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Triazine Dendrimers for Drug Delivery: Evaluation of Solubilization Properties, Activity in Cell Culture, and *In Vivo* Toxicity of a Candidate Vehicle

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Three criteria are evaluated to assess the potential of a dendrimer based on triazines, 1, for use as a vehicle for drug delivery. These criteria are: (1) its ability to solubilize small hydrophobic guests as measured spectrophotometrically; (2) its ability to deliver a drug *in vitro* as evaluated using a gene reporter assay; and (3) its *in vivo* toxicity in mice as determined by autopsy and screens of liver and kidney function.

The Future of Supramolecular Chemistry

Where is supramolecular chemistry going? Bigger. Smarter. Longer. Faster. Cheaper. Useful. These adjectives, or a subset of them with perhaps the most important and most elusive being "useful", lend themselves to the goals of the community in the diverse areas that the field encompasses. Imagination, creativity and innovation remain key components to success. Ourselves? In hindsight, my own experience with Rinehart as an undergraduate assaying marine natural products using the L1210 antitumor assay, with Whitesides in graduate school studying self-assembly using melamine, and with Wong for post-doctoral studies in drug (and drug paradigm) discovery foreshadowed this area of research. Our dream is to produce a versatile scaffold that will selectively home in on tumors to deliver drugs. The dream provides direction and inspiration. Such a vehicle could increase the therapeutic index of known agents, allow for the use of even more cytotoxic drugs, and ultimately—since this is a dream—enhance and extend the quality of life for those besieged.

Eric E. Simanek was born in Tuscola, IL in 1969. He obtained a BS in Chemistry from the University of Illinois in Urbana-Champaign in 1991. Research experience in the laboratories of Professor Kenneth L. Rinehart, Jr and at Abbott Laboratories as well as interactions with Professor Eric N. Jacobsen led him to pursue graduate study. He obtained his PhD in 1996 from Harvard University under the direction of Professor George M. Whitesides. After post-doctoral study with Professor Chi-Huey Wong at Scripps Research Institute in La Jolla, he joined the faculty at Texas A&M University in 1998. His group focuses primarily on dendrimer chemistry, with efforts directed towards separation science and drug delivery.

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Vehicle 1 solubilizes pyrene to a similar extent to dendrimers based on poly(arylether)s, 4, encapsulating approximately 0.2 molecules of pyrene per dendrimer. This activity is approximately 10-fold greater than that of the more polar poly(propyleneimine) and poly(amidoamine) dendrimers, 2 and 3. Gas-phase computational models reveal that both 1 and 4 have cores that are accessible to solvent, suggesting that these dendrimers can occupy much greater volumes than 2 and 3 whose cores are confined toward the interior of the structure. Electrostatic potential maps can be used to rationalize differences in solubilization between 1 and 4. Precipitation results from mixing cationic 1 with the anionic indomethacin, but not with methotrexate, suggesting that the composition of the drug may dictate the scope of delivery applications. Dendrimer 1 solubilizes 10-hydroxycamptothecin and a novel bisindolemethane; approximately four and five molecules of drug per dendrimer are solubilized, respectively. In cell-culture experiments using a luciferase reporter gene assay, the dendrimer:bisindolemethane conjugate shows comparable activity to the bisindolemethane delivered in aqueous DMSO, suggesting that the dendrimer does not preclude delivery of the molecule to an intracellular target. Preliminary toxicology studies of 1 in mice show that this molecule has no adverse toxicity to the kidneys or the liver in single doses delivered intraperitoneally up to 10 mg/kg.

Keywords: Dendrimer; Polymer; Drug delivery; Melamine; Triazine; Toxicity; Cell culture; Solubilization

INTRODUCTION

The non-covalent architectures reported from numerous laboratories confirm that the study of host–guest chemistry in dendrimers lags far behind the study of self-assembly in these systems [1–10]. Two approaches have been taken to studying molecular recognition events in dendrimers. The first approach focuses on the incorporation of specific recognition domains into the dendrimer scaffold or at the core [11–13]. This strategy is consistent with past efforts where synthetic receptors were rationally designed to recognize specific small molecule guests. Engineering these host–guest architectures often focused narrowly on the proper disposition of functional groups to maximize intermolecular interactions without incurring entropic costs from restricting free rotation. From these inquiries, design criteria including Etter's rules [14], preorganization [15] and peripheral crowding [16,17] emerged. Adapting this strategy to dendrimers offers significant challenges. In general, unless the recognition domain is rigid and/or self-contained, the dynamic environment of a dendrimer limits opportunities for preorganization. Discrete recognition domains are not easily incorporated into a dendrimer with the exception of the core or periphery due to synthetic constraints of most dendrimer systems. Notable success has been reported, however, and includes the recognition of barbiturates with dendritic branches containing 2,6-diaminopyridine domains [11], the encapsulation of cholesterol within cyclophanes buried at the core of a dendrimer [12], and, more recently, the recognition of molecules of C_{60} through a process by which recognition of the first C_{60} leads to preorganization of a binding domain for subsequent molecules [13].

The second approach for pursuing molecular recognition in dendrimers treats the dendrimer as a unimolecular micelle [18,19]. As such, the dendrimer represents a phase distinct from solvent into which small molecules can partition. This strategy alleviates many of the challenging design criteria faced in small-molecule host–guest chemistry, but selectivity is often sacrificed—many hydrophobic guests will partition into a hydrophobic dendrimer phase from water. The issue of host engineering is precluded by the synthetic constraints imposed by the dendrimer system being explored with the notable exception that peripheral groups can be stoichiometrically modified. These limitations notwithstanding, this strategy has excited the drug-delivery community [20–23] due in large part to Meijer's seminal work with the "dendritic box" into which guests can be trapped until surface groups are removed [24,25]. The efforts described here relate to this latter (micellar) approach.

As the products of covalent synthesis, dendrimers offer advantages over micelles. Dendrimers often exhibit no critical micellar concentration (cmc) under which the architecture is unstable. As a result, drug-laden drug dendrimers could presumably be delivered at significantly lower concentrations than the micellar constructs, and should remain stable for longer periods of time. The polymeric nature of dendrimers offers opportunities for "smart targeting" using two strategies. First, these architectures can be covalently functionalized with useful ligands that could direct the drug-delivery vehicle to specific sites in a magic-bullet sense to facilitate uptake by specific cell populations. Significant energies have been invested in the discovery of peptides, carbohydrates, and small molecules that accomplish these goals [26–28]. The size of dendrimers may also play an important role in their use as drug vehicles. Linear polymers display an enhanced permeability (EP) at sites of nascent or corrupt vasculature in tumors and prolong retention (R) due to an underdeveloped lymphatic drainage system [29]. This effect may also hold true for dendrimers of appropriate dimensions.

The studies presented here address three issues of drug delivery using a candidate vehicle, **1**. First, the ability of this triazine dendrimer, **1**, to sequester pyrene is described and compared with the three major classes of dendrimers including the poly(amidoamine) (PAMAM) dendrimers of Tomalia [30], Fréchet's poly(arylethers) [31,32], and the poly(propyleneimine) (PPI) dendrimers of Vogtle and Meijer [33]. The origins of differences between the solubilization efficiency of these architectures were explored with the aid of molecular mechanics/dynamics and semi-empirical computations. These studies were extended by examining whether **1** solubilized pharmacophores including indomethacin, methotrexate, 10-hydroxycamptothecin and a bisindolemethane. Second, the ability of **1** to deliver the bisindolemethane was evaluated in a cell-culture assay using a luciferase gene reporter assay. Third, preliminary inquiries into the toxicity of the triazine dendrimers *in vivo* were performed in mice.

EXPERIMENTAL

General

Triazine trichloride (99%, Acros), piperazine (99%, Acros), *N*,*N*-diisopropylethylamine (98%, Acros), trifluoroacetic acid (99%, Acros), tetraethyleneglycoldiamine (98%, Molecular Biosciences, Inc.), and di-*t*-butyl dicarbonate (99%, Acros) were used as received. None of the intermediates or products proved to be crystalline, and accordingly, melting points are not reported. Silica gel (EM Science, Germany) was mesh 230–400. NMR spectra ^{1}H (300 MHz) and ^{13}C (75 MHz) were recorded on a Varian Mercury 300 Spectrometer.

Dendrimer 1

Intermediate **12** (31 mg, 0.003 mmol) was dissolved in 2 mL of CH_2Cl_2–TFA (1:1). After stirring for 12 h, solvent was removed to yield a transparent film. ^{1}H NMR (CD_3OD) δ 3.90 (br, 168 H), 3.66 (m, 288 H), 3.12 (m, 96 H). ^{13}C NMR (CD_3OD) δ164.50, 70.25, 70.05, 68.74, 66.67, 43.85, 43.03, 40.58, 39.43. MS: Calcd. 8067.30 $(M)^+$. Found (MALDI-TOF): 8068.41 $(M + H)^+$.

Intermediate 5

Boc-diamine (4.40 g, 15.0 mmol) was dissolved in 100 mL of THF before triazine trichloride (1.38 g, 7.50 mmol), and 3.0 mL of *N*,*N*-diisopropylethyl amine (2.2 g, 17 mmol) were added in 100 mL of THF. The mixture was stirred for 12 h at room temperature. After removing the solvent, the residue was purified by column chromatography using CH_2Cl_2–CH_3OH (100:12, 100:3) to afford a colorless oil (3.92 g, 75%). ^{1}H NMR ($CDCl_3$) δ6.10 (br, 1 H), 5.98 (br, 1 H), 5.19 (br, 2 H), 3.63 (m, 24 H), 3.54 (m, 4 H), 3.30 (d, 4 H), 1.42 (s, 18 H). ^{13}C NMR (75 MHz) δ168.33, 166.10, 156.25, 78.24, 70.43, 70.28, 70.19, 69.53, 69.36, 69.11, 41.07, 28.88, MS: Calcd. 696 (M^+). Found ($^+$LSIMS): 696 (M^+).

Intermediate 6

Intermediate **5** (2.40 g, 3.45 mmol) was dissolved in 100 mL of THF before piperazine (0.89 g, 10.3 mmol) was added to the solution. The solution was bubbled with nitrogen, sealed in a Parr vessel, and stirred at 70°C for 12 h. After removing the solvent, the residue was purified by column chromatography using CH_2Cl_2–CH_3OH (25:1, 10:1) to afford the product as an oil (2.11 g, 82%). ^{1}H NMR ($CDCl_3$) δ5.22 (br, 4 H), 3.95 (br, 4 H), 3.63 (m, 24 H), 3.51 (m, 4 H), 3.30 (d, 4 H), 3.06 (br, 4 H), 2.50 (br, 1 H), 1.43 (s, 18 H). ^{13}C NMR ($CDCl_3$) δ166.21, 165.16, 156.25, 79.35, 70.76, 70.48, 44.37, 43.26, 40.65, 28.64. MS: Calcd. 746 (M^+). Found ($^+$LSIMS): 746(M^+).

Intermediate 7

Intermediate **6** (1.61 g, 2.16 mmol) was dissolved in 30 mL of THF before triazine trichloride (199 mg, 1.08 mmol) and 0.5 mL of *N*,*N*-diisopropylethyl amine were added in THF. After stirring for 24 h at room temperature, the solid was filtered, and the solvent was removed. The residue was purified by chromatography with CH_2Cl_2–CHOH (25:1) to give **5** as an oil (1.45 g, 84%). ^{1}H NMR ($CDCl_3$) δ5.20 (br, 8 H), 3.79 (br, 16 H), 3.60 (m, 48 H), 3.51 (m, 8 H), 3.29 (d, 8 H), 1.41 (m, 36 H). ^{13}C NMR ($CDCl_3$) δ169.91, 164.78, 156.23, 79.32, 70.77, 70.58, 70.46, 70.21, 68.13, 43.56, 43.16, 40.73, 28.63. MS: Calcd. 1601.90 $(M)^+$. Found (MALDI-TOF): 1602.64 $(M + H)^+$,1624.63 $(M + Na)^+$, 1640.62 $(M + K)^+$.

Intermediate 8

Intermediate **7** (1.04 g, 0.65 mmol) was dissolved in 30 mL of THF before piperazine (168 mg, 1.95 mmol) was added. The solution was bubbled with nitrogen, sealed in a Parr vessel, and stirred at 70°C for 12 h. The solid was removed by filtration, and the solvent was removed. The residue was dissolved in a mixed solution of CH_2Cl_2–CH_3OH (20:1) and then purified by column chromatography using CH_2Cl_2–CH_3OH (20:1, 16:1) to afford the product as an oil (870 mg, 81%). ^{1}H NMR ($CDCl_3$) δ5.22 (br, 8 H), 3.98 (br, 4 H), 3.75 (m, 16 H), 3.60 (m, 48 H), 3.52 (m, 8 H), 3.29 (d, 8 H), 3.10 (br, 4 H), 2.54 (br, 1 H), 1.41(s, 36 H). ^{13}C NMR ($CDCl_3$) δ166.00, 165.58,

165.24, 156.23, 79.32, 70.76, 70.48, 44.40, 43.32, 41.83, 40.67, 28.64, MS: Calcd. 1652.94 $(M)^+$; Found (MALDI-TOF): 1653.18 $(M)^+$, 1675.17 $(M + Na)^+$, 1691.17 $(M + K)^+$.

Intermediate 9

Intermediate **8** (463 mg, 0.28 mmol) was dissolved in 10 mL of THF before triazine trichloride (25.8 mg, 0.14 mmol) and 0.2 mL of *N*,*N*-diisopropyl ethyl amine were added. After stirring at room temperature for 24 h, the solvent was removed, and the residue was purified by chromatography with CH_2Cl_2–CH_3OH (20:1, 16:1) to give **9** as a transparent film (378 mg, 79%). ^1H NMR ($CDCl_3$) δ5.22 (br, 16 H), 3.79 (br, 32 H), 3.60 (m, 96 H), 3.52 (m, 16 H), 3.29 (d, 16 H), 1.41 (m, 72 H). ^{13}C NMR ($CDCl_3$) δ169.92, 165.65, 164.85, 156.22, 79.31, 70.78, 70.59, 70.47, 70.30, 43.63, 43.32, 40.73, 28.64. MS: Calcd. 3417.36 $(M)^+$. Found (MALDI-TOF): 3418.66 $(M + H)^+$, 3441.51 $(M + Na)^+$.

Intermediate 10

Monoprotected piperazine (320 mg, 1.72 mmol) was dissolved in 10 mL of THF. To the solution, triazine trichloride (105 mg, 0.57 mmol) and 0.2 mL of *N*,*N*-diisopropyl ethyl amine were added. The reaction mixture was stirred at room temperature for 2 h and then at 80°C for 12 h. The solvent was removed, and the residue was purified by chromatography with CH_2Cl_2–CH_3OH (20:1, 16:1) to give protected **10** as a white solid (307 mg, 85%). ^1H NMR (DMSO-d_6) δ3.71 (br, 12 H), 3.41 (br, 12 H), 1.44 (m, 27 H). ^{13}C NMR (DMSO-d_6) δ165.22, 154.99, 80.05, 43.75, 43.38, 28.61. MS: Calcd. 634 (M^+). Found (+FAB/DP): 635 $(M + H^+)$.

Intermediate 11

Intermediate **10** (120 mg, 0.19 mmol) was dissolved in 2 mL of CH_2Cl_2–TFA (1:1). The solution was stirred for 5 h. After removing solvent and TFA, the white solid (salt of TFA) was obtained. The solid was dissolved in water, and the solution was adjusted to pH 11 by adding 1 M NaOH. Intermediate **11** was obtained as white solid upon removal of the solvent (62 mg, 98%). ^1H NMR (CD_3OD) δ3.56 (m, 12 H), 2.75 (m, 12 H), ^{13}C NMR (CD_3OD) δ165.40, 43.21, 39.98. MS: Calcd. 333 (M^+). Found (+FAB/DP): 334 $(M + H^+)$.

Intermediate 12

Intermediate **9** (103 mg, 0.03 mmol) was dissolved in 3 mL of THF and 0.1 mL of *N*,*N*-diisopropylethyl amine before intermediate **11** (3.3 mg, 0.01 mmol) was added to the solution. The solution was bubbled with nitrogen and sealed in a Parr vessel. The mixture was stirred at 80°C for 24 h. After the solvent was evaporated, the residue was purified by column chromatography on silica gel using CH_2Cl_2–CH_3OH (17:1, 12:1) to afford the product (78.5 mg, 75%). ^1H NMR ($CDCl_3$) δ5.32 (br, 48 H), 3.77 (br, 168 H), 3.59 (m, 288 H), 3.51 (m, 48 H), 3.28 (d, 48 H), 1.41 (m, 216 H). ^{13}C NMR ($CDCl_3$) δ165.62, 156.27, 79.32, 70.72, 70.49, 43.36, 40.64, 28.66 MS: Calcd. 10,470 $(M)^+$. Found (ESI-TOF), 3492.9 $(M + 3H)^{3+}$, 2619.0 $(M + 4H)^{4+}$, 2096 $(M + 5H)^{5+}$, 1746.8 $(M + 6H)^{6+}$, 1497.4 $(M + 7H)^{7+}$, 1310.4 $(M + 8H)^{8+}$, 1164.9 $(M + 9H)^{9+}$.

Solubilization

The solubilization experiment was performed according to a modified literature method using an excess of pyrene deposited as a thin film on the bottom of flasks and adding dendrimer in a solution of 20 mM phosphate buffer (pH 7.5). After sonication for 5 min, the solution was allowed to stand overnight. Excess pyrene was removed using a 0.45 μm syringe filter. The concentration of pyrene in the solution was determined spectrometrically at 340 nm on a SPECTROMAX PLUS spectrophotometer. Indomethacin was monitored at 320 nm. Concentrations of both the bisindolemethane and 10-hydroxycamptothecin were determined by slow dissolution (with heat and sonication) of a known amount of material in dendrimer solution. This strategy precludes issues of environment-sensitive extinction coefficients.

Cell-culture Studies

MCF-7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were maintained in MEM media with phenol red and supplemented with 0.22% sodium bicarbonate, 10% fetal bovine serum (FBS) albumin, 0.011% sodium pyruvate, 0.1% glucose, 0.24% HEPES, 10^{-6}% insulin and 10 mL/L antibiotic solution. Cells were grown in 150 cm^2 culture plates in an air:carbon dioxide (95:5) atmosphere at 37°C, and passaged every 6 days.

Transfection and Luciferase Activity Assay

MCF-7 cells were seeded I 5% FBS DME/F12 media in 12 well plates 1 day before transfection using calcium phosphate-DNA co-precipitation method or SuperFect transfection kit. GAL4Luc reporter plasmid (0.5 μg), gPPARγ (0.05 μg) and β-Gal DNA (0.1 μg) were used for transfection. After incubation for 16 h with calcium phosphate, or 3 h with SuperFect, cells were washed with

PBS and treated with compounds as indicated for 16–20 h in fresh media. Cells were then lysed with 200 μL of 1 × Reported Lysis Buffer; 30 μL of cell extract were used for luciferase and β-Gal assays. LumiCount was used to quantitate luciferase and β-Gal activities. The luciferase activities were normalized to β-Gal activity. The bisindole compound (10 mM) induced a three- to fourfold increase in luciferase activity compared with the solvent (DMSO) control. Using equimolar concentrations of the dendrimer, no effects on the indole-induced response were observed.

Computations

Computational results for the gas-phase low-energy structures of **1–4** and the electrostatic potential surface maps for the guests were obtained using the software package Cerius^2 4.6 by Accelrys, Inc. Minimizations and dynamics were performed with the Open Force Field (OFF) program, using the pcff second-generation force field [34]. The dendrimers were initially minimized in the fully extended conformation. Constant volume and temperature (NVT) molecular dynamics (MD) calculations were then performed on the minimized extended structures via simulated annealing. The simulated annealing was carried out for 840.0 ps, over a temperature range of 300–1000 K, with $\Delta T = 50$ K, using the Nosé temperature thermostat, a relaxation time of 0.1 ps, and a time step of 0.001 ps. The dendrimers were minimized after each anneal cycle, resulting in 300 minimized structures of **1–4**. Electrostatic potential [35] surface maps, at the AM1 [36] geometry optimized structures, were generated for the building block fragments of **1**, **2**, **3**, and **4**, and the solvents, pyrene and indole, using MOPAC 6.0 as implemented in Cerius^2 4.6, by Accelrys, Inc. Figure 2 was generated with Persistence of Vision Ray Tracer (POV-Ray). Figure 3 was generated using Cerius^2 4.6 by Accelrys, Inc.

RESULTS AND DISCUSSION

Synthesis

The tri(ethylene glycol) amine periphery was chosen to provide water solubilition and a reactive group for future post-synthetic modifications of the dendrimer. Piperazine groups are used as linkers because of their high reactivity, low cost, and enhanced stability in comparison with *p*-aminobenzylamine, a linker that was used previously [37,38]. The synthesis of **1** proceeds convergently in six linear steps in a 33% overall yield (Scheme 1). The intermediates are readily separable by column chromatography. The iterative nature of the synthesis is reflected in the piperazine groups (E, F, H) of the ^{1}H and ^{13}C NMR spectra (Fig. 1), and the line corresponding to the chlorinated carbon of the monochlorotriazine (L). The MALDI-TOF MS spectrum of the dendrimer **1** shows a single line corresponding to the desired parent ion.

Solubilization of Pyrene

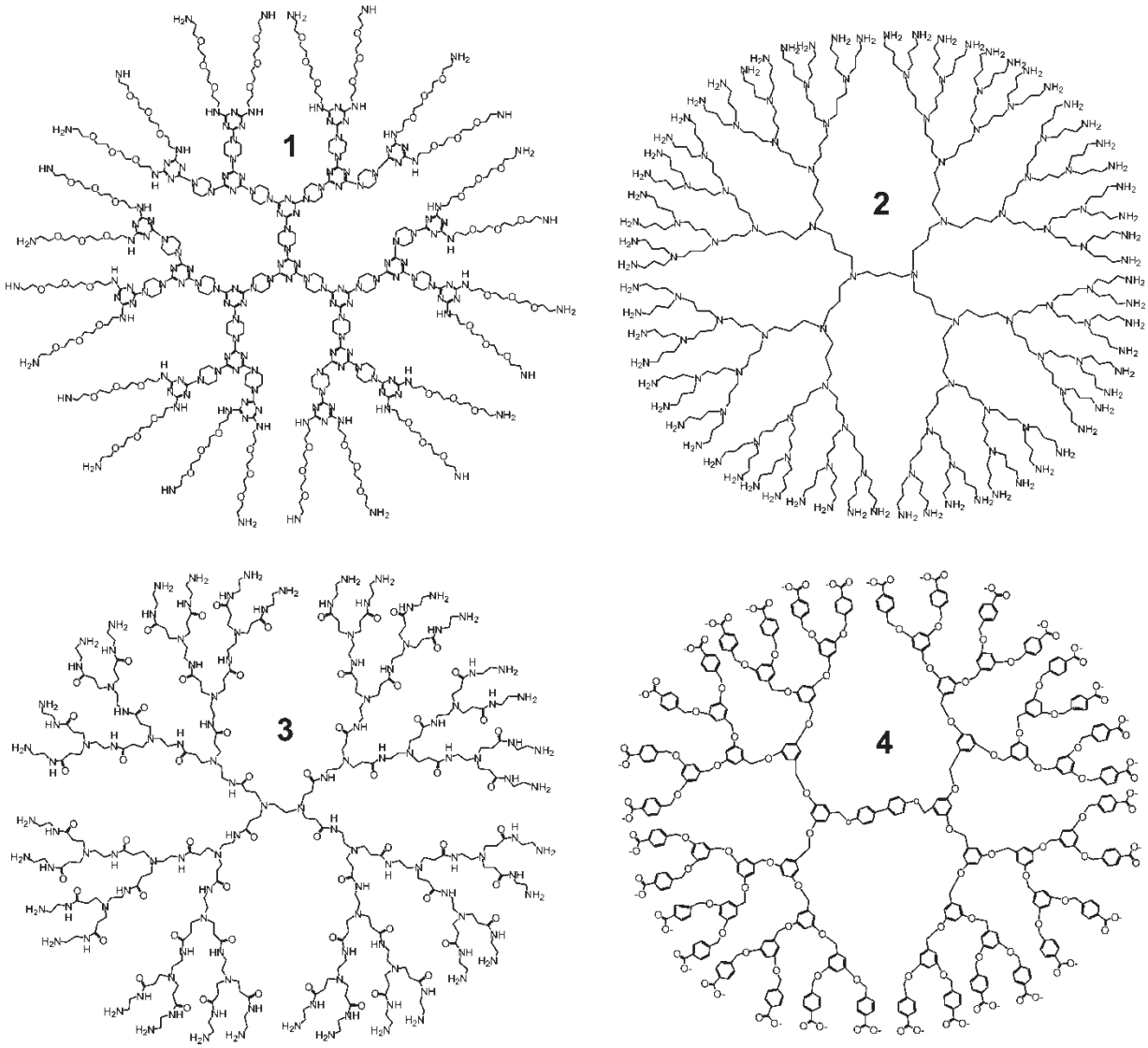
Pyrene has been used to evaluate solubilization of hydrophobic guests in a variety of dendrimers. The linear relationship between the concentrations of **1** and solubilized pyrene observed spectrophotometrically is consistent with the absence of a critical micellar concentration for **1**. The solubility limit of pyrene in phosphate buffer solution (pH 7.5) is 1.02×10^{-6} M. Addition of dendrimer increases the solubility markedly to 2.6×10^{-5} M, corresponding to an average of 0.2 molecules of pyrene sequestered/**1**. The ability of dendrimers **2**, **3** [33], and **4** [31] to solubilize pyrene is shown in Table I, although conditions for the experiment vary slightly. All these dendrimers have approximately the same molecular weight.[†]

The ability of **1** to sequester pyrene is similar to that of Frechet-type dendrimers, **4**, and exceeds the ability of poly(amidoamine) (PAMAM) or poly(propyleneimine) (PPI) dendrimers, **2** and **3**. Because the molecular weights for **1–4** are similar, we attribute this difference in sequestration ability to differences in composition. This composition dependence is more than a simple surface group effect: **1–3** have cationic surfaces, while **4** has an anionic surface. We believe that the hydrophobic aromatic interiors that offer the potential for π–π interactions of **1** and **4** are responsible for the 10-fold differences in solubilization. The interiors of **2** and **3** contain polar tertiary amine groups.

Computation

The gas-phase low-energy conformations of **1–4** derived from simulated annealing computations appear strikingly similar (Fig. 2).

[†]The strategy adopted combines elements described in Refs [30–33] and was performed in our laboratory.

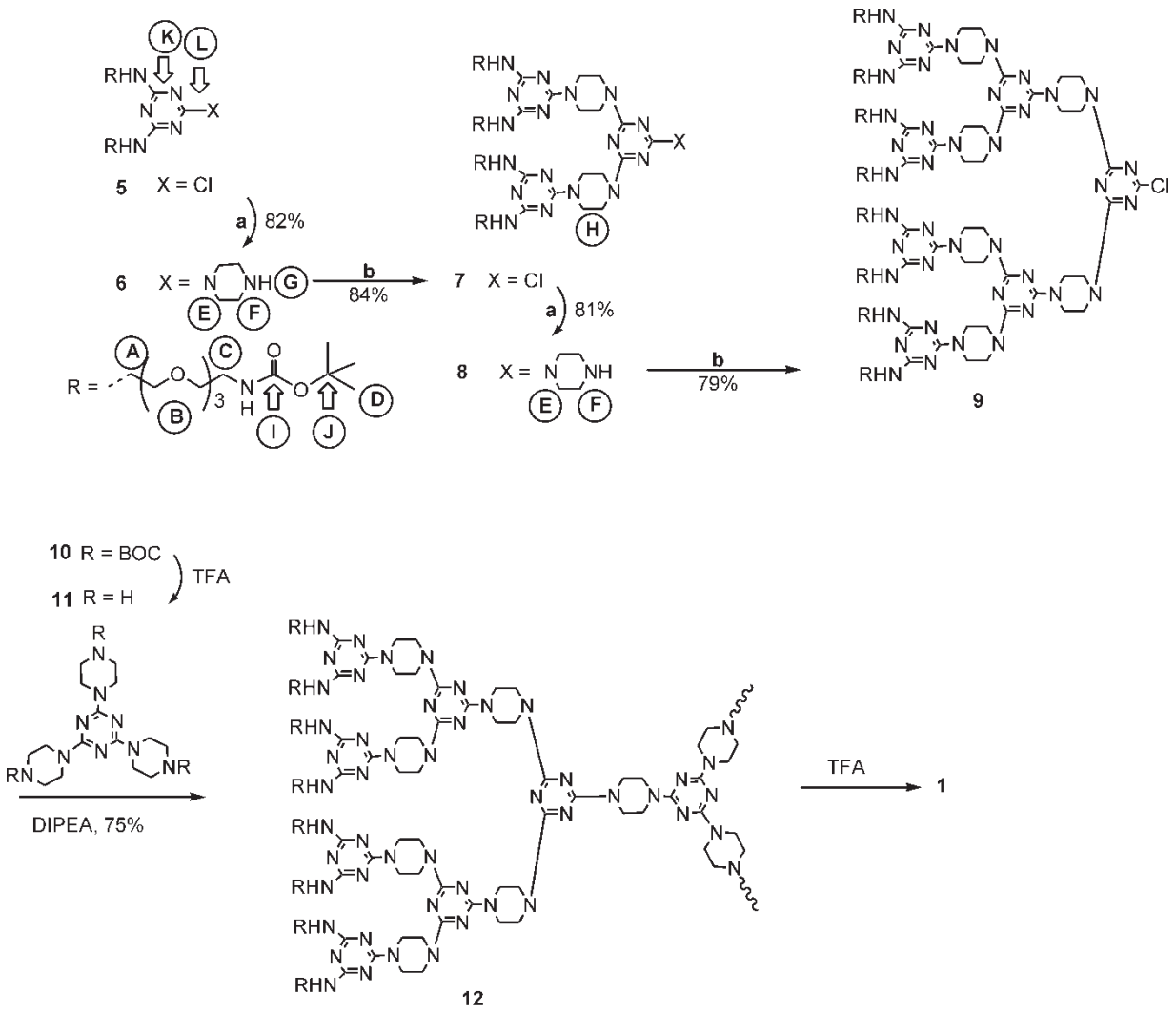


Each dendrimer appears globular with comparable dimensions resulting from both the similar molecular weights of the architectures and the flexibility inherent to the building blocks. Closer inspection of the structures, however, suggests a potential difference between the dendrimers that solubilize pyrene poorly (**2** and **3**) and those that solubilize pyrene more effectively (**1** and **4**). The cores (shown in maroon) of **2** and **3** appear closer to the center of the globule, than those of **1** and **4** that are closer to the surface. Similar dimensions result from hydrophobic collapse. However, similarities in the free volumes of these architectures may be artificial—both **1** and **4** may be able to expand to accommodate guest, owing to their less globular nature.

While the ability of **1** and **4** to solubilize pyrene is quite similar, the origins of enhanced solubilization by **4** might be attributed to electrostatics. Electrostatic potential surface maps at the AM1 optimized geometry of the building blocks show that the arylether ring of **4** is more electron-rich than the triazine of **1** and, accordingly, corroborate our intuition that the former may interact better with electropositive pyrene (Fig. 3).

Solubilization of Drugs: Indomethacin, Methotrexate, and 10-Hydroxycamptothecin

There are relatively few examples of the solubilization of complex pharmacophores by dendrimers. Solubilization studies using indomethacin, a derivatized indoleacetic acid with anti-inflammatory activity, have been described using **4** following a similar procedure to that of pyrene. These drug-dendrimer constructs showed slow-release potential [32]. Using cationic **1**, we saw no increased solubilization of indomethacin. Instead,

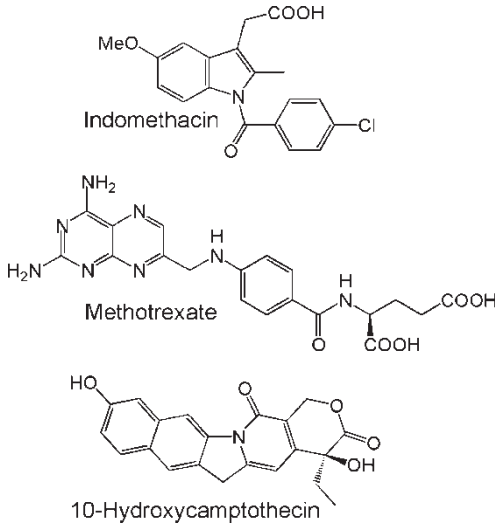


SCHEME 1 Synthesis of candidate **1**. Labels identify groups (H or C) that appear in the NMR spectra. Reagents: (a) excess piperazine, 70°C, THF; (b) $C_3N_3Cl_3$, Hunig's base, 25°C, THF.

a precipitate appeared in dendrimer–indamethacin solutions (a buffered solution of indomethacin or dendrimer remains homogeneous). NMR analysis revealed that the precipitate was the complex between the dendrimer and indomethacin. We conclude that the composition of the dendrimer may play important and potentially limiting roles in solubilization. Here, we attribute the precipitation event to the anionic drug and cationic surface groups of the dendrimer. To this end, we note that **4** comprises carboxylic acid groups on the surface. Methotrexate, a diacid with excellent solubility in buffer, yielded no precipitate; nor did **1** appear to enhance its solubility significantly. Compound **1** also solubilizes 10-hydroxycamptothecin with an average of 3.7 molecules solubilized/**1**. The evaluation of this and other pharmacophores requires significant additional work and will be reported elsewhere.

Solubilization of Drugs: Bisindolemethanes

Bisindolemethanes are inhibitors of estrogen-induced growth of T47D cells and mammary tumors



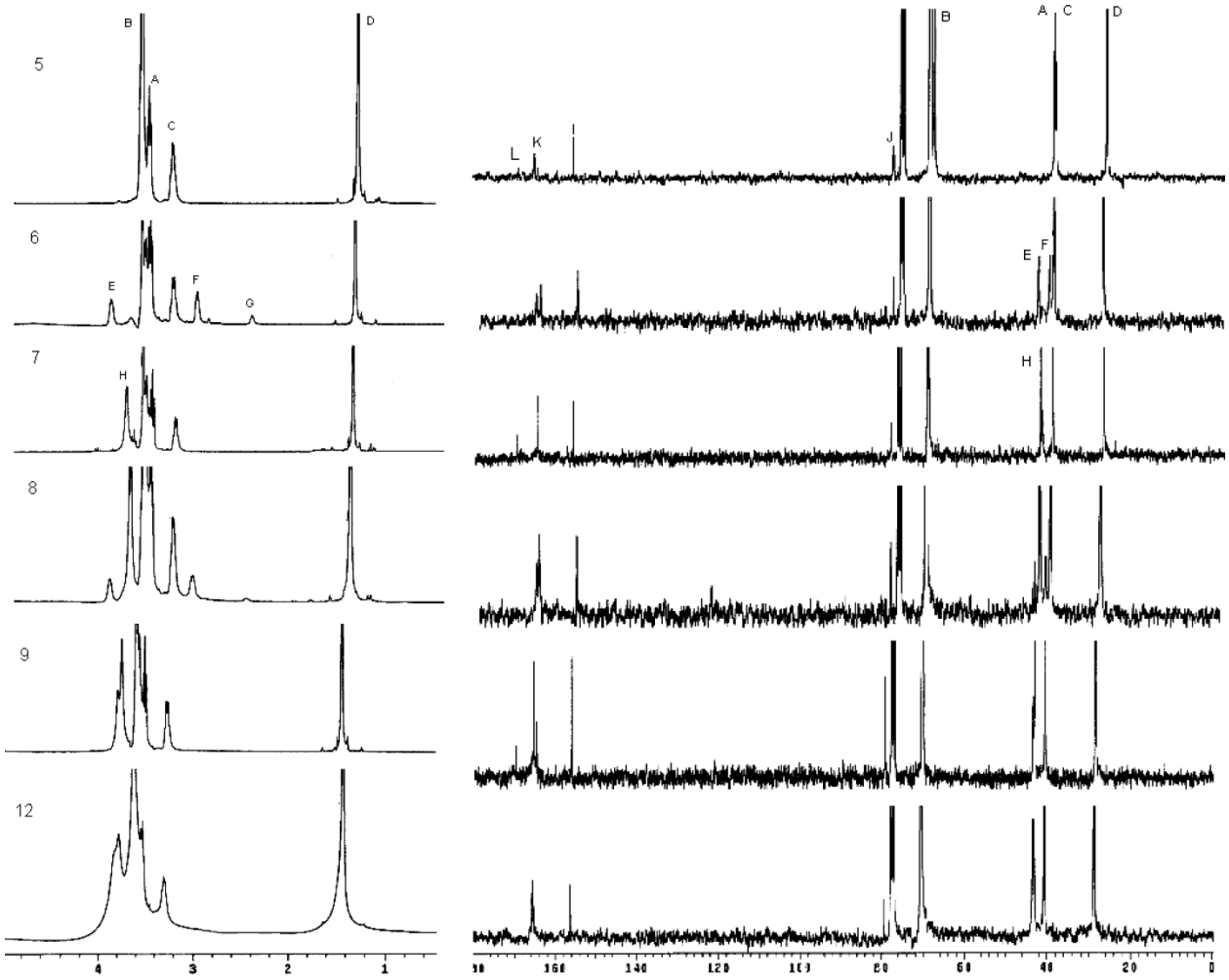


FIGURE 1 ^{1}H and ^{13}C NMR of **1** and intermediates showing the iterative nature of the synthesis. Labeled groups are identified in Scheme 1.

in rats, and are currently being considered for clinical trials.[‡] Descriptively, these compounds have no solubility in water. We find that **1** (8.9×10^{-4} M) dramatically increases the solubility of 1,1-bis(3-indolyl)-1-(*p*-trifluoromethyphenyl)methane to up to 4.0×10^{-3} M, corresponding to each dendrimer solubilizing 4.5 drug molecules. Of greater importance, the **1**–bisindolemethane complex is as active in cell culture. The bisindolemethane is delivered to cells by **1** or aqueous DMSO with equal efficacy, as determined by activation of a reporter gene activity in MCF7 breast cancer cells transfected with expression plasmids for a GAL4-peroxisome proliferation-activated receptor γ fusion protein and a reporter containing five tandem GAL4 response elements [39]. While the mechanism of delivery of the **1**–bisindolemethane complex is undetermined, we conclude that the dendrimer does not preclude cellular uptake and response to the solubilized ligand.

Toxicology of 1

While the use of melamine-based dendrimers as model systems for studying host–guest interactions will undoubtedly yield interesting findings as to the relationship between structure and function, our long-term optimistic outlook is that these vehicles could themselves be used for drug delivery. Accordingly, we need to determine if these architectures possessed inherent toxicities that could preclude their use *in vivo*. Table II shows the results of *in vivo* dosing of male C3H

[‡]We investigated a G3 PAMAM dendrimer under the same conditions. The saturated concentration of pyrene in 1.49×10^{-4} buffer solution of PAMAM was found to be 1.42×10^{-6} M. On average, a single dendrimer molecule can dissolve only 0.0095 molecules of pyrene, which is 19 times lower than **1** (0.18 pyrene/dendrimer).

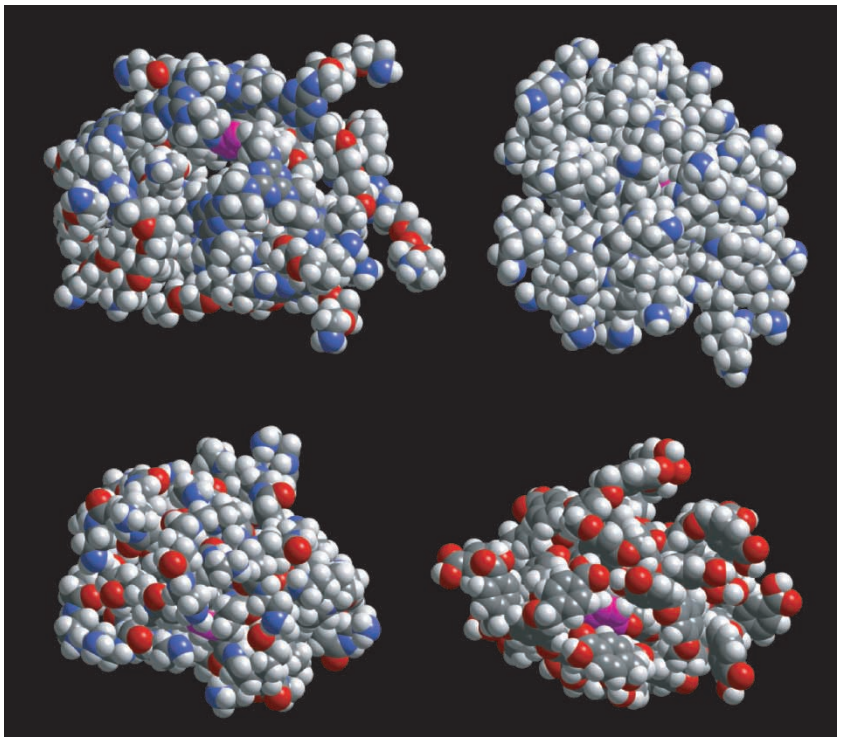


FIGURE 2 Computational models of the low-energy gas-phase structures of **1–4** obtained via simulated annealing. The core of the dendrimers appears maroon. (See colour plate 9 at the end of this issue.)

mice challenged (*i.p.* injection) with 1 mg/kg, 2.5 mg/kg, or 10 mg/kg of **1** using four mice per group. Forty-eight hours after injection, the mice were sacrificed. Visual inspection upon autopsy revealed no abnormalities; organ color and texture were normal. Liver and kidney weight were not significantly increased. No increases in alanine aminotransferase [40] or glutamic pyruvic transaminase [41], both indicators of hepatic dysfunction, were measured, and no increases in blood urea nitrogen, an indicator of renal dysfunction, were seen.

CONCLUSIONS AND FUTURE DIRECTIONS

The present studies suggest that a dendrimer based on melamine, **1**, can effectively sequester a range of hydrophobic guests, including candidate anticancer drugs. This ability matches, or greatly exceeds, the ability of other classes of dendrimers. However, the ionic character of both the guest and dendrimer host may emerge as a critical design feature for these vehicles. We are greatly encouraged by the absence of any vehicle effects on the efficacy of the bisindolemethane in cell-culture assays, as well as the apparent absence of toxicity

TABLE I Solubilization of pyrene by **1–4**

Cmpd	Gn (MW, Da)	[Cmpd] (M)	[Pyrene] (M)	[Pyrene]/[Cmpd]
1	G3 (8104)	1.5×10^{-4}	2.6×10^{-5}	0.2
2	G4 (7166)	1.2×10^{-3}	3.3×10^{-5}	0.03
3	G3 (6907)	1.5×10^{-4}	1.4×10^{-6}	0.01
4	G4 (8100)	2.1×10^{-4}	9.5×10^{-5}	0.5

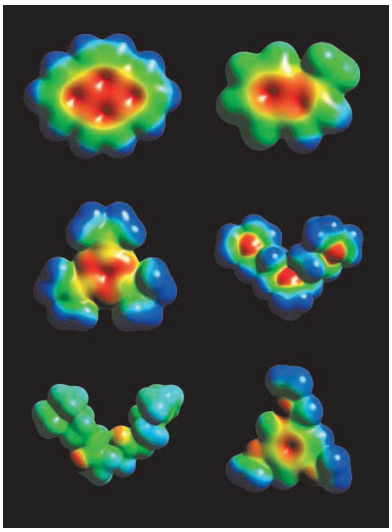


FIGURE 3 Electrostatic maps of pyrene, indole, and models of the building blocks of **1–4**. Electrostatic potentials mapped on the 0.017 contour electron density surface for the AM1 geometry optimized building blocks of **1–4**, pyrene, and indole. (See colour plate 9 at the end of this issue.)

TABLE II *In vivo* dosing with **1**. Four groups of four mice each were dosed. No significant change in liver or kidney weight, alanine aminotransferase activity (ALT), or blood urea nitrogen (BUN) levels was observed

Dose (mg/kg)	Liver weight (g)	Kidney weight (g)	ALT (SF units/ml)	BUN (mg/dl)
0	1.279(.075)	0.542(0.046)	114.5(25.9)	53.5(8.4)
1	1.381(.127)	0.531(0.061)	131.5(6.8)	50.9(4.7)
2.5	1.380(.070)	0.517(0.047)	91.8(28.1)	47.6(5.8)
10	1.319(.090)	0.504(0.038)	95(40.8)	49.1(11.7)

in preliminary animal models. Additional cell-culture and animal studies including tumor models are warranted.

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