate dendritic polymers for use in basic drug delivery studies and eventually in the clinic.

## Acknowledgments

Received 6/6/2006; revised 9/19/2006; accepted 10/19/2006.

**Grant support:** In part by the Johnson and Johnson Focused Giving Program and the NIH. Partial support via a Research Scholar Grant (RSG-02-024-01-CDD) from the American Cancer Society (N.H. Oberlies) and NIH grant R01 CA104286 from The National Cancer Institute (D.J. Kroll).

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The authors wish to thank Anthony Ribeiro for help with the NMR experiments.

## References

- Anonymous. Cancer facts & figures 2002–2005 American Cancer Society. Available from: <http://www.cancer.org>, 2002.
- Cragg GM, Newman DJ, Snader KM. Natural products in drug discovery and development. *J Nat Prod* 1997;60:52–60.
- Wall M, Wani M, Cook C, Palmer K, McPhail AT, Sim GA. Plant antitumor agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from *Camptotheca acuminata*. *J Am Chem Soc* 1966;88:3888–90.
- Oberlies NH, Kroll DJ. Camptothecin and taxol: historic achievements in natural products research. *J Nat Prod* 2004;67:129–35.
- Sriram D, Yogeewari P, Thirumurugan R, Bal TR. Camptothecin and its analogues: a review on their chemotherapeutic potential. *Nat Prod Res* 2005;19:393–412.
- Thomas CJ, Rahier NJ, Hecht SM. Camptothecin: current perspectives. *Bioorg Med Chem Lett* 2004;12:1585–604.
- Garcia-Carbonero R, Supko JG. Current perspectives on the clinical experience, pharmacology, and continued development of the camptothecins. *Clin Cancer Res* 2002;8:641–61.
- Del Bino GD, Lassota P, Darzynkiewicz Z. The S-phase cytotoxicity of camptothecin. *Exp Cell Res* 1991;193:27–35.
- Ma MK, McLeod HL. Lessons learned from the irinotecan metabolic pathway. *Curr Med Chem* 2003;10:41–9.
- Hsiang YH, Liu LF. Identification of mammalian DNA topoisomerase I as an intracellular target of the anticancer drug camptothecin. *Cancer Res* 1988;48:1722–6.
- Uehara Y. Natural product origins of Hsp90 inhibitors. *Curr Cancer Drug Targets* 2003;3:325–30.
- Hatefi A, Amsden B. Camptothecin delivery methods. *Pharm Res* 2002;19:1389–99.
- Hecht J. Gastrointestinal toxicity of irinotecan. *Oncology* 1998;12:72–8.
- Armstrong DK. Topotecan dosing guidelines in ovarian cancer: reduction and management of hematologic toxicity. *Oncologist* 2004;9:33–42.
- Greenwald RB, Choe YH, McGuire J, Conover CD. Effective drug delivery by PEGylated drug conjugates. *Adv Drug Deliv Rev* 2003;55:217–50.
- Driver RW, Yang LX. Synthesis and pharmacology of new camptothecin drugs. *Mini Rev Med Chem* 2005;5:425–39.
- Kopecek J. Soluble biomedical polymers. *Polym Med* 1977;7:191–221.
- Uhrich KE, Cannizzaro SM, Langer R, Shakesheff KM. Polymeric systems for controlled drug release. *Chem Rev* 1999;99:3181–98.
- Boas U, Christensen JB, Heegaard PMH. Dendrimers in medicine and biotechnology. Cambridge (UK): RSC Publishing; 2006.
- Svenson S, Tomalia DA. Dendrimers in biomedical applications—reflections on the field. *Adv Drug Deliv Rev* 2005;57:2106–29.
- Schultz LG, Zimmerman SC. Dendrimers: potential drugs and drug delivery agents. *Pharm News* 1999;6:25–9.
- Liu H, Farrell S, Uhrich KE. Drug release characteristics of unimolecular polymer micelles. *J Control Release* 2000;68:167–74.
- Liu M, Kono K, Fréchet MJM. Water-soluble dendritic unimolecular micelles: their potential as drug delivery agents. *J Control Release* 2000;65:121–31.
- Malik N, Wimattanapatapee R, Klopsch R, et al. Dendrimers: relationship between structure and biocompatibility *in vitro*, and preliminary studies on the biodistribution of I-125-labelled polyamidoamine dendrimers *in vivo*. *J Control Release* 2000;65:133–48.
- Esfand R, Tomalia DA. Poly(amidoamine) (PAMAM) dendrimers: from biomimicry to drug delivery and biomedical applications. *Drug Discov Today* 2001;6:427–36.
- Liu MJ, Fréchet MJM. Designing dendrimers for drug delivery. *Pharm Sci Technol Today* 1999;2:393–401.
- Jansen J, Debrabandervandenberg EMM, Meijer EW. Encapsulation of guest molecules into a dendritic box. *Science* 1994;266:1226–9.
- Naylor A, Goddard W, Kiefer G, Tomalia D. Starburst dendrimers. Molecular shape control. *J Am Chem Soc* 1989;111:2339–41.
- Pistolis G, Malliaris A. Study of poly(propylene imine) dendrimers in water, by exciplex formation. *Langmuir* 2002;18:246–51.
- Hawker CJ, Wooley KL, Fréchet MJM. Unimolecular micelles and globular amphiphiles: dendritic macromolecules as novel recyclable solubilization agents. *J Chem Soc [Perkin 1]* 1993;12:1287–97.
- Richter-Egger D, Tesfai A, Tucker S. Spectroscopic investigations of poly(propyleneimine) dendrimers using the solvatochromic probe phenol blue and comparisons to poly(amidoamine) dendrimers. *Anal Chem* 2001;73:5743–51.
- Yokoyama S, Otomo A, Mashiko S. Laser emission from high-gain media of dye-doped dendrimer. *Appl Phys Lett* 2002;80:7–9.
- Kolhe P, Misra E, Kannan RM, Kannan S, Lieh-Lai M. Drug complexation, *in vitro* release and cellular entry of dendrimers and hyperbranched polymers. *Int J Pharm* 2003;259:143–60.
- Carnahan MA, Grinstaff MW. Synthesis and characterization of poly(glycerol-succinic acid) dendrimers. *Macromolecules* 2001;34:7648–55.
- Carnahan MA, Middleton C, Kim J, Kim T, Grinstaff MW. Hybrid dendritic-linear polyester-ethers for *in situ* photopolymerization. *J Am Chem Soc* 2002;124:5291–3.
- Luman NR, Grinstaff MW. Synthesis and aqueous aggregation properties of amphiphilic surface-block dendrimers. *Org Lett* 2005;7:4863–6.



37. Söntjens SHM, Nettles DL, Carnahan MA, Setton LA, Grinstaff MW. Biodendrimer-based hydrogel scaffolds for cartilage tissue repair. *Biomacromolecules* 2006;7:310–6.
38. Carnahan MA, Grinstaff MW. Synthesis of generational polyester dendrimers derived from glycerol and succinic or adipic acids. *Macromolecules* 2006;39:609–16.
39. Grinstaff MW. Biodendrimers: new polymeric materials for tissue engineering. *Chemistry* 2002;8:2839–46.
40. Velazquez AJ, Carnahan MA, Kristinsson J, Stinnett S, Grinstaff MW, Kim T. New dendritic adhesives for sutureless ophthalmic surgeries: *in vitro* studies of corneal laceration repair. *Arch Ophthalmol* 2004;122:867–70.
41. Kang C, Carnahan MA, Wathier M, Grinstaff MW, Kim T. Novel tissue adhesives to secure laser *in situ* Keratomileusis flaps. *J Cataract Refract Surg* 2005;31:1208–12.
42. Wathier M, Jung PJ, Carnahan MA, Kim T, Grinstaff MW. Dendritic macromers as *in situ* polymerizing biomaterials for securing cataract incisions. *J Am Chem Soc* 2004;126:12744–5.
43. Morgan MT, Carnahan MA, Immoos CE, et al. Dendritic molecular capsules for hydrophobic compounds. *J Am Chem Soc* 2003;125:15485–9.
44. Wani MC, Wall ME. Plant antitumor agents. II. The structure of two new alkaloids from *Camptotheca acuminata*. *J Org Chem* 1968;34:1364–7.
45. Adams DJ, da Silva MW, Flowers JL, et al. Camptothecin analogs with enhanced activity against human breast cancer cells. I. Correlation of potency with lipophilicity and persistence in the cleavage complex. *Cancer Chemother Pharmacol* 2006;57:135–44.
46. Alali FQ, El-Elimat T, Li C, et al. New colchicinoids from a native Jordanian meadow saffron, *Colchicum brachyphyllum*: isolation of the first naturally occurring dextrorotatory colchicinoid. *J Nat Prod* 2005;68:173–8.
47. Kroll DJ, Rowe TC. Phosphorylation of DNA topoisomerase II in a human tumor cell line. *J Biol Chem* 1991;266:7957–61.
48. Nellans HN. Paracellular intestinal transport: modulation of adsorption. *Adv Drug Deliv Rev* 1991;7:339–64.
49. Wiwattanapatapee R, Carreno-Gomez B, Malik N, Duncan R. Anionic PAMAM dendrimers rapidly cross adult rat intestine *in vitro*: a potential oral delivery system? *Pharm Res* 2000;17:991–8.
50. Kohle P, Khandare J, Pillai O, Kannan S, Lieh-Lai M, Kannan RM. Preparation, cellular transport, and activity of polyamidoamine-based dendritic nanodevices with high drug payload. *Biomaterials* 2006;27:660–9.