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Mode of action investigation for the antibacterial cationic anthraquinone analogs

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ABSTRACT

Reported previously by our group, we have developed a novel class of antibacterial cationic anthraquinone analogs with superb potency (MIC <1 μg/mL) against Gram positive (G+) pathogens including Methicillin-resistant *Staphylococcus aureus* (MRSA). However, most of these compounds only manifest modest antibacterial activity against Gram negative (G−) bacteria. Further investigation on the antibacterial mode of action using fluorogenic dyes reveals that these compounds exert two different modes of action that account for the difference in their antibacterial profile. It was found that most of the compounds exert their antibacterial activity by disrupting the redox processes of bacteria. At high concentration, these compounds can also act as membrane disrupting agents. This information can help to design new therapeutics against various bacteria.

In light of the epidemic of antibiotic resistant bacteria,[1] our group has been devoting effort in the development of new antibacterial agents.[2] We have recently reported the discovery of a new class of antibacterial cationic anthraquinone analogs derived from 1-alkyl-1*H*-naphtho[2,3-*d*]triazole-4,9-diones (Fig. 1).[3] Our studies show that the compound attached with an octyl group (**3a**) displays significant antibacterial activity. Further shortening the alkyl chain length leads to the decrease of antibacterial activity. Interestingly, increasing the alkyl chain length to dodecyl (C12) and hexadecyl (C16) groups, such as in compounds **3e** and **3f**, similar activities against both G+ and G− bacteria were noted. Although compound **3a** has highly activity toward G+ bacteria, it is only modestly active against G− bacteria. Therefore, we were interested in studying more details on the possible mode of antibacterial action and structure activity relationship (SAR) of these compounds. For this purpose, four additional cationic anthraquinone analogs were synthesized (Scheme 1).

Compounds **2b**, **2c** and **2d** were prepared from the cycloaddition/oxidation approach using naphthoquinone and the corresponding alkyl bromides in a one-pot protocol.[4] Methylation of the N-3 nitrogen provided the desired products **3b**, **3c** and **3d** with nonyl, decyl, and undecyl groups, respectively. In order to investigate the effect of the chain length on N-3 nitrogen, ethylation of **2a** was conducted using ethyl triflate to yield **3g**. Ion-exchange using Dowex resin offered the final products with chloride as the counter ion.

All of the newly synthesized compounds along with **3a**, **3d** and **3e**, which were prepared previously, were tested against *Escherichia coli* (G−, ATCC 25922) and *Staphylococcus aureus* (G+, ATCC 25923). The minimum inhibitory concentrations (MIC's) were determined (Table 1). From the antibacterial testing results, it is apparent that these cationic compounds are much more active against G+ bacteria than G− bacteria which is consistent with the antibacterial profile of cationic antiseptic agents, such as hexadecyltrimethylammonium bromide (HTB) and cetrimonium bromide.[5] However, unlike most of the antiseptic agents that often contain much longer linear alkyl chain (C12-C18), compounds we synthesized that incorporated with octyl, nonyl and decyl groups provide the highest activity, especially, against *S. aureus*. An increase in the chain length greater than C12 results in a decrease in the compounds' antibacterial activity. To our surprise, the MIC's of **3e** and **3f** were higher than what we previously reported.[3] We noted some induced resistance in *E. coli* when the experiments were observed for 10–18 h. This could be accounted for the discrepancies in MIC's. Interestingly, the incorporation of ethyl group

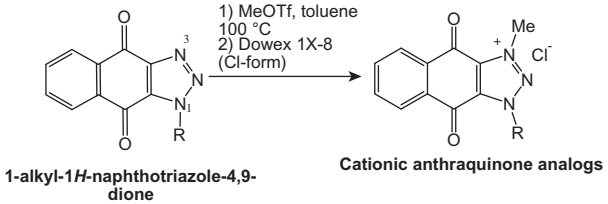

Figure 1. Cationic anthraquinone analogs.

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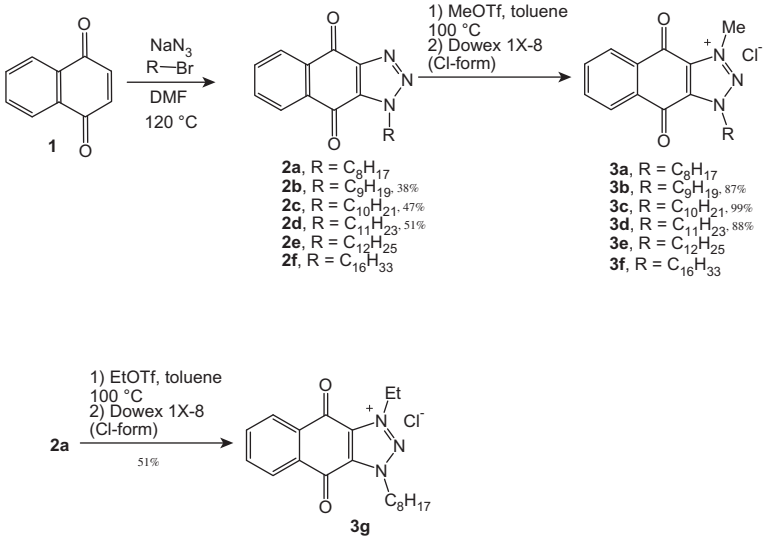

Scheme 1.

Table 1
MIC's of cationic anthraquinone analogs (unit: μg/mL)

Compound	*E. coli*	*S. aureus*
3a	16–32	0.032–0.064
3b	64	0.032–0.064
3c	32	0.032
3d	32–64	0.25–0.5
3e	16–32	0.064–0.125
3f	⩾250	0.25–0.5
3g	⩾250	0.25–0.5

instead of methyl group (**3a** vs **3g**) results in an eight-fold decrease in the antibacterial activity. It seems that the antibacterial activity is sensitive to the lipophilicity of the incorporated alkyl groups. Further investigation using compounds with various chain lengths at both nitrogen atoms and quantity structure activity relationship (QSAR) analysis has been carried out.

The cationic anthraquinone compounds have two distinct structural motifs: the cationic anthraquinone core and the lipophilic alky chain(s). Molecules bearing naphthoquinone or anthraquinone cores are known to uncouple mitochondria oxidative phosphorylation via redox chemistry.[6] Antiseptic agents with lipophilic alkyl chain(s) are noted for their ability to disrupt the bacterial membrane.[7] Therefore, we postulate that these cationic anthraquinone compounds exert their antibacterial activity through one mode or a combination of these modes of action. To evaluate the hypothesis of membrane disruption, a fluorescence dye, Sytox was used.[8] Sytox dye cannot penetrate the intact bacterial membrane unless the membrane is damaged by the membrane disrupting agents. Once inside the cells, Sytox dye can bind to nucleic acid and emit strong fluorescence. Therefore, the observation of fluorescence is an evidence of membrane disruption. To investigate the other hypothesis involving disruption of the redox processes inside the cell, a fluorogenic redox indicator, 5-cyano-2,3-ditolyltetrazolium chloride (CTC) dye was employed.[9] CTC dye can penetrate the bacterial membrane and, once inside the cells, CTC can be readily reduced by non-specific electron carriers. The reduced form of CTC dye will emit strong fluorescence indicating the cell redox processes are operating normally. Therefore, an observation of no fluorescence is an indication of cell death as a result of inhibited redox processes.

Two concentrations of cationic anthraquinone analogs were used: one was above MIC to provide the bactericidal condition and the other was below MIC to evaluate the effect of compounds with some live bacteria. To simplify the task, we selected MIC's of 2 mg/mL and 4 μg/mL for *E. coli*, and 8 and 0.064 μg/mL for *S. aureus*. For the Sytox dye experiment, the results were observed following 10 min and 2 h of incubation of bacteria with the compounds. A known membrane disrupting agent HTB was used as the control. For the CTC dye experiment, the results were observed following 2 and 6 h of incubation of bacteria with the compounds. Neomycin was used as the control. Neomycin interferes with the bacterial protein synthesis, which is a slower than the process of redox inhibition. Thus, we expect to observe fluorescence from neomycin treated cells during the designed time frames even at a concentration above the MIC of neomycin. For all the experiments, two types of photos were taken for comparison: fluorescence and phase modes.

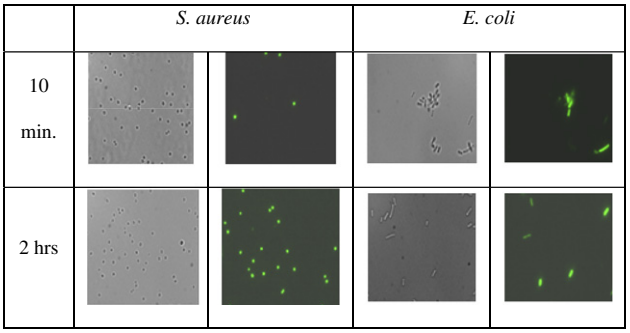

Figure 2. Bacteria treated with HTB (2 mg/mL) and Sytox dye.

From the Sytox dye experiments, cells that were treated with HTB (2 mg/mL) produced green fluorescence after 10 min of incubation and more cells with fluorescence appeared following 2 h of incubation (Fig. 2). When *E. coli* was treated with compound **3a** at a concentration below MIC, very few cells emitted green fluorescence (Fig. 3). In contrast, when *E. coli* was treated with compound **3a** at a concentration above MIC, many cells emitted green fluorescence. The results suggest that the bacterial membrane can only be disrupted by cationic anthraquinone analogs at high concentration but remain largely unaffected with compounds at lower concentration. When *S. aureus* was treated with cationic anthraquinone analogs, very few cells emitted fluorescence at concentrations both above and below the MIC's (Fig. 4). The results

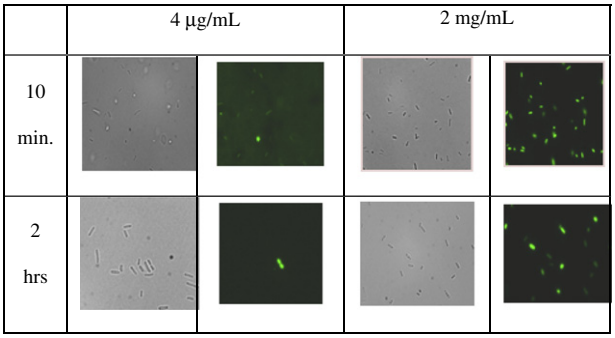

Figure 3. *E. coli* treated with **3a** and Sytox dye.

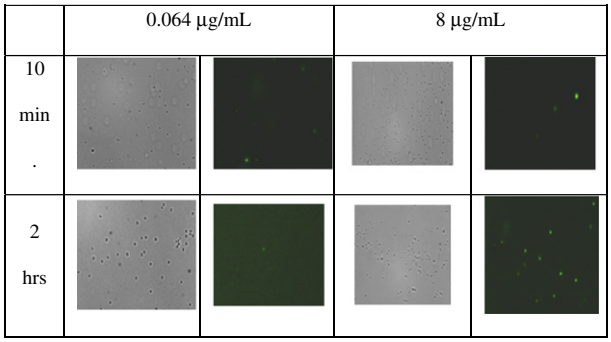

Figure 4. *S. aureus* treated with **3a** and Sytox dye.

imply that the bacterial membrane is largely unaffected at concentrations both above and below the MIC's. This suggests that different target(s) inside the G+ bacteria are responsible for the antibacterial activity of these cationic anthraquinone analogs. Similar trends occurred using other compounds.[11]

From the CTC dye experiments, red fluorescence appeared for both *E. coli* and *S. aureus* that were treated with neomycin at concentrations both above and below its MIC's following the designed incubation time (Fig. 5). The bactericidal effect of neomycin occurs in a relatively long time frame (10–18 h). Therefore, this observation of fluorescence indicated that the redox processes remained active and most of the cells were still alive even in the presence of bactericidal neomycin after 6 h.

For the assay using CTC against *E. coli*, significant fluorescence was observed after a 2 and 6 hrs of incubation with the concentration below the compounds' MIC indicating that the majority of the bacteria were still alive (Fig. 6). In contrast, very little or no fluoresce was noted when the concentration was above the compounds' MIC indicating cell death as a result of the inhibited redox processes. Combining the results from Sytox dye experiments, it confirms that

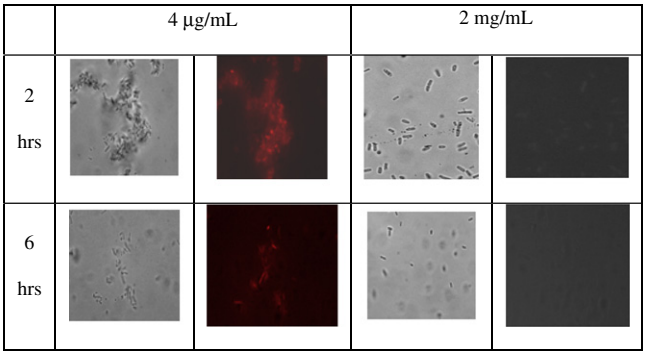

Figure 6. *E. coli* treated with **3a** and CTC dye.

these cationic anthraquinone compounds kill bacteria by membrane disruption which occurs much faster than the antibacterial mode of neomycin. For the assay against *S. aureus*, some fluorescence was observed with the concentration below the compounds' MIC suggesting that the redox processes were hampered even while cells were still alive (Fig. 7). Finally, almost no fluorescence was seen when cells were treated with concentration above the compounds' MIC since the redox processes had been inhibited. Therefore, it is clear that the redox process(s) can be disrupted by these cationic anthraquinone analogs. Again, similar trends occurred using other compounds.[11]

G− bacteria differ from G+ bacteria by the presence of outer membrane and the lipopolysaccharides (LPS), which contain various negatively charged functional groups.[10] The cationic anthraquinone analogs exert their antibacterial activity predominately by inhibiting or disrupting the redox processes inside the cells. To inhibit the redox processes, these cationic compounds need to diffuse across the bacterial membrane. The outer membrane and LPS of G− bacteria can form the additional barrier that account for the relatively lower antibacterial activities of these cationic compounds.[5] However, at high concentration, these cationic compounds can still function as membrane disrupting agents like HTB. Nevertheless, increasing the chain length (lipophilicity) will make it more difficult for the compounds to pass through the bacterial membrane, and thus results in lower antibacterial activity.

In conclusion, we have identified the optimal chain length for antibacterial activity of the cationic anthraquinone analogs. We have also shown that these compounds can inhibit the redox processes inside the cells which could be the cause of bactericidal effect of these compounds. These compounds can also disrupt the bacterial membrane at a higher concentration. The unique combination of these two antibacterial modes of action can help to design better antibiotics that can be either selective or broad spectrum.

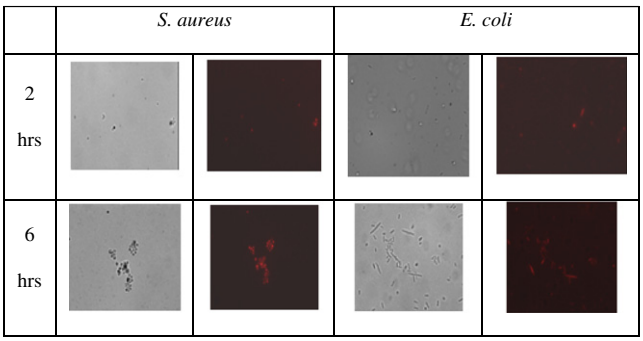

Figure 5. Bacteria treated with neomycin (2 mg/mL) and CTC dye.

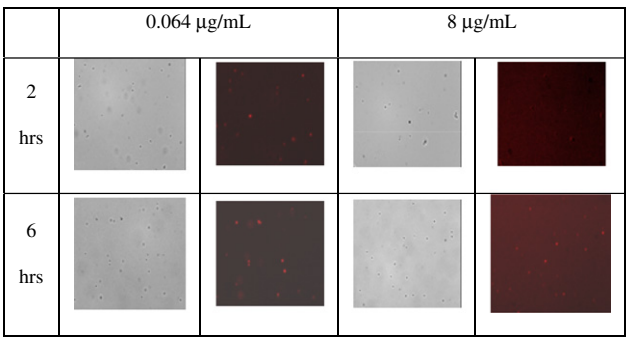

Figure 7. *S. aureus* treated with **3a** (NQM108) and CTC dye.

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Supplementary data

Supplementary data (experimental procedures, spectroscopic information for the synthesized compounds and additional photos from the antibacterial experiments) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.08.107.

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