Communication

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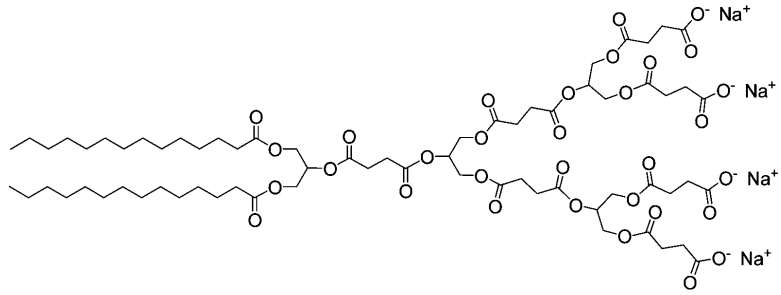
J. Am. Chem. Soc., **2008**, 130 (44), 14444-14445 • DOI: 10.1021/ja806912a • Publication Date (Web): 09 October 2008



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Published on Web 10/09/2008

Anionic Amphiphilic Dendrimers as Antibacterial Agents

Steven R. Meyers, Frank S. Juhn, Aaron P. Griset, Nathanael R. Luman, and Mark W. Grinstaff*

Departments of Biomedical Engineering and Chemistry, Metcalf Center for Science and Engineering, Boston University, Boston, Massachusetts

Received September 1, 2008; E-mail: mgrin@bu.edu

Dendritic macromolecules, due to their structure, unique properties, and precise compositions, are of significant interest[1] and are finding uses in an ever-increasing number of medical applications.[2] This is especially evident in the drug delivery area where the dendritic structure enables the attachment of a multitude of drugs or targeting moieties as well as the opportunity to control pharmacokinetics through alterations in generation number.[3] Our interest lies in the synthesis and evaluation of dendritic macromolecules composed of building blocks that are natural metabolites or known to be biocompatible for ocular tissue repair,[4] cartilage tissue engineering,[5] and drug delivery.[6] To expand the biomedical applications of dendrimers and our understandings of the resulting structure−activity relationships, we are investigating anionic dendritic macromolecules as antibacterial agents. Herein, we report the antibacterial activity of an anionic amphiphilic dendrimer and the striking selectivity in its cytotoxicity toward a prokaryotic Gram-positive bacterium compared to a eukaryotic human cell.

There is a significant global need for new antibacterials and alternative mechanisms of action given the rise in resistance among bacteria.[7] Of the various known antibacterial agent classes, amphiphilic compounds act through perturbation and disruption of the prokaryotic membrane.[8] We hypothesized that amphiphilic anionic dendrimers may exhibit antibacterial activity with minimal eukaryotic cell cytotoxicity, since dendrimers with terminal anionic charges are generally noncytotoxic and have low toxicity in zebrafish whole animal development studies.[9] On the other hand, cationic dendrimers, some of which have antibacterial properties if the positive charge is properly shielded,[10] have repeatedly shown cytotoxicity against a variety of eukaryotic cell lines.[3e,11] In addition, there are many reports of linear polycationic agents but only a few descriptions of linear polyanionic antibacterial agents (e.g., sulfonated polystyrene).[12] Consequently, we synthesized a series of surface-block anionic amphiphilic dendrimers composed of succinic acid, glycerol, and myristic acid possessing various numbers of acid and alkyl functionalities.[13] Based on the physicochemical properties of these amphiphilic anionic dendrimers, we identified two potential candidates, dendrimers **1** and **2** (Figure 1). Both dendrimers were synthesized in 9 steps with an overall yield of 30 and 28%, respectively, for evaluation of antibacterial activity (see Supporting Information). Additionally, linear anionic amphiphile sodium dodecyl sulfate (SDS), **3**, and neutral-charge amphiphile Triton X-100, **4**, were added to the evaluation as positive controls with known antibacterial activity (i.e., disruption of the cytoplamic membrane and protein solublization).[8]

Cytotoxicological experiments were conducted against a wild-type Gram-positive bacterial strain (*Bacillus subtilis* AG174). Bacteria were cultured until logarithmic growth was achieved, and then dilutions were added to LB broth with various concentrations of compounds **1**−**4** and the constituents of the dendrimers: glycerol, succinic acid, and myristic acid along with an untreated negative control. The turbidity of the wells was monitored for 9 h, and the resulting cytotoxicities are shown in Figure 2. As expected,

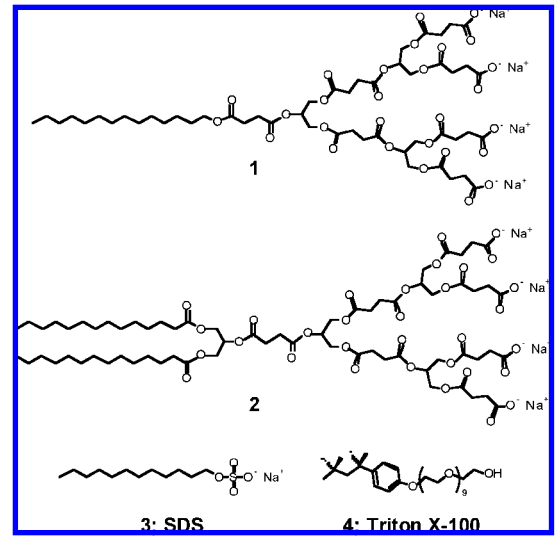

Figure 1. Structures of the two dendritic anionic amphiphiles, **1** and **2**, SDS, **3**, and Triton X-100, **4**.

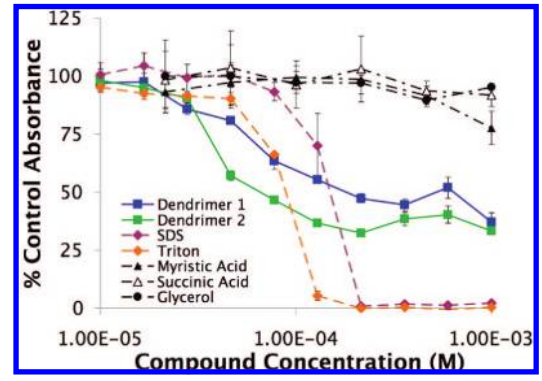

Figure 2. Cytotoxicity of the compounds against Gram-positive *B. subtilis*. Absorbances determined by measuring the turbidity of the cell-containing medium and reported as a fraction of untreated bacteria turbidity over the same 9 h period ($n = 3$; mean ± SD).

commercial amphiphiles **3** and **4** proved to be toxic while myristic acid, succinic acid, and glycerol were not toxic to the *B. subtilis* strain over the concentration range tested. Significantly, we observed antibacterial activity for the synthesized anionic amphiphilic dendrimers **1** and **2**, though the amplitude of the sigmoidal curve was comparatively compressed. The half-maximal effective concentration (EC_{50}) for **1** and **2** are 6.0×10^{-5} and 4.1×10^{-5} M, respectively. A partial explanation for this effect was obtained from further kinetic studies which suggested a bacteriostatic mechanism of action that required ∼1.5 h to slow the growth significantly compared to an untreated control.

We next examined the eukaryotic cytotoxicity by evaluating all the compounds against a primary cell line of human umbilical vein

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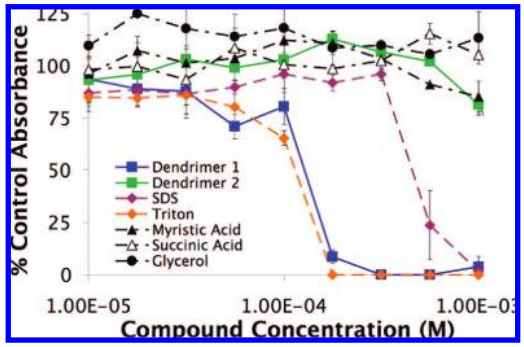

Figure 3. Cytotoxicity of the compounds against HUVEC. Absorbances are calculated as a percentage of the untreated cells over a 24 h time period ($n = 3$; mean ± SD).

Table 1. Experimental Properties of the Dendritic Amphiphiles **1** and **2** as well as SDS and Triton X-100[a]

	1	**2**	SDS	Triton
CAC (M)	2.0×10^{-4}	1.1×10^{-5}	1.0×10^{-3}	2.4×10^{-4}
HUVEC EC_{50}(M)	1.3×10^{-4}	1.5×10^{-3}	5.4×10^{-4}	1.1×10^{-4}
B. Subtilis EC_{50}(M)	6.0×10^{-5}	4.1×10^{-5}	1.4×10^{-4}	8.8×10^{-5}
ratio EC_{50}	2.2	≥36	3.8	1.3

[a] CMCs for SDS and Triton are from ref 15.

endothelial cells (HUVECs). Low-passage number HUVECs were equilibrated in a subconfluent monolayer and challenged with varied concentrations of the compounds for 24 h. The resultant cell viabilities were determined using a tetrazolium assay (Figure 3). As seen before with the Gram-positive bacteria, glycerol, myristic acid, and succinic acid were not cytotoxic, while both **3** and **4** were cytotoxic. **1** also showed cytotoxicity; however, **2** did not show any lethality in the concentration range tested. Subsequent experiments at higher values up to its aqueous solubility limit of 2×10^{-3} M produced a reduction to ~50% of the negative untreated control, but a complete sigmoidal shape was never obtained and so the EC_{50} for **2** was estimated to be greater than $\sim 1.5 \times 10^{-3}$ M. Importantly, **3**, **4**, and dendrimer **1** affected the viability of both prokaryotes and eukaryotes at similar concentrations with the compounds always having a ratio of eukaryotic/prokaryotic EC_{50} less than a factor of 3.8, which is nonideal for an antibacterial compound (Table 1). Dendrimer **2** however exhibited a ≥36-fold eukaryotic/prokaryotic EC_{50} ratio.

Upon further examination, the cytotoxicity of these compounds appears to be correlated with the formation of supramolecular structures in solution. Amphiphilic dendrimers are known to form a variety of supramolecular structures based on generation number, charge, hydrophilic/hydrophobic ratio, MW, etc., and such structures are actively investigated.[14] The critical aggregation concentrations (CAC) for compounds **1** and **2** were measured tensiometrically to be 2.0×10^{-4} and 1.1×10^{-5} M, respectively, values similar to their EC_{50} against *B. subtilis* and in the case of **1**, close to the EC_{50} against HUVEC as well. However, with **2** there is minimal lethality against HUVECs, and there appears to be no correlation between toxicity and CAC in this case (Table 1). We have observed that **2** can form vesicles of ~100 nm in diameter by TEM. Further experiments are underway to investigate the mechanism of action and supramolecular assemblies for these antibacterial dendrimers and the resulting eukaryotic/prokaryotic EC_{50} ratio.

In summary, we report the discovery of an anionic amphiphilic dendrimer that possesses Gram-positive antibacterial activity and minimal eukaryotic cell toxicity. This selectivity, as denoted by the lack of overlap in the cytotoxicological curves, is of chemical, biological, and clinical interest, as antibacterials such as these would be maximally effective against microbial infections without harming the host. Moreover, **2** can be prepared easily in good yield and in the future may provide a cost-effective route for preparation. Continued efforts in the synthesis of new dendritic macromolecules, characterization of their unique properties, and evaluation in clinically important indications will lead to new solutions for a variety of health care needs.

Acknowledgment. The authors would like to thank the NIH and BU for partial support. We also thank Dr. Tim Gardner.

Supporting Information Available: Experimental materials and methods for all procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA806912A