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Synthesis and biological evaluation of novel 1,3,5-triazine derivatives as antimicrobial agents

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Abstract—Numerous studies have contributed to the development of natural and synthetic antimicrobial peptides as a prospective source of antibiotic agents. Based on the concept that cationic charge, bulk, and lipophilicity are major factors determining antibacterial activity in these peptides, we designed and screened several combinatorial libraries based on 1,3,5-triazine as a template. A set of compounds were identified to show potent antimicrobial activity together with low hemolytic activity.
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As multidrug-resistant bacterial strains proliferate, the need for new kinds of antibiotics is growing. In the past two decades it has been found that a wide range of antimicrobial peptides (AMPs) are secreted by innate immune system of multicellular organisms in response to infection by foreign bacteria, viruses, or fungi.[1–3] These host defense peptides have been considered as prospective antibiotic agents because their effect is rapid, broad spectrum, and indifferent to resistance to standard antibiotics such as penicillin.[4] AMPs differ dramatically in size, sequence, and structure, apparently sharing only amphiphilicity and positive charge.[1,2] The proposed mechanism of action of AMPs focuses on the interaction between the peptides and the negatively charged cell wall and/or plasma membranes of bacterial cells, although certain antimicrobial peptides can also employ more sophisticated mechanisms.[5] Recently, a pharmacophore for short cationic antimicrobial peptides of the cathelicidin family including indolicidin and tritrpticin has been identified.[6,7] A range of very short cationic peptides containing only positively charged Arginine (R) and lipophilic Tryptophan (W) residues have been found to serve as effective antimicrobial agents with MIC values in the low micromolar range.[8,9]

Despite some potential advantages over conventional antibiotics, practical applications of antimicrobial peptides, however, are limited by several factors. Peptides tend to be expensive to synthesize in bulk, are vulnerable to protease degradation, and have relatively high cytotoxicity to red blood cells. A number of mimetics constructed from unnatural β-amino acids, cyclization of peptides, peptoid mimics, or other peptide analogs have been investigated in efforts to overcome these problems.[10–13] Here we have designed and screened several combinatorial libraries of small compounds to mimic the hydrophobic and charge pattern detected in the pharmacophore of short cationic antimicrobial peptides. The present work focuses on stepwise design and optimization of functional groups selected to reproduce the RW pharmaceutical properties based on 1,3,5-triazine as a template. The results reveal potential new antimicrobial leads, as well as provide some insight into the structure–function relationship of these agents.

1,3,5-Triazine possessing threefold symmetry was chosen in our libraries as the scaffold because they allow for versatile modifications uncomplicated by regiochemical concerns and have proven themselves to be useful biological targets.[14–16] The first two libraries were created in order to screen various functional groups for their antimicrobial activity. A total of 79 compounds in library 1 and 72 compounds in library 2 (Scheme 1) were synthesized utilizing an orthogonal synthetic approach based on the triazine scaffold as documented previously.[17,18] Two mono-substituted triazine templates were used for these libraries, in which amantadine

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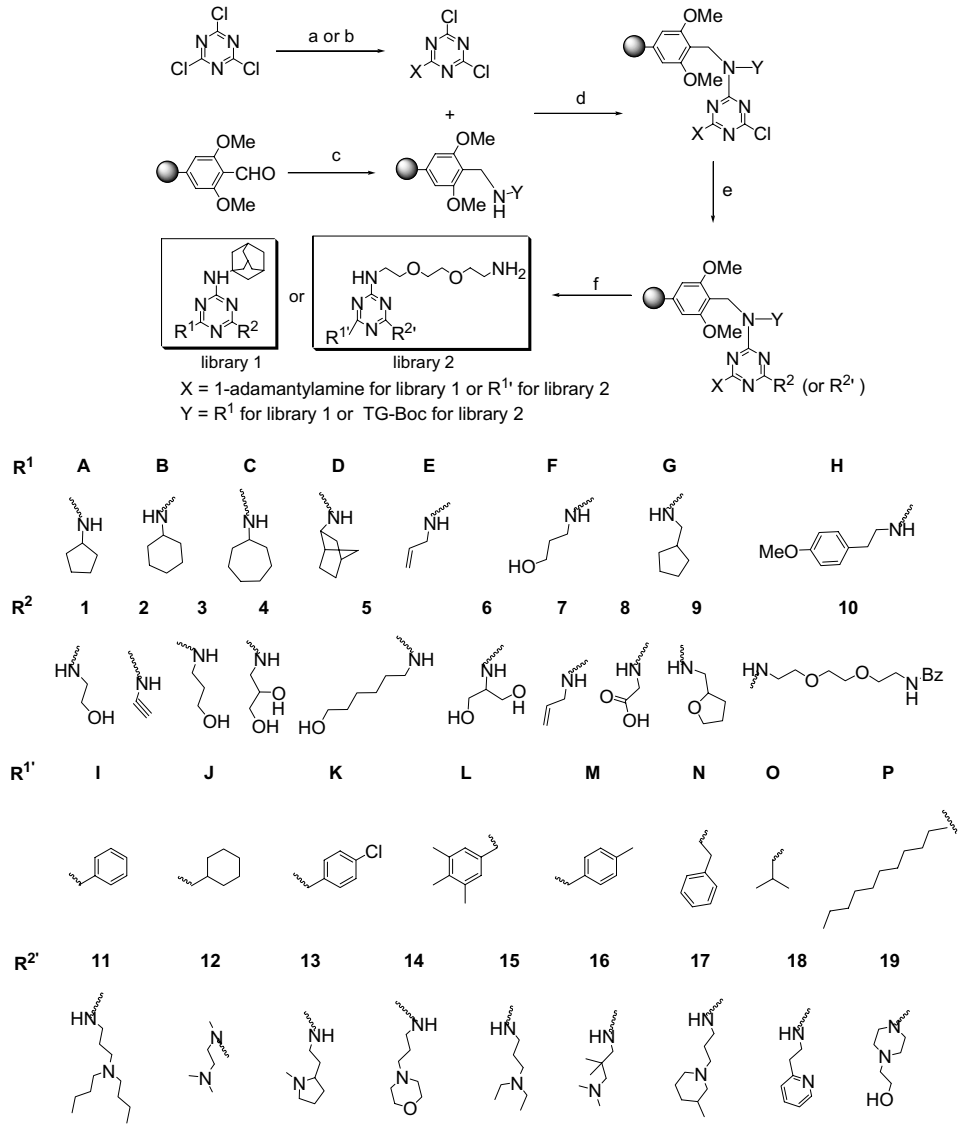

Scheme 1. General synthetic scheme for libraries 1 and 2. Reagents and conditions: (a) 1-Adamantylamine, DIEA, THF, rt, 1 h, for library 1; (b) $R^{1'}MgX$, THF, 0 °C, 2 h, for library 2; (c) R^1 (for library 1) or TG-Boc (for library 2), 2% acetic acid in THF, rt, 1 h, followed by NaB(OAc) 3H, rt, 12 h; (d) THF, DIEA, 60 °C, 1 h; (e) R^2 or $R^{2'}$, DIEA, NMP:*n*-BuOH = 1:1, 120 °C, 3 h; (f) 10% TFA in dichloromethane, rt, 1 h.

or 2,2′-(Ethylenedioxy)bis(ethylamine) (TG-Boc) were prefixed onto the triazine scaffold to represent one lipophilic/bulky group and one charged group, respectively. In addition to the prefixed groups, series of compounds were synthesized to introduce two more groups (hydrophobic, bulky, positively charged, etc.) to positions R^1 and R^2. Antimicrobial activities were tested against the Gram-positive bacteria, *Bacillus subtilis*, which showed library 1 with amantadine to be superior to library 2 in general (data not shown).

The subsequent library 3 with 2×8 compounds (Fig. 1) was designed based on eight functional groups with highest frequency of effectiveness screened in libraries 1 and 2.[19] C11 was identified to be the most active antimicrobial with IC_{50} = 2.5 μM in library 3, see Ref. [20] for assay results. A further set of 22 triazine compounds was synthesized with optimization of R^1 and R^2 groups based on C11, then purified and confirmed by NMR.[21]

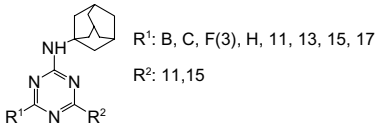

Figure 1. Design of library 3. B, C, F(3), H, 11, 13, 15, and 17 are functional groups shown in Scheme 1.

Some of these show the highest antimicrobial activity we have obtained so far, for example TZ-4 and TZ-12. The antimicrobial test data for these compounds revealed some interesting trends and various degrees of activities against *B. subtilis* (Fig. 2). In general, increased bulk (ring size) on one side chain of the triazine compounds enhanced antimicrobial activity, with ring sizes 7 and 8 being optimal. But if the ring size was increased to 12 (TZ-5), activity decreased almost eightfold relative to rings of size 7 or 8. On the other hand, increasing the amine chain length did not show any obvious trend.

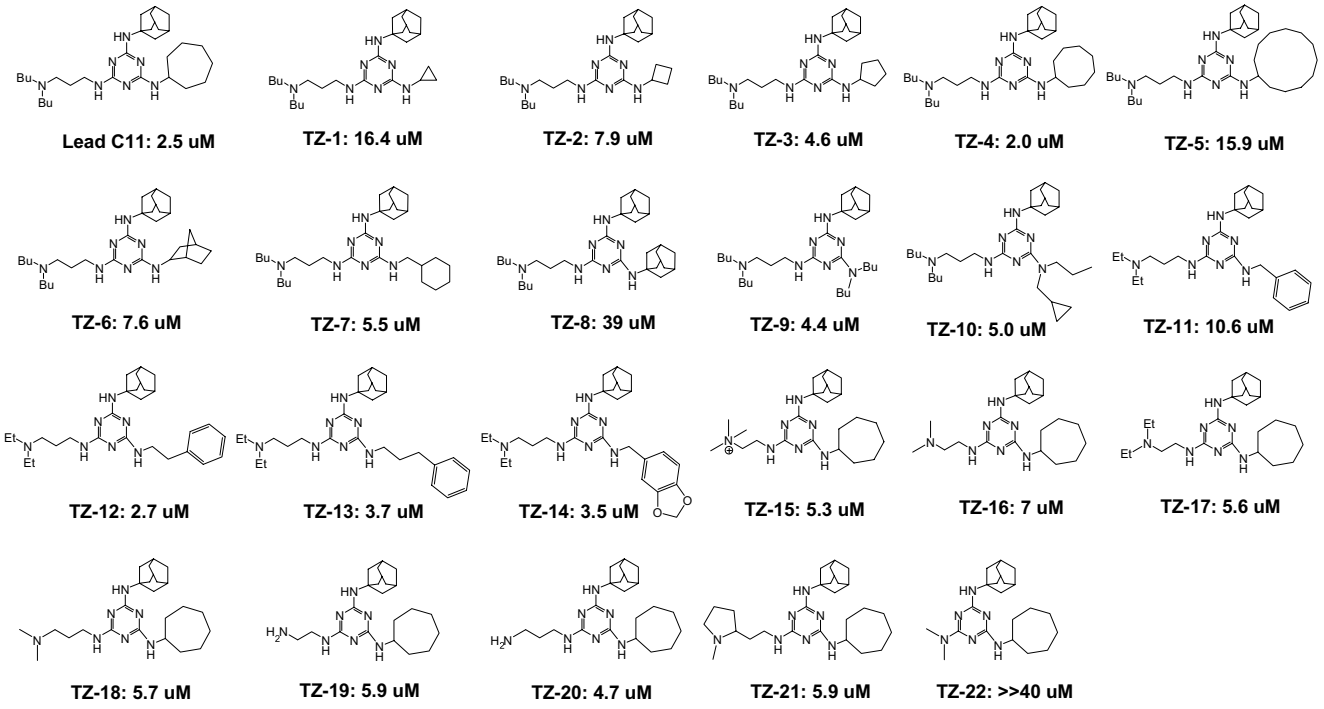

Figure 2. Optimization of R^1 and R^2 groups based on lead compound C11 from library 3. The activity data are IC_{50} which are the means of three independent experiments performed in parallel against *B. subtilis*.

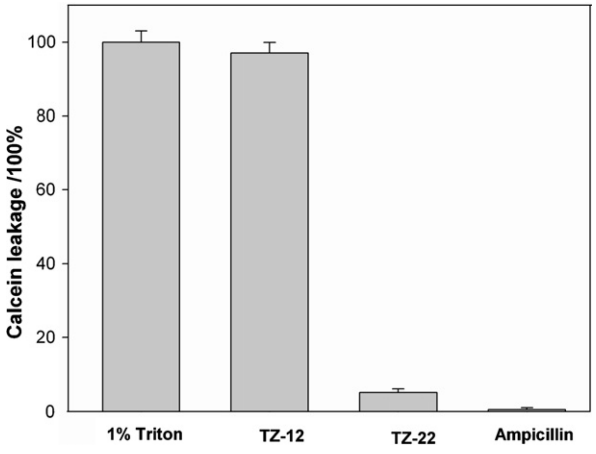

Figure 3. Calcein leakage induced by 1% Triton, TZ-12 (50 μM), TZ-22 (90 μM), and ampicillin (70 μM). Fraction of leakage was calculated from the fluorescence intensity at 515 nm, with 100% leakage calibrated by addition of 1 % Triton X-100.

Interestingly, triazines with two amantadine groups had much lower antimicrobial activity relative to compounds having only one amantadine group.

To test spectrum of antibacterial activity, the three most active compounds C11, TZ-4, and TZ-12 were further tested against both Gram-positive and Gram-negative bacteria, including ampicillin- and streptomycin-resistant *E. coli* (D31) and multidrug-resistant *S. aureus*. Hemolytic activity was assayed against red blood cells (Table 1).[22] The results showed that these compounds were generally more selective against Gram-positive bacteria, with IC_{50} value in the low micromolar range and that none was hemolytic even at high concentration.

Finally, to test whether the active triazine compounds kill bacteria via disrupting membrane integrity, a calcein dye leakage experiment was carried out on TZ-12 with ampicillin and one of the triazine compounds with little antimicrobial activity (TZ-22) as control (Fig. 3).[23] TZ-12 causes 100% dye leakage at 50 μM. The results confirmed that the antimicrobial active triazine compound could cause dye leakage from negatively charged 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (POPG) lipid vesicle, while neither ampicillin nor compound TZ-22 caused dye leakage.

In summary, we have designed and screened privileged libraries consisting of tri-substituted triazine compounds

Table 1. Spectrum of antibacterial activity for triazine compounds C11, TZ-4, and TZ-12

Compounds	IC_{50}[a] (μM)				HD_{50}[b] (μM)
	Escherichia coli	*Acinetobacter baumannii*	*Bacillus anthracis*	*Staphylococcus aureus*	
C11	620	53	41	68	>3500
TZ-4	550	84	86	70	>3500
TZ-12	200	22	21	17	>3500

[a] The results are means of three independent experiments performed in parallel.
[b] HD_{50} determined from dose–response curve is peptide concentrations corresponding to 50% hemolysis.

as antimicrobials. Several lead compounds possessing potent antimicrobial and low hemolytic activity were identified. Further, these compounds preferentially kill Gram-positive bacteria relative to gram-negative ones and probably kill them via disrupting membrane integrity as shown by a dye leakage assay. These responses are similar to those of most natural or synthetic AMPs reported in the literature. These data open a possible route to the elaboration of novel antibiotics derived from AMP mimetics that afford more drug-like pharmaceutical properties than peptides. Further investigation on the mechanism of these triazine compounds will be needed to distinguish their mode of action from that of other triazine compounds.

Acknowledgments

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19. Frequency of functional group effectiveness from libraries 1 and 2. The number of occurrences of the top eight side chain groups in triazines at 100 μM versus *B. subtilis* (>90% inhibition): functional group B(four times), C(3), F(4), H(3), 11(6), 13(3), 15(3), and 17(4). These functional groups were used to design library 3.
20. Assay results of screening library 3. Triazine compounds: B11(IC_{50} 7.5 μm), C11(2.5 μm), C15(7.5 μm), H11(7.5 μm), H15(3.0 μm), 11–11(7.5 μm), 11–15(7.5 μm), and 11–17(7.5 μm). All these compounds cause less than 10% hemolysis at 100 μM.
21. The purity and identity of all the products were monitored by LC–MS at 250 nm (Agilent 1100) and more than 90% of the compounds demonstrated >90% purity. *NMR and ESI-MS characterization data for example*: C11 ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.81 (br, 2H), 3.96 (brs, 1H), 3.42 (m, 2H), 2.67 (m, 1H), 2.57 (m, 4H), 2.10 (m, 8H), 1.99 (m, 2H), 1.83 (m, 3H), 1.69–1.45 (m, 22H), 1.34 (m, 4H), 0.93 (t, 6H, J = 7.5 Hz). ESI-MS $(M+H)^+$ calcd, 526.4; Found, 526.2. *Data for TZ-4*: ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.79 (br, 2H), 4.02 (brs, 1H), 3.41 (m, 2H), 2.61 (m, 1H), 2.50 (t, 4H, J = 6.8 Hz), 2.10 (m, 8H), 1.88 (m, 2H), 1.78 (m, 3H), 1.68–1.46 (m, 24H), 1.32 (m, 4H), 0.92 (t, 6H, J = 7.3 Hz). ESI-MS $(M+H)^+$ calcd, 540.4; Found, 540.2. *Data for TZ-12*: ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.29–7.13 (m, 6H), 4.70 (br, 2H), 3.52 (m, 2H), 3.33 (m, 2H), 2.78 (m, 2H), 2.54 (m, 6H), 2.02 (m, 8H), 1.70 (m, 3H), 1.60 (m, 6H), 1.02 (t, 6H, J = 6.8 Hz). ESI-MS $(M+H)^+$ calcd, 478.3; Found, 478.1.
22. Growth inhibition and hemolysis assays here and mentioned above were carried out using a microdilution method as previously described in Ref. 9. Bacterial strain: *A. baumannii* (ATCC BAA-747; Rockville, MD); *B. subtilis* (ATCC 6633; Rockville, MD); Ampicillin- and streptomycin-resistant strain *E. coli* (D31; *E. coli* Genetic Resource Center, Yale University, New Haven, CT); multi-drug resistant strain *S. aureus* (ATCC BAA-44); *B. anthracis* (32F2 Sterne) (gift from Dr. Martin Blaser, NYU medical school).
23. The method of preparation of dye-encapsulated vesicles has been reported in detail: Lasch, V.; Weissig, M. In *Bran in Liposomes: a Practical Approach*; Taylor, K. M. G., Craig, D. Q. M., Eds.; Oxford University Press: Oxford, 2003; p 10.