Comparing anti-HIV, antibacterial, antifungal, micellar, and cytotoxic properties of tricarboxylato dendritic amphiphiles

Richard V. Macri a, Janka Karlovská b, Gustavo F. Doncel c, Xiaosong Du a, Bhadreshkumar B. Maisuria a, André A. Williams a, Eko W. Sugandhi a, Joseph O. Falkinham III d, Alan R. Esker a, Richard D. Gandour a,*

a Department of Chemistry (0212), Virginia Tech, Blacksburg, VA 24061, United States
b Department of Physical Chemistry of Drugs, Faculty of Pharmacy, Comenius University, Odbojárov 10, SK-832 32 Bratislava, Slovakia
c CONRAD, Department of Obstetrics and Gynecology, Eastern Virginia Medical School, Norfolk, VA 23507, United States
d Department of Biological Sciences (0406), Virginia Tech, Blacksburg, VA 24061, United States

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Three series of homologous dendritic amphiphiles—RCONH(CH 2 CH 2 COOH) n , 1(n); RCONH(CH 2 CH 2 COOH) 2 , 2(n); RNHCONH(CH 2 CH 2 COOH) 3 , 3(n), where R = n-C 3 H 2n+1 , and n = 13–22 carbon atoms—were assayed for their potential to serve as antimicrobial components in a topical vaginal formulation. Comparing epithelial cytotoxocities to the ability of these homologues to inhibit HIV, Neisseria gonorrhoeae, and Candida albicans provided a measure of their prophylactic/therapeutic potential. Measurements of the ability to inhibit Lactobacillus plantarum, a beneficial bacterium in the vagina, and critical micelle concentrations (CMCs), an indicator of the potential detergency of these amphiphiles, provided additional assessments of safety. Several amphiphiles from each homologous series had modest anti-HIV activity (EC 50 = 110–130 μM), Amphiphile 2(18) had the best anti-Neisseria activity (MIC = 65 μM), while 1(19) and 1(21) had MICs against C. albicans of 16 and 7.7 μM, respectively. Two measures of safety showed promise as all compounds had relatively low cytotoxic activity (EC 50 = 210–940 μM) against epithelial cells and low activity against L. plantarum, 1(n), 2(n), and 3(n) had MICs ≤ 490, 1300, and 940 μM, respectively. CMCs measured in aqueous triethanolamine and in potassium hydroxide showed linear dependences on chain length. As expected, the longest chain in each series had the lowest CMC—in triethanolamine: 1(21), 1500 μM; 2(22), 320 μM; 3(22), 340 μM, and in potassium hydroxide: 1(21), 130 μM; 3(22), 40 μM. The CMC in triethanolamine adjusted to pH 7.4 was 400 μM for 1(21) and 3900 μM for 3(16). The promising antifungal activity, low activity against L. plantarum, relatively high CMCs, and modest epithelial cytotoxicity in addition to their anti-Neisseria properties warrant further design studies with dendritic amphiphiles to improve their safety indices to produce suitable candidates for antimicrobial vaginal products.

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1. Introduction

Preventing the spread of sexually transmitted pathogens, especially HIV, has come to the forefront as a global challenge. Medicinal chemists have addressed this challenge by designing compounds that can be used as topical vaginal microbicides, including contraceptive microbicides. Several promising HIV-inhibitory candidates have emerged that act in various ways—inhibiting virus cell entry, reverse transcription, integration, and/or maturation. Yet, developing new topical microbicides remains urgent as underscored by the recent halting of human trials with Ushercell and with SAVVY, a mixture of surfactants. The latest approach is to formulate gels that contain multiple agents from different classes of HIV inhibitors. Combining approaches that include changing behaviors, using barriers (condoms and microbicides), and treating vaginal infections, which represent serious risk factors for HIV acquisition, is required to prevent the spread of pathogens. Further, a microbicide should not harm resident microflora, in particular lactobacilli. In the final analysis, successful microbicides must be safe, acceptable, efficacious, and affordable.

Most amphiphiles are surfactants that act as detergents. Often dismissed as possible topical microbicides, these detergent amphiphiles disrupt membranes and damage tissue; hence, they are not safe. Yet, several detergent amphiphiles (e.g., SAVVY) have been studied as microbicides because they have anti-HIV activity at concentrations below those needed for detergency. In addition, they also display antimicrobial activity against other sexually transmitted pathogens such as Chlamydia trachomatis and Neisseria gonorrhoeae. This promising profile has led to human trials, in...
which SAVVVY\textsuperscript{8} was judged as harmless to the reproductive tract but unsuccessful in protecting against HIV infection.\textsuperscript{15,16}

Not all amphiphiles are good detergents. Amphiphiles that act as cell-signaling molecules interact with proteins in lipid bilayer membranes without causing membrane disruption.\textsuperscript{17} Furthermore, novel synthetic amphiphiles that do not denature membrane proteins can stabilize proteins outside a membrane.\textsuperscript{18} Therefore, it would appear possible to design a non-detergent amphiphile that is an effective antimicrobial agent while being completely safe for the vaginal environment. In addition, such an amphiphile must have good aqueous solubility to enable formulation into hydrogels.

With the focus on designing amphiphiles as antimicrobials, we prepared one-tailed, long-chain, water-soluble, dendritic tricarboxylato amphiphiles\textsuperscript{19–22} that selectively inhibited the growth of several bacteria and fungi.\textsuperscript{21} These dendritic amphiphiles demonstrated species, linker, and chain-length specificities. Very long fatty chains, up to 22 carbon atoms, were included in the homologous series of one-tailed amphiphiles—3CAmn (1(n)), 3CCbn (2(n)), 3CUr3(n)], where 3C = three carboxyl groups, Am = amido linker, Cb = carbamato linker, Ur = ureido linker, and n = the number of carbon atoms in the fatty chain.

Could these dendritic amphiphiles have anti-HIV activity? The rationale for assaying them as potential anti-HIV agents drew on previous work. By attaching lipid anchors (e.g., cholestanyl ethyl and nonacosanyl (C29)) to multi-carboxyl pharmacophores, Cushman and co-workers\textsuperscript{22–28} explored cosalane and analogues as antimicrobials.\textsuperscript{21} These dendritic amphiphiles demonstrated species, linker, and chain-length specificities. Very long fatty chains, up to 22 carbon atoms, were included in the homologous series of one-tailed amphiphiles—3CAmn (1(n)), 3CCbn (2(n)), 3CUr3(n)], where 3C = three carboxyl groups, Am = amido linker, Cb = carbamato linker, Ur = ureido linker, and n = the number of carbon atoms in the fatty chain.

By using a pendent-drop analyzer to measure surface tension,\textsuperscript{19} the CMCs of 1(15)–1(21) and 2(16)–2(22) were measured in triethanolamine/water [0.9% (wt/vol)]. The CMCs of 3(16)–3(22) in aqueous triethanolamine were previously reported.\textsuperscript{19} The pH of the solutions ranged from 8.0 to 9.5 depending on the concentration of the amphiphiles. The surface tension data for both 1(13), 2(14), and 3(14) failed to exhibit a clear CMC for concentrations below 10,000 μM, suggesting the absence of micelles. Our previous study\textsuperscript{19} suggested that, compared to results for the 1(n) and 2(n) series, the homologous series 3(n) in aqueous triethanolamine displayed an unusually gradual decrease in log CMC versus n. To confirm these results, CMCs of all three series were measured by a second method.
CMCs of 1(15)–1(21), 2(16)–2(22), and 3(16)–3(22) in triethanolamine/water [0.9% (wt/vol)] were measured by using pyrene fluorescence as an indicator of micelle formation. The log CMCs for the three series displayed nearly identical dependencies on chain length (Fig. 1). Additionally, all the CMCs were within 20% of those measured by surface tension, except for those previously reported for 3(16) and 3(22). Re-examination of the data collected previously for these two amphiphiles showed breaks in the plots of surface tension versus log concentration at values similar to those CMCs obtained by fluorescence measurements. Plotting log CMC measured by both methods versus n (Fig. 1, open symbols) revealed that all three series have similar dependences on chain length.

To probe the effect of counterion on micelle formation, CMCs of 1(13)–1(21) and 3(14)–3(22) in potassium hydroxide solutions were measured by conductivity methods. The pH of the solutions ranged from 10.0 to 11.0 depending on the concentration of the amphiphiles. Plots of log CMC versus n (Fig. 1, solid symbols) showed different dependencies on chain length.

Several observations emerge from examining Figure 1. In aqueous triethanolamine, CMCs of 1(n) are higher than those of 2(n) and 3(n). In aqueous potassium hydroxide, CMCs of 1(n) are higher for the longer chains and lower for the shorter chains when compared to those of 3(n). The linear dependencies of log CMC on n for 1(n) and 3(n) in aqueous potassium hydroxide differ, −0.175 ± 0.004 and −0.30 ± 0.02, respectively. Comparing the CMCs for a given series in aqueous potassium hydroxide to those in aqueous triethanolamine reveals the expected decrease (see Section 1). For series 1(n), the decrease averages 12-fold; the dependence of log CMC on chain length is virtually identical with the different counterions. For series 3(n), the decrease ranges from 3- to 13-fold as chain length increases. The dependence of log CMC on n in aqueous potassium hydroxide is significantly steeper than that in aqueous triethanolamine.

The CMC data were measured at high pH to ensure that only the trianion (Fig. 2) was present. However, the microbiological assays were performed at pH 7.4. As values of CMC depend on pH when pH ~ pK_a, we needed to know the pK_a for ionizing the di- and trianion. The ionization equilibria in water at pH 7.4 for 1(21) were calculated to give the pK_a’s shown above and % microspecies shown below the equilibrium arrows in Figure 2. The three calculated pK_a’s were very close, ranging from 3.7 to 4.7. Previous titration studies of the tricarboxylato headgroup showed that the trianion dominates the equilibrium at pH ≥ 7, consistent with the calculations.

Amphiphiles 1(21) and 3(16) were selected to study how pH affects CMC. This choice, based on the strong anti-C. albicans activity (see below), enabled a direct comparison with CMC values determined with potassium and triethanolammonium counterions, respectively. The choice also reflected the extremes in chain length. As the biological assays were performed with triethanolamine stock solutions of the amphiphiles, the pH of an aqueous triethanolamine solution was adjusted with phosphoric acid. Values of CMC were measured by using pyrene fluorescence at 7.4 and 6.4 (Fig. 3). Amiphile 3(16) had a CMC of 3900 μM compared to 7100 μM at higher pH; 1(21) had a CMC of 400 μM compared to 1500 μM. Adjusting the pH to 6.4 dropped the CMC to 480 μM for 3(16) and 150 μM for 1(21).

The twofold and fourfold decrease in CMC for 3(16) and 1(21), respectively, suggests that at a pH of 7.4 the dianion is present at
a high enough concentration to affect micelle formation. The values of CMC are still quite high compared to the biological activity (see below) measured at this pH. This result validates using these amphiphiles at neutral pH. Lowering the pH to 6.4 lowers the values of CMC even further, however. The trend in decreasing CMC with decreasing pH in the acidic range is clear and warrants further studies with these triheaded amphiphiles, especially with different counterions to explore limitations on the useful pH range for these amphiphiles.

### 2.2. Biological activity

Being aware that HIV-1 R5 predominates over HIV-1 IIIB (X4-tropic) during the primary infection,\(^\text{31}\) not because only R5 viruses are transmitted but rather because a bottleneck exists at the mucosal level that selects R5,\(^\text{32}\) we first screened against HIV-1 IIIB (X4-tropic) because this assay is more robust and consistent. Anti-viral compounds, preferably, should be active against both types of viruses. Any compound selected for further development would also be tested against R5 and cell-associated viruses.

Dendritic amphiphiles displayed different patterns for anti-HIV, antibacterial, antifungal, and cytotoxic activities (Table 1). The susceptibilities of *L. plantarum*, *C. albicans*, and *N. gonorrhoeae*, expressed as minimal inhibitory concentration (MIC\(_{99}\)) for complete inhibition of growth, varied with respect to chain length and linker. Assays for anti-HIV and cytotoxicity, expressed as 50% effective concentration (EC\(_{50}\)), showed similar patterns. The ranges of cytotoxic (210–940 \(\mu M\)) and anti-HIV (110–740 \(\mu M\)) activities were narrower than those for antibacterial (65–12000 \(\mu M\)) and antifungal (7.7–2800 \(\mu M\)) activities. The headgroup more so than the linker or alkyl chain length influences cytotoxic and anti-HIV activities (Table 1). In contrast, antibacterial and antifungal activities displayed chain-length, linker, and species selectivities. Perhaps these differences in activities reflect membrane selectivity by dendritic amphiphiles. The membrane of human cells and the envelope of HIV differ from those of bacteria and fungi.

#### 2.2.1. Comparison of anti-HIV and cytotoxic activities

The anti-HIV activities of the three homologous series showed a slight dependence on chain length (Fig. 4, green open symbols); the cytotoxicity did not (Fig. 4, red symbols). The cytotoxicities occurred at slightly higher concentrations than the anti-HIV activities. The largest difference occurred for 1\((15)\), where [cytotoxicity EC\(_{50}\)/anti-HIV EC\(_{50}\)] = 4. The cytotoxicities of the 2\((n)\) (Fig. 4, compare green open circles and red circles) and 3\((n)\) (Fig. 4, compare green open triangles and red triangles) series occurred, on average, at 2.5-fold and 1.6-fold higher concentrations than the anti-HIV activities, respectively. The safety indices for anti-HIV activity are too low to warrant further testing. Target values for these ratios should be \(\geq 1000\).\(^\text{37}\) Compounds displaying low safety indices might induce mucosal irritation and inflammation, and may facilitate, rather than decrease, HIV acquisition.\(^\text{33–35}\) Lower safety indices, however, may still be acceptable for a short-term treatment of vaginal infections,\(^\text{2}\) especially in populations with low prevalence of sexually transmitted infections.

#### 2.2.2. Comparison of antibacterial activities

The susceptibilities of *N. gonorrhoeae* and *L. plantarum* to the three homologous series show that the longer homologues in all series inhibit growth more effectively than the shorter homologues (Fig. 5). Further, *N. gonorrhoeae* was more susceptible than *L. plantarum*. From our previous studies,\(^\text{21}\) the susceptibility of *L. plantarum* to 1\((n)\) showed no chain-length dependence (Fig. 5, orange squares). Susceptibilities of *L. plantarum* to 2\((n)\) and 3\((n)\) showed values of MIC\(_{99}\) within the range of the CMCs in triethanolamine. Susceptibility of *N. gonorrhoeae* was highest for the longer chains.

### Table 1

<table>
<thead>
<tr>
<th>Amphiphile</th>
<th>EC(_{50})^a ((\mu M))</th>
<th>MIC(_{99})^b ((\mu M))</th>
<th>EC(_{50})^a ((\mu M))</th>
<th>KOH</th>
<th>Amine pyrene</th>
<th>Amine, surface tension</th>
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<tr>
<td>Anti-HIV</td>
<td>L. plantarum(^c)</td>
<td>N. gonorrhoeae</td>
<td>C. albicans(^d)</td>
<td>Cytotoxicity</td>
<td></td>
<td></td>
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<tr>
<td>1((13))</td>
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<td>12,000</td>
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<td>300</td>
<td>3500 n/d</td>
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<td>12,000</td>
<td>290 n/d</td>
<td>940</td>
<td>1500 18,000 17,000</td>
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<tr>
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<td>520</td>
<td>130</td>
<td>16 n/d</td>
<td>340</td>
<td>71 11,000 11,000</td>
</tr>
<tr>
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<td>120</td>
<td>7.7 n/d</td>
<td>250</td>
<td>13 1500 2000</td>
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<td>6400</td>
<td>580</td>
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<td>620</td>
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<tr>
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<td>6000</td>
<td>280</td>
<td>47 n/d</td>
<td>440</td>
<td>230 1800 1800</td>
</tr>
<tr>
<td>2((16))</td>
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<td>1400</td>
<td>65</td>
<td>180 n/d</td>
<td>350</td>
<td>71 2700 2800</td>
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<tr>
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<td>120</td>
<td>1400 n/d</td>
<td>280</td>
<td>71 800 500</td>
</tr>
<tr>
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<td>1300</td>
<td>120</td>
<td>1300 n/d</td>
<td>360</td>
<td>71 330 320</td>
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<tr>
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<td>1600 n/d</td>
<td>290</td>
<td>8400 n/d</td>
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<tr>
<td>3((16))</td>
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<td>6000</td>
<td>280</td>
<td>47 n/d</td>
<td>330</td>
<td>2300 7100 4900</td>
</tr>
<tr>
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<td>1400</td>
<td>130</td>
<td>1400 n/d</td>
<td>280</td>
<td>130 2300 2200</td>
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<tr>
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<td>120 1300 1400</td>
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<tr>
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<td>940</td>
<td>120</td>
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<td>260</td>
<td>40 400 340</td>
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</table>

Data for anti-HIV activity and cytotoxicity are expressed as EC\(_{50}\)'s, while data on inhibition of *L. plantarum*, *N. gonorrhoeae*, and *C. albicans* are expressed as minimal inhibitory concentrations (MIC\(_{99}\)).

\(^a\) Standard deviation ±10%.

\(^b\) Error ±1 twofold dilution.

\(^c\) Data from Ref. 21.

\(^d\) Not determined.
in all series (Fig. 5, blue open symbols), with the MIC$_{99}$ for 2(18) slightly better than those of the other homologues.

The MIC$_{99}$ against _N. gonorrhoeae_ for all series reach a plateau in activity for the long chains in each series (Fig. 5, blue open symbols). This contrasts with natural saturated fatty acids in which hexadecanoic acid had the lowest IC$_{50}$ and acids longer than C$_{17}$ showed no activity. The authors of that study drew the conclusion that the undissociated fatty acid was the active form. If so, the susceptibility of _N. gonorrhoeae_ to dendritic amphiphiles would increase in the vagina where pH (~4) is comparable to the calculated pK$_a$'s (Fig. 2).

### 2.2.3. Comparison of anti- _C. albicans_ activities

The susceptibility of _C. albicans_ to the amphiphiles showed different chain-length dependencies among the series. Activity improved with increasing chain length for 1(n), while the activities of 2(16) and 3(16) were considerably lower than other homologues in the respective series (Fig. 6). These cutoff effects for the 2(n) and 3(n) series could not be explained by insolubility or low values of CMC. The MIC$_{99}$ for 1(21) against _C. albicans_ was the lowest.

### 2.3. Comparing CMCs, cytotoxicities, and biological activity

The CMCs in aqueous triethanolamine (Fig. 1) define the upper limits of antimicrobial activity. Presuming that all homologues would show a decrease in CMC of two- to fourfold when measured at pH 7.4 as observed for 1(21) and 3(16), the majority of antimicrobial and anti-HIV activities expressed as MIC$_{99}$ or EC$_{50}$ are lower than the CMCs in triethanolamine at pH = 7.4. However, CMCs in potassium hydroxide are lower than the MIC$_{99}$s and EC$_{50}$s for all series reach a plateau in antimicrobial activity. Presuming that all homologues maintain aqueous solubility, increase the CMC, and reduce the cytotoxicity, one objective will be to find a headgroup that gives much lower than those against _L. plantarum_. The relative resistance of _L. plantarum_ to the amphiphiles means that these beneficial microbes would survive any low-dose treatment with these compounds. The promising anti- _C. albicans_ activity offers possibilities for optimizing a lead that may be developed as a component of an antimicrobial formulation to treat vaginal candidal infections. From these studies, the best candidate is 3(16) because of high CMCs (>2000 M), modest cytotoxicity (EC$_{50}$ = 350 μM), and anti- _C. albicans_ activity (MIC$_{99}$ = 47 μM).

The challenge going forward will be to make structural changes that maintain aqueous solubility, increase the CMC, and reduce the cytotoxicity, while improving antifungal, antibacterial, and anti-HIV activities. One objective will be to find a headgroup that gives a high CMC and is insensitive to changes in pH in the range of 4–7.5. Other objectives will be to find the mechanism of action and study how these amphiphiles interact with bilayer membranes. Our current efforts are focused on exploring these challenges.

### 3. Methods

#### 3.1. General

Amphiphiles were synthesized and purified as described previously. All reagents and solvents were used as received, except water, which was purified by reverse osmosis.

#### 3.2. Measuring CMCs

##### 3.2.1. Surface-tension measurements

A video system (FTA-200, First Ten Ångstroms), mounted on a vibration isolation platform, captured images of a pendant drop from an 18-gauge stainless-steel needle (1.27 mm) to determine surface tension. To help maintain humidity levels and ensure that the drop size did not vary significantly during the measurements, the pendant drop was enclosed in a standard glass cuvette that contained 0.5 mL of aqueous triethanolamine (~9 mg/mL) dissolved in ultrapure (Type I) water. A hole (2.54 mm) was drilled in the Teflon lid to accommodate the needle. Calibration of the instrument entailed measuring the tip width of the needle with a
3.3. HIV inhibition and cytotoxicity assays

Surface tension values for an individual static drop were measured for each solution by drop-shape analysis, from 20 images of each drop (one image was taken by the software every 0.5 s) to produce an average surface tension. Reproducibility of the measurements was determined by performing the drop-shape analysis on five different drops of the same solution to obtain values with standard deviations of approximately 0.2 mN/m. Plots of surface tension versus log[amphiphile] were made; linear least-squares analyses of both the points before and the points after the break were used to determine the CMC.

3.2.2. Fluorescence measurements

Aliquots of pyrene in methanol were first transferred to empty vials, then the solutions of amphiphiles at given concentrations were added to vials after the methanol evaporated. All mixtures were shaken gently for at least 24 h. The emission fluorescence spectra of pyrene were recorded by a spectrometer with the excitation wavelength = 334 nm. Excitation and emission bandpasses were set at 5 nm and 2.5 nm, respectively. The emission intensities of the first (I1 = 373 nm) and third (I3 = 384 nm) peaks were used to determine the CMC value. Plots of log concentration of amphiphile versus I1/I3 of were made. The CMC was taken as point where a distinct change in the decrease of I1/I3 occurred.56

3.2.3. Conductivity measurements

The dry dendritic amphiphile was added to a KOH solution. The suspension was heated to 60 °C, vortex-mixed, and supplemented by KOH powder until a transparent solution was obtained. For each solution, pH at 30 °C was measured before starting and after finishing measurements of conductivity. The conductivity measurements were made at 30.0 ± 0.1 °C with the digital WTW inoLab® conductimeter (Wissenschaftlich Technische Werkstätten), equipped with an WTW Tetra Con 325 electrode (Wissenschaftlich Technische Werkstätten). The solutions were stirred during measurements. The value of the CMC was estimated by least-squares analysis of the linear portions of conductivity-versus-concentration plots both before and after a break in the conductivity is observed.

3.3. HIV inhibition and cytotoxicity assays

Anti-HIV activity was evaluated by incubating serial dilutions of the dendritic amphiphiles with HIV-1 (IIIB) and transfected CD4+, β-galactosidase-expressing HeLa cells for 2 h in 12-well plates.5 After this incubation, virus and compounds were washed away, and the cells were suspended and cultured in fresh RPMI 1640 medium supplemented with 10% fetal bovine serum for 48 h at 37 °C and 5% CO2. These cells express β-galactosidase under the control of the HIV long terminal repeat. Thus, expression of this enzyme is linked to HIV infection. After the 48 h incubation, cells were washed in phosphate-buffered saline, lysed, and the β-galactosidase activity of the lysate was measured by using the GalactoStar β-galactosidase reporter gene assay following the manufacturer’s directions (Applied Biosystems, Foster City, CA). Chemiluminescence of the β-galactosidase hydrolysis product was measured in a luminometer. Cells incubated with virus and no compounds provided values representing 100% infection. Compound cytotoxicity was evaluated on a separate plate, under the same conditions, by using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Promega, Madison, WI). This assay assesses cell viability based on the mitochondrial reduction of MTT, and has been used to determine the irritating potential for surfactants on skin.58 Fifty percent effective concentrations (EC50’s) were calculated by using a linear regression model.

3.4. Measurement of minimal inhibitory concentrations (MIC) against N. gonorrhoeae

N. gonorrhoeae was grown on Chocolate Agar medium (BBL Microbiology Systems, Sparks, MD) containing 1% (w/v) hemoglobin. Single colonies of N. gonorrhoeae strain ATCC 31426 were picked and used to inoculate 2 mL of GC broth medium (BBL Microbiology Systems, Sparks, MD) and grown at 37 °C in an anaerobic jar containing a CO2 gas generator (Gas Pak, BBL Microbiology Systems, Sparks, MD). After 4 d incubation, 5 mL of buffered saline gelatin (BSG)—gelatin (0.1 g/L), NaCl (8.5 g/L), KH2PO4 (0.3 g/L), and Na2HPO4 (0.6 g/L) in H2O—was added to each culture and vortexed. The brown insoluble contents of the GC medium were allowed to settle to the bottom of the tube for 4 h at room temperature. The supernatant cell suspension was transferred to a fresh sterile tube without collecting the insoluble contents and the turbidity of the suspension was diluted with BSG to equal a McFarland No. 1 standard. The resulting suspension was streaked on Chocolate Agar to confirm colony morphology and look for contaminants. For the work reported here, all cultures and suspensions that were used as inocula were uncontaminated and the colonies had the expected morphologies. Sensitivity of the N. gonorrhoeae cells grown as described above were measured by broth microdilution in 96-well plates as described.21 Aqueous triethanolamine without amphiphile was also tested for antimicrobial activity by using the same protocol; no antimicrobial activity was found. The concentration of amphiphile ranged from 6.25 mg/mL to 0.55 μg/mL. After the 96-well plates were incubated at 37 °C for 4 d, the MICs were read. The MIC of each compound was measured in triplicate and was defined as the lowest concentration of drug resulting in a prominent visible decrease in turbidity.

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References and notes
