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Synthesis, Biological Activity, and QSAR Studies of Antimicrobial Agents Containing Biguanide Isosteres

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Analogues of chlorhexidine and chemically related antimicrobial compounds were synthesized, based on a model in which the bisbiguanide moieties were replaced by conformationally restricted cyclic isosteres. This model was tested by measuring the antimicrobial activities of the compounds. Quantitative structure–activity relationship (QSAR) studies showed a parabolic dependence of antimicrobial activity on the lipophilicity of the compounds. The basicity of the functional groups in the molecules was also very important, as uncharged molecules were not able to disrupt the microbial phospholipid bilayer and cause an antimicrobial effect. We compared our QSAR results to those reported in other studies of antimicrobials of diverse structure. We found very similar QSAR models for all compounds studies with a log *P* (octanol/water partition constant) optimum at 5.5 (neutral log *P* value). The form of the QSAR equations were similar, suggesting a common mode of action for these agents.

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Introduction

Pathogenic bacteria and fungi are of great economic significance in health care and agriculture. Bacterial and fungal infections acquired by patients undergoing hospital treatment (nosocomial infections) represent severe health care problems, and the rising incidence of multiply resistant strains is of major concern. Crop losses and damage due to attack by fungi and insects amount to billions of dollars per year and create large markets for effective agrochemical agents.

There are some classes of chemical agents that exhibit a relatively broad spectrum of activity against bacteria, fungi, and insects. Alkyl and aryl guanides, biguanides, bisbiguanides, and other lipophilic cationic compounds, such as bispyridinamines and (pyrrolylimino)-cyclohexadienes, show this type of activity, albeit through different modes of action in different species. The broad-spectrum fungicides Dodine, Guazatine, and Iminoctadine are guanidines and have been used in agriculture for over a decade.[1] They show activity against several economically significant fungi. Chlorhexidine **1**, a bisbiguanide, is a widely used topical antimicrobial compound exhibiting a broad spectrum of activities (Diagram 1).[2] Azarole, a (pyrrolylimino)-cyclohexadiene, shows potent antitubercular and antibacterial activity.[3] Bispyridinamines, which may be considered a type of 'masked guanide', have been developed as antimicrobial agents against dental plaque.[4]

The antimicrobial mode of action of many of these compounds is not well understood. Some guanidinium compounds that show antimycotic activity inhibit Δ^{14}-reductase and Δ^{8}-Δ^{7}-isomerase enzymes in the ergosterol biosynthetic pathway.[5] Octenidine, a bispyridinamine, inhibits extracellular polysaccharide-producing enzymes of some microbes.[6] Although chlorhexidine was patented 50 years ago, surprisingly little published work exists. The mechanism by which chlorhexidine kills bacteria is still ill-defined.[7]

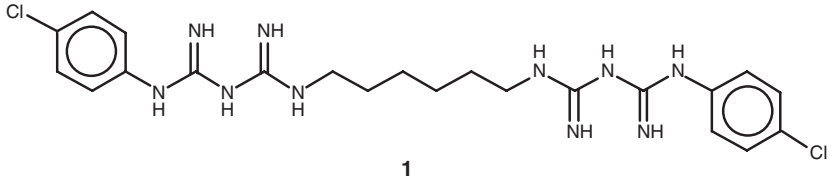

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Diagram 1.

 10.1071/CH03146 0004-9425/04/010077

Chlorhexidine may possibly act by several mechanisms:[8–10]

- adsorption to the surface of bacteria;
- damage to permeability barriers, facilitating entry of the bactericide to the cytoplasm and leakage of ions from the cell;
- precipitation of the cytoplasm and prevention of repair of the cell wall and membrane;
- potential interaction with cellar ATPases.

There are relatively few reports describing structure–activity relationships in this or similar types of compounds. Warner and coworkers reported a QSAR study of biguanides, carbamimidates and bisbiguanides exhibiting activity against *Streptococcus mutans*.[11] Lindholm synthesized antibacterial pyridylguanidines and screened them against several bacteria and yeasts, carrying out comprehensive QSAR analyses on these data.[12] The QSAR analyses found a parabolic relationship between the logarithm of the antimicrobial activity and log *P* in each single chemical class. In two recent reviews Denyer[8,9] noted that the study of biocide mechanisms of action offers an, as yet, largely untapped initiator of novel development directions and called for more QSAR studies of these classes of bioactive agents.

We have an ongoing interest in the application of QSAR to bioactive agent design.[13–15] We aim to design more potent, efficacious analogues of these compounds for use as agents against *Staphylococcus aureus*, particularly the resistant strain *MRSA*, and against dental plaque.[16,17] This paper describes the synthesis and antimicrobial activity of analogues of chlorhexidine **1** and related compounds that contain guanide or biguanide isosteres. These compounds exhibit activity against a range of bacteria and yeasts. We also report a QSAR analysis of these compounds, a comparison with other QSAR studies of antimicrobial agents, and a discussion of the implications of the QSAR models for the mode of action of these agents.

Design Rationale

It is clear that many topical antimicrobial agents (disinfectants) have one or more basic structural features (often guanides, biguanides, or tertiary amines) that are positively charged under physiological conditions and linked to a lipophilic chain.[4,11,18] The basicity (and tautomerism) of the biguanide and related moieties, intramolecular hydrogen bonding, and lipophilicity are potential modulators of antimicrobial activity. Many antimicrobial agents are also conformationally flexible molecules. Guanide and biguanide moieties can adopt several tautomeric forms, as illustrated in the structural work of Fabrizzi et al.,[19] and Pinkerton and Schwarzenbach,[20] and described in the theoretical paper by Jordan and Gready.[21] To design topographical mimics of chlorhexidine it was necessary to make several assumptions about the basicity, tautomeric state, geometry, and conformational preferences of the active compounds such as chlorhexidine:

- the biguanide moiety adopts an essentially planar conformation in its biologically active form[19,20] which can be mimicked by conformationally restricted cyclic analogues;
- in bisbiguanides, the length of the linker between the two biguanide moieties is important;
- differences in the basicities of these conformationally restricted isosteres would not dramatically affect their antimicrobial activities.

The validity of these assumptions was tested experimentally by observing the antimicrobial activities of analogues designed on the basis of these assumptions.

We devised potential antimicrobial agents based on chlorhexidine and other antimicrobial agents, where the biguanide or guanide moieties were replaced by potential bioisosteres or bioanalogues.[22] Our conformationally restricted analogues were guanylpyrimidines and aminopyrimidines, in which part of the guanide or biguanide is incorporated into a planar pyrimidine ring.

Chemistry

The synthesis of the bisguanidino compounds **1**–**7**, Table 1, has recently been reported.[23] The alkanebisaminopyrimidines **10** and **11** are known structures.[24,25] Other compounds reported here were made by modification of the general synthesis or adaptation of an existing method for the synthesis of bisguanidinoalkanes.[23]

Compounds **8** and **9** were prepared from the diamine and 2-chloropyrimidine by a method[26] based on that using triethylamine as the base in dioxan (Scheme 1, Method A). 4-Chlorphenoxy-4-pyrimidine **13** was prepared by a regioselective synthesis[27] in which none of the bis 2,4-substituted isomer was detected. Treatment of **13** with 1,6-diaminohexane in dioxane gave compound **10** (Scheme 1, Method B). Treatment of 4,6-dichloro-2-methylthiopyrimidine with 4-chloroaniline in acetic acid and concentrated hydrochloric acid as catalyst afforded **14**, which was oxidized to the sulfone **15**. Catalytic hydrogenolysis with hydrogen in the presence of an acid scavenger yielded **16** (Scheme 1, Method C). Compound **11** was prepared by the nucleophilic displacement of the sulfone group from the intermediate **16** by 1,6-diaminohexane in a similar way to that reported.[23] The 2,4-bisanilinopyrimidine **12** was prepared following a literature method.[28]

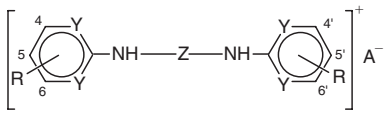
Antimicrobial Activities

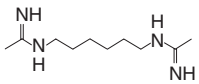
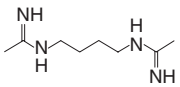
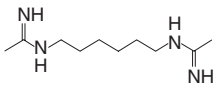
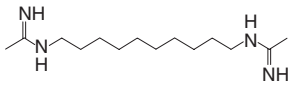
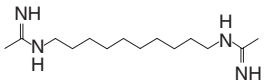
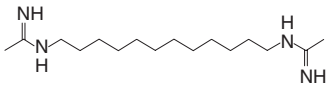
The structures and antimicrobial activities of the twelve compounds studied are reported in Table 1. The activities of the most active compounds **6**, **7**, and **12** approach that of chlorhexidine **1**. It is clear that all compounds except **8**, **9**, and **10** showed substantial antimicrobial activity against all strains except *Pseudomonas*. This microbe is known to contain hundreds of different proteins in the cell wall of the bacterium that pump material out of the cell, allowing *P. aeruginosa* to resist the effects of many antibiotics. *P. auriginosa* also excretes an exopolysaccharide biofilm that protects it from antimicrobial agents better than other bacteria.[29]

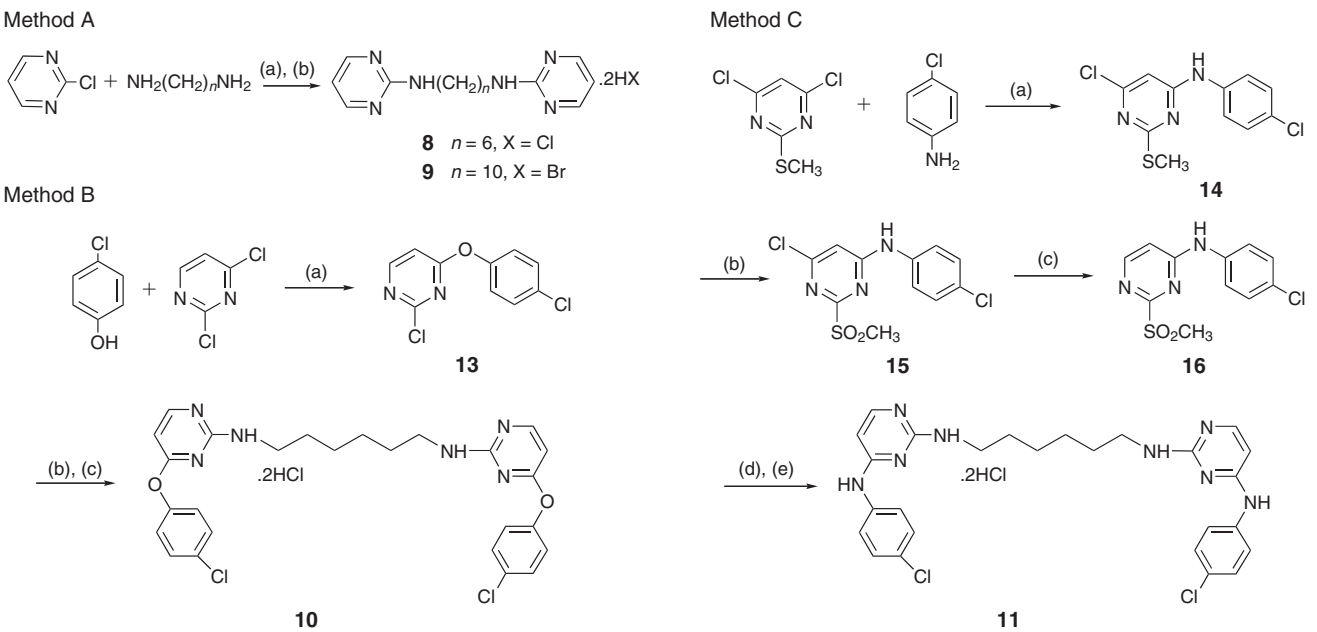
QSAR Analyses

The physicochemical properties and molecular descriptors used in the QSAR study are reported in Table 2. The multiple

Table 1. Antimicrobial activities (MIC) of bisguanides and bisbiguanides [μM]



Compound	R	Y	Z	A	*S. aureus*	*MRSA*	*E. coli*	*P. aeruginosa*	*C. albicans*
1			chlorhexidine		8	8	16	63	32
2	4,6-dimethyl	N		Cl^-	155	620	1240	>1200	310
3	5-chloro	N		Cl^-	640	640	1300	>1300	>1300
4	5-chloro	N		Cl^-	600	600	1200	>1200	>1200
5	5-chloro	N		Br^-	33	33	66	>1000	130
6	5-chloro	N		Cl^-	17	17	33	>1000	66
7	5-chloro	N		Cl^-	16	16	63	>1000	63
8	H	N	$(CH_2)_6$	Cl^-	>3000	>3000	>3000	>3000	>3000
9	H	N	$(CH_2)_{10}$	Br^-	>3000	>3000	>3000	>3000	>3000
10	4-chlorophenoxy	N	$(CH_2)_6$	Cl^-	>1000	>1000	>1000	>2000	>1000
11	4-chloroanilino	N	$(CH_2)_6$	Cl^-	61	61	120	>1000	500
12	5-chloro	CH		Cl^-	12	12	>1000	>1500	25



Scheme 1. Method A: (a) Et_3N, dioxane; (b) HX, MeOH. Method B: (a) NaOH, acetone, H_2O; (b) Et_3N, dioxane; (c) HCl, MeOH. Method C: (a) HOAc, HCl; (b) H_2O_2, HOAc; (c) H_2, Pd/C, MgO; (d) $NH_2(CH_2)_6NH_2 \cdot DMSO$; (e) HCl, MeOH.

Table 2. SAR data for bisguanides and bisbiguanides [μM]

Compound	log *P*	I_{N+}	I_{bis}	*S. aureus* −log MIC	*MRSA* −log MIC	*E. coli* −log MIC	*C. albicans* −log MIC
1	4.78	1	1	5.10	5.10	4.80	4.50
2	3.79	1	0	3.81	3.21	2.91	3.51
3	1.73	1	0	3.19	3.19	2.89	2.30
4	2.81	1	0	3.22	3.22	2.92	2.33
5	4.97	1	0	4.48	4.48	4.18	3.88
6	4.97	1	0	4.78	4.78	4.48	4.18
7	6.08	1	0	4.80	4.80	4.20	4.20
8	2.05	0	0	1.83	1.83	1.83	1.83
9	4.21	0	0	1.91	1.91	1.91	1.91
10	8.30	0	0	2.42	2.42	2.42	2.42
11	7.54	1	0	4.21	4.21	3.91	3.61
12	5.40	1	0	4.92	4.92	2.52	4.62

regression analyses yielded parabolic relationships between lipophilicity and the antimicrobial activity for all species except *P. aeruginosa* for which there was insufficient biological activity for a QSAR model. It was clear from Tables 1 and 2 that only those compounds with basic nitrogen atoms that were likely to be positively charged at the pH of the screen media show significant activity. Compounds **8**, **9**, and **10**, with weakly basic anilino nitrogen atoms in the linker chain, were inactive at the highest concentrations tested. An indicator variable for charged molecules was employed. The bisbiguanide-containing compound chlorhexidine **1** showed enhanced activity and this moiety was accounted for by another indicator variable, as previous studies have done. This was applied cautiously as the indicator variable was of marginal statistical significant ($t > 1.3$, $P > 0.75$) especially given only one example of this type of compound. However, the QSAR models suffered substantially when the variable was omitted.

QSAR equations were derived for two scenarios: (a) all active compounds included in the model and compounds inactive at the highest concentration tested excluded; and (b) all compounds included, with inactive compounds given a value four times that of the highest concentration at which they were tested. The following structure–activity relationships were derived.

(a) Inactive compounds excluded:

$$-\log \text{MIC}^{*}\,[\mu\text{M}]\,\{\textit{S. aureus}\} = 1.177(\pm 0.357)\log P - 0.098(\pm 0.038)(\log P)^2 + 0.604(\pm 0.396)I_{bis} - 1.107(\pm 0.772)$$

n 9, s 0.36, r^2 0.85, log P(opt) 6.0

$$-\log \text{MIC}\,[\mu\text{M}]\,\{\textit{MRSA}\} = 1.048(\pm 0.518)\log P - 0.081(\pm 0.056)(\log P)^2 + 0.725(\pm 0.575)I_{bis} - 1.224(\pm 1.121)$$

n 9, s 0.52, r^2 0.74, log P(opt) 6.4

$$-\log \text{MIC}\,[\mu\text{M}]\,\{\textit{E. coli}\} = 1.829(\pm 1.028)\log P - 0.148(\pm 0.098)(\log P)^2 + 0.835(\pm 0.657)I_{bis} - 1.392(\pm 2.535)$$

n 7, s 0.57, r^2 0.76, log P(opt) 6.1

$$-\log \text{MIC}\,[\mu\text{M}]\,\{\textit{C. albicans}\} = 1.601(\pm 0.334)\log P - 0.155(\pm 0.036)(\log P)^2 + 0.153(\pm 0.748)$$

n 7, s 0.33, r^2 0.86, log P(opt) 5.2

where I_{bis} is an indicator variable for bisbiguanides, n is the number of compounds in the analysis, s is the standard error, r^2 is the squared correlation coefficient, F is the F-statistic, and log P (opt) is the value of log P at which the activity is maximum, derived from the regression equation.

(b) Inactive compounds included at four times their highest tested concentration:

$$-\log \text{MIC}\,[\mu\text{M}]\,\{\textit{S. aureus}\} = 1.009(\pm 0.314)\log P - 0.071(\pm 0.025)(\log P)^2 + 1.882(\pm 0.335)I_{N+} - 0.640(\pm 0.804)$$

n 12, s 0.45, r^2 0.89, F 22.6, log P(opt) 7.1

$$-\log \text{MIC}\,[\mu\text{M}]\,\{\textit{MRSA}\} = 1.034(\pm 0.370)\log P - 0.072(\pm 0.029)(\log P)^2 + 1.816(\pm 0.395)I_{N+} - 0.736(\pm 0.946)$$

n 12, s 0.53, r^2 0.86, F 15.8, log P(opt) 7.2

$$-\log \text{MIC}\,[\mu\text{M}]\,\{\textit{E. coli}\} = 0.895(\pm 0.378)\log P - 0.061(\pm 0.030)(\log P)^2 + 1.473(\pm 0.396)I_{N+} - 0.408(\pm 0.961)$$

n 11 (**12** excluded), s 0.53, r^2 0.82, F 10.5, log P(opt) 7.3

$$-\log \text{MIC}\,[\mu\text{M}]\,\{\textit{E. coli}\} = 0.826(\pm 0.330)\log P - 0.056(\pm 0.026)(\log P)^2 + 1.569(\pm 0.320)I_{N+} - 0.327(\pm 0.897)$$

n 12 (**12** not ionized), s 0.51, r^2 0.83, F 12.9, log P(opt) 7.4

$$-\log \text{MIC}\,[\mu\text{M}]\,\{\textit{C. albicans}\} = 1.252(\pm 0.387)\log P - 0.091(\pm 0.031)(\log P)^2 + 1.131(\pm 0.413)I_{N+} - 1.160(\pm 0.990)$$

n 12, s 0.56, r^2 0.79, F 10.1, log P (opt) 6.9

where I_{N+} is an indicator variable for charged nitrogen atoms and other terms are as defined above.

*Minimum inhibitory concentration.

Discussion

It is clear from Table 1 that several of the topographical analogues of chlorhexidine show substantial broad-spectrum antimicrobial activity. In particular, the activities of **6** and **7** approach to that of chlorhexidine, except against *P. aeruginosa*. The conformational restriction introduced by the pyrimidine ring, and the changes in basicity on incorporation into a ring appear to have a relatively small influence on activity as long as the nitrogen atom is protonated at physiological pH. Compounds **8**–**10** do not meet this criterion and are inactive in all microbial strains tested.

However, as the QSAR equations show, the overriding dependence of antimicrobial activity is on the lipophilicity of the compounds not the length of the central linker chain (defining distances between positively charged groups) or any other structural feature. The dependence on lipophilicity (log *P*) is parabolic in each case, resulting in optimal values of log *P* at which activity is greatest. As compound **12** shows, it is possible to have high antimicrobial activity with compact molecules provided the log *P* value is near the optimum and the compound is charged. However, the smaller molecular dimensions of this compound and its high antimicrobial activity were useful in eliminating the molecular size as a significant structural variable in the QSAR. The indicator variable for the presence of the biguanide moiety in the molecule was significant, as others have found in QSAR studies. However, this must be viewed cautiously in our models as this feature only occurred once in the dataset.

The QSAR models that include the poorly active/inactive compounds in particular are consistent with the mechanism that antimicrobial compounds of this type interact with and disrupt phospholipid bilayers in microbes. In this model proposed by Ikeda et al.[30] for oligomerized biguanide compounds similar to those we studied, positively charged groups on the biocide interact with the acidic phospholipid (anionic) head groups, which, while being present in the membrane in relatively low concentrations, fulfill vital roles in stabilizing the membrane. The calcium counterions normally present at the surface of the cell membrane are displaced by the cationic moieties of the antimicrobial agent. The hydrophobic linker regions can insert into the hydrophobic interior of the bilayer, helping stabilize the bond between the charged head group of the phospholipids and the delocalized positive charge on the biocide. The linker is not required to span the phospholipid membrane, consistent with a lack of linker length dependence in the QSAR models. Phospholipid phase separation occurs causing ion leakage from the cytoplasm and cell death. This mechanism is illustrated in Fig. 1.[31]

The indicator variable denoting charged/uncharged molecules shows that there is a very large loss in activity when the molecule is not charged (large positive coefficient in the QSAR model). Compound **12** has a log *P* value very similar to that of chlorhexidine but a weaker basicity than the guanide and biguanide moieties. Calculations on the ionic speciation of this compound as a function of pH show that it has the lowest conjugate acid pK_a and is the compound whose ionization state is most sensitive to the effect of pH under

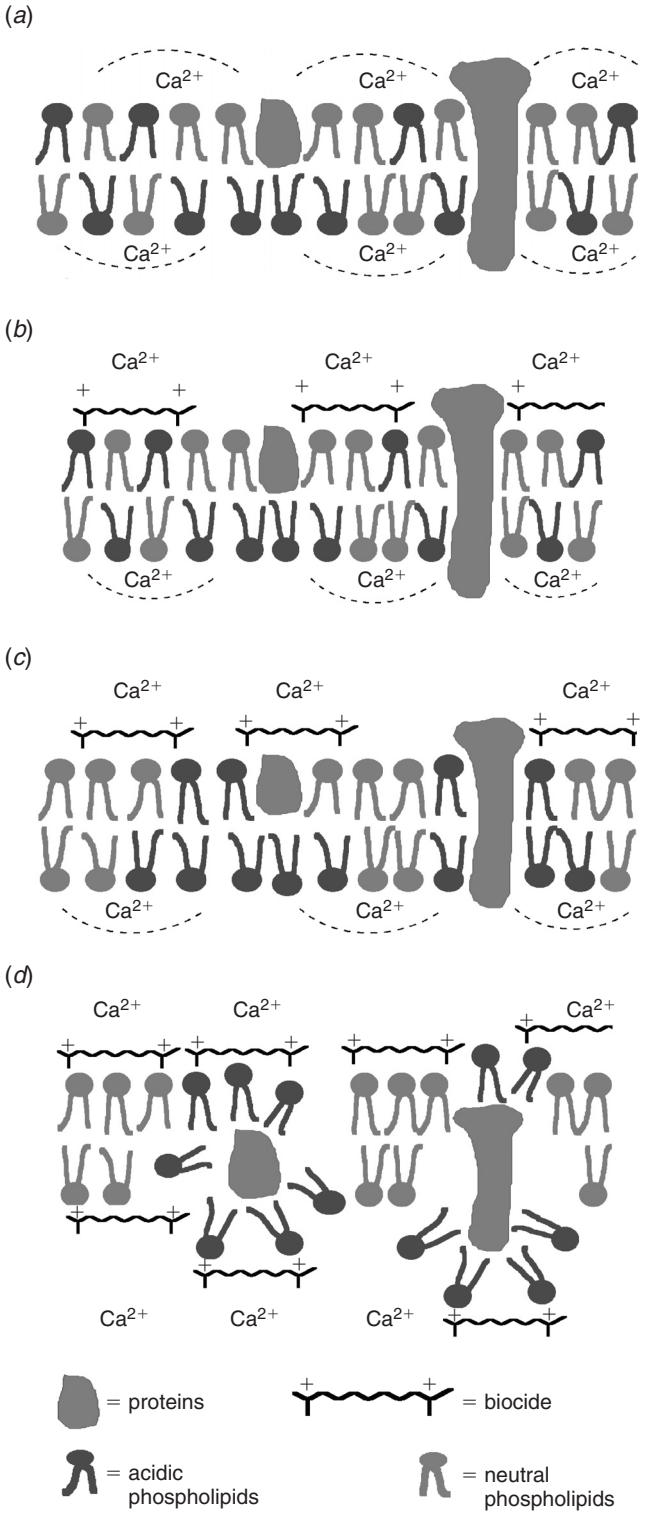


Fig. 1. (*a*) Bacterial cytoplasmic membrane conforming to fluid mosaic model; stabilized by calcium ions and phospholipid mixture and distribution. (*b*) Initial wave of biocide displaces surface cations, binds to acidic phospholipids, causing change in packing. (*c*) Biocide induces a phospholipid phase separation, affects concentrate in the area of integral proteins; causes increase in membrane permeability, K^+ efflux, loss of enzyme function, i.e. bacteriostatic level. (*d*) Destabilized zones aggregate into favourable hexagonal phase by building excess of biocide (electrostatic and hydrophobic); complete loss of membrane function, i.e. bactericidal level.

the conditions of testing. The compound showed inhibitory activity below 10 μM concentration against *S. aureus* and *C. albicans*, activity of the same order as chlorhexidine. However it showed a narrower spectrum of activity as it was inactive against the Gram-negative *E. coli* and *P. aeruginosa.* The weaker basicity may be a factor in its low activity against *E. coli*, if this bacterium has a different cytoplasmic pH than other microbes, or a different ratio of acid and neural phospholipids in its cytoplasmic membrane. It is interesting that the models containing all twelve compounds show a slightly higher log *P* optimum than the models based on just active compounds.

Global or Consensus QSAR Analyses

Our QSAR analyses yielded relationships between antimicrobial activity and log *P* that are similar for each species. Interestingly, the equations we derived for active compounds are essentially the same as those derived by several other studies of antimicrobial activities of other structural types. Lindholm[12] studied QSAR in pyridylguanidines and obtained an optimum log *P* value of 5.4–5.5 for antimicrobial agents, consistent with our findings. This value is also similar to that derived by Warner et al.,[11] whose QSAR analyses, like ours, used an indicator variable to account for the higher activity of bisbiguanides compared with biguanides. This may indicate that biguanides can interact more effectively with the negatively charged phospholipid head groups due to the larger size of the cationic biguanide moiety. Recently, Gasparrini and coworkers[32] found a log *P* optimum of 5.2–5.4 for their acyl-L-carnitine alkyl ester antimicrobials. A very similar log *P* optimum was reported by Tsubouchie et al. for biguanide antiseptics in a recent publication.[33] We calculated log *P* values for the compounds in this paper, and carried out a QSAR analysis of the antimicrobial activity using their reported activities. We obtained a parabolic dependence of antimicrobial activity on the lipophilicity with QSAR equations and log *P* values for optimum activity very similar to those from other studies, including ours. These QSAR equations and optimum log *P* values for current, and previous studies, are summarized in Table 3.

The close correspondence of the QSAR equations, and very similar values for the optimum log *P* value in all these studies, across a wide range of structures, suggests that a common mechanism of action exists for all of these chemical classes as discussed in the previous section. However, the mechanistic analyses are complicated by the fact that some compounds have several mechanisms of toxicity towards microbes. For example, the antimicrobial biocides dinitrophenol, tribrominated salicylanilide, polymyxin, and chlorhexidine all collapse the proton membrane potentials at inhibitory concentrations. Other agents, including biguanides also inactivate or inhibit ATPase. However, it appears to be the cytoplasmic membrane disruption, and not ATPase inactivation or disruption of electron transport, which is the main lethal event in chlorhexidine action.[10,34,35] In addition, adherence is an important pathogenic mechanism in *Candida*

Table 3. QSAR equations from current and previous studies

	Compound	Organism	QSAR equation	log P_0
1	carnitine esters[32]	Gram+	$-0.17\log P^2 + 1.81\log P + 0.58$	5.2
2	carnitine esters[32]	Yeasts	$-0.15\log P^2 + 1.57\log P + 0.95$	5.4
3	biguanides[11]	*S. mutans*	$-0.17\log P^2 + 1.94\log P - 0.29$	5.8
4	carbamimidates[11]	*S. mutans*	$-0.19\log P^2 + 2.13\log P - 0.60$	5.5
5	3 + 4[11]	*S. mutans*	$-0.17\log P^2 + 1.97\log P - 0.32$	5.7
6	bisbiguanides[11]	*S. mutans*	$-0.08\log P^2 + 1.04\log P + 2.17$	6.6
7	3 + 4 + 6[11]	*S. mutans*	$-0.12\log P^2 + 1.54\log P + 0.56$	6.5
8	3 + 4 + 6[11]	*S. mutans*	$-0.13\log P^2 + 1.56\log P + 0.34I_{bis} + 0.48$	6.2
9	2-pyridylguanidines[12]	*S. aureus*	$-0.18\log P^2 + 1.83\log P - 0.57\sigma_p - 5.73$	5.1
10	2-pyridylguanidines[12]	*P. aerug.*	$-0.11\log P^2 + 0.93\log P - 0.35\sigma_p - 4.27$	4.2
11	2-pyridylguanidines[12]	*E. coli*	$-0.20\log P^2 + 1.95\log P - 6.05$	4.9
12	2-pyridylguanidines[12]	*P. vulgaris*	$-0.17\log P^2 + 1.40\log P - 0.28\pi - 5.03$	6.0
13	2-pyridylguanidines[12]	*C. albicans*	$-0.11\log P^2 + 1.33\log P - 7.00$	6.0
14	bispyridylguanidines[12]	*S. aureus*	$-0.08\log P^2 + 0.83\log P - 2.53\sigma_m - 3.26$	5.2
15	bispyridylguanidines[12]	*P. aerug.*	$+0.28\log P - 3.27$	…
16	bispyridylguanidines[12]	*E. coli*	$-0.12\log P^2 + 1.12\log P - 3.60$	4.7
17	bispyridylguanidines[12]	*P. vulgaris*	$+0.23\log P + 2.34\sigma_m - 3.23$	…
18	bispyridylguanidines[12]	*C. albicans*	$-0.07\log P^2 + 0.80\log P - 2.93$	5.7
19	biguanides[33]	*S. aureus*	$-0.26\log P^2 + 2.92\log P - 3.61$	5.5
20	biguanides[33]	*MRSA*	$-0.30\log P^2 + 3.45\log P - 5.02$	5.7
21	biguanides[33]	*E. coli*	$-0.21\log P^2 + 2.42\log P - 1.82$	5.7
22	biguanides[33]	*P. aerug.*	$-0.21\log P^2 + 2.21\log P - 1.10$	5.2
23	biguanides[33]	*B. cepacia*	$-0.54\log P^2 + 5.67\log P - 9.99$	5.2
24	this work	*S. aureus*	$-0.10\log P^2 + 1.18\log P + 0.60I_{bis} + 1.11$	6.0
25	this work	*MRSA*	$-0.08\log P^2 + 1.05\log P + 0.73I_{bis} + 1.22$	6.4
26	this work	*E. coli*	$-0.15\log P^2 + 1.83\log P + 0.84I_{bis} - 1.39$	6.1
27	this work	*C. albicans*	$-0.16\log P^2 + 1.60\log P + 0.15$	5.2

infections and interference with this process may represent a major component of the mode of action of antifungal drugs.[36]

Conclusions

The results of this study provide insight into the physicochemical requirements for antimicrobial activity. Although most of these agents contain linear chains, the molecular length is not an important factor in the structure–activity relationships as compound **12** and several of the most active biguanides reported by Tsubouchi et al.[33] show. Rather, there is a consistent parabolic dependence of activity on the lipophilicity of the compounds. The optimum log *P* of 5.5 for antimicrobial activity is similar for all compound classes studied and for most species. The activity may also be increased by the presence of a biguanide group (as in chlorhexidine). It is essential for antimicrobial activity that the molecule contains a basic functional group that can become positively charged in the microbe environment. Our findings are consistent with current theories of microbe phospholipid biolayer interaction and destabilization.

This suggests considerable scope for use of topographical mimics of the biguanide moiety in antimicrobial agents of this type. It appears that antimicrobial agents require a log *P* value near 5.5 and a nitrogen atom capable of carrying at least a partial positive charge at physiological pH.[37] Conformational restriction of the biguanide analogue within the cyclic analogues does not seriously affect antimicrobial activity.

Experimental

General

Antimicrobial design was based on a model structure for chlorhexidine derived from the crystal structure of biguanide[38] using the molecule building capabilities of the Sybyl modelling package.[39]

Chemical Synthesis

Melting points were measured (uncorrected) on an Electrothermal apparatus. ^{1}H NMR spectra were measured on a Varian EM 360 instrument or a Bruker WM 250 spectrometer with trimethylsilane as the internal standard. All spectra quoted in the text were measured at 250 MHz unless otherwise specified. ^{13}C NMR spectra were measured on a Bruker WM 250 MHz instrument at 62.9 MHz. Infrared spectra were measured on a Perkin–Elmer 783 instrument as KBr discs unless otherwise reported in the text. Mass spectra were obtained with a Finnigan 3300 instrument or a JEOL JMS-DX 303 instrument as chemical ionization spectra with methane as the reagent gas. High-resolution chemical ionization mass spectra were recorded on a Micron SS 77OF instrument using methane as the reagent gas. Microanalyses were undertaken at the National Analytical Laboratory, Melbourne. Elemental analyses are within ±0.4% of the calculated values. Dioxane was passed through a column of aluminium oxide before use. 2-Chloropyrimidine (Janssen) was purified by recrystallization from petroleum spirit before use. DMSO was dried by stirring over calcium hydride for 48 h. and then distilling under vacuum. HPLC was carried out using a Waters 501 pump and a Du Pont Zorbax C18 analytical column and acetonitrile/water (45 : 55) as the eluting solvent system. Column Chromatography was carried out by using aluminium oxide (Merck, Art 1077, 90 Activ. neutral 0.063–0.2 mm). Petroleum Spirit was distilled before use and refers to the 60–80°C fraction.

2,2′-N,N′-(hexane-1,6-diyl)bis(2-aminopyrimidinium) Dihydrochloride ***8***

A solution of 2-chloropyrimidine (900 mg, 8 mmol) and 1,6-diaminohexane (690 mg, 6 mmol) in dioxane (12 mL) containing triethylamine (1.6 mL, 1.16 g, 11 mmol) was heated under reflux for 8 h. The cooled mixture was evaporated in vacuo, mixed as a slurry in water and the solid collected and well washed with water. Crystallization from ethanol afforded the free base as an off-white powder (430 mg, 40%), mp 178–179.5°C. δ_H ($CDCl_3$) 1.28–1.5 (4H, m, 2 × CH_2), 1.5–1.70 (4H, m, 2 × CH_2), 3.39 (2H, q, *J* 6, 2 × $NHCH_2$), 5.06–5.36 (2H, br s, 2 × $NHCH_2$), 6.49 (2H, t, *J* 5, pyrimid H-5), 8.24 (4H, d, *J* 5, pyrimid H-6). MS (CI) *m*/*z* 273 (MH^+, 100%).

A solution of the free base (400 mg, 1.5 mmol) in warm ethanol (14 mL) was treated with an excess of concentrated hydrochloric acid (0.6 mL). The solution was treated with ethyl ether until precipitation was complete. The precipitate was collected and as a solution in methanol (25 mL), treated with activated carbon followed by addition of excess ethyl ether until precipitation was complete. The solid was collected to afford the dihydrochloride **8** as off white crystals (335 mg, 65%), mp 233–234°C. δ_H ([D_4]MeOH) 1.53–1.75 (4H, m, 2 × CH_2), 1.75–1.98 (4H, m, 2 × CH_2), 3.69 (4H, t, 2 × $NHCH_2$), 4.97–5.20 (8H, br s, 4 × NH and NH^+ and H_2O), 7.12 (2H, t, *J* 6, pyrimid H-5), 8.61–8.86 (4H, bs, pyrimid H-6). ν_{max} (KBr disc)/cm^{-1} 3600–2200, 1625, 1460, 1330, 1200, 1100, 1080, 1050, 1030, 975, 790, 770. MS(CI) *m*/*z* 273 (MH^+ – 2HCl, 100).

2,2′-N,N′-(decane-1,6-diyl)bis(2-aminopyrimidinium) Dihydrochloride ***9***

A solution of 2-chloropyrimidine (1.8 g, 8 mmol) and 1,10 diaminodecane (2.06 g, 12 mmol) in dioxane (25 mL) containing triethylamine (3.2 mL, 22 mmol) was treated in an identical manner to **8** to yield, after recrystallization from ethanol, **9** as colourless crystals (1.19, 45%), mp 105–106°C. δ_H ($CDCl_3$) 1.10–1.35 (12H, br s, 6 × CH_2), 1.35–1.64 (4H, m, 2 × CH_2), 3.35 (4H, q, *J* 6, 2 × $NHCH_2$), 5.2–5.6 (2H, br s, 2 × NHCH2), 6.49 (2H, t, *J* 5, pyrimid H-5), 8.26 (2H, d, *J* 5, pyrimid H-6). MS(CI) *m*/*z* 329 (MH^+, 100).

A solution of the free base (490 mg, 1.5 mmol) in ethanol (6 mL) was treated with concentrated hydrobromic acid (0.7 mL, 6.2 mmol) by an identical procedure to **8** to afford the dihydrobromide **9** as colourless crystals (606 mg, 82%), mp 138–139°C. δ_H ($CDCl_3$) 1.0–1.5 (12H, br s, 6 × CH_2), 1.5–1.71 (4H, m, 2 × CH_2), 3.58 (4H, q, *J* 6, 2 × $NHCH_2$), 6.50–6.84 (2H, m, *J* 5, pyrimid H-5), 8.13–6.32 (2H, m, *J* 5, pyrimid H-6), 8.51–8.86 (2H, m, 2 × NH). ν_{max} (KBr disc)/cm^{-1} 3150, 3050, 2920, 2850, 1640, 1535, 1470, 1420, 1320, 1210, 1080, 1000, 780. MS(CI) *m*/*z* 329 (MH^+ – 2HBr, 100), 108(100).

2-Chloro-4-(4-chlorophenoxy)pyrimidine ***13***

4-Chlorophenol (15.42 g, 0.12 mol) was dissolved in a solution of sodium hydroxide (4.8 g, 0.12 mol) in water (30 mL). Acetone (90 mL) was added and the stirred mixture maintained at ice-bath temperature. To this was added, dropwise, a solution of 2,4-dichloroprymidine (17.88 g, 0.12 mol) in acetone (40 mL). The stirred mixture was maintained at ice-bath temperature for 4 h. during which time a precipitate formed. The mixture was extracted with ethyl ether (1 × 450 mL) and the ether layer washed with 2.5 M NaOH (3 × 30 mL), water (3 × 30 mL), dried (Na_2SO_4), and the ether evaporated in vacuo to yield a moist solid. Crystallization from light petroleum afforded **13** (17.40 g, 60%) as colourless flakes, mp 111–113°C. δ_H ($CDCl_3$) 6.83 (2H, d, *J* 5.5, pyrimid H-5), 7.08–7.13 (2H, m, phenyl), 7.36–7.42 (2H, m, phenyl). 8.45 (2H, d, *J* 5.5, pyrimid H-6). ν_{max} (KBr disc)/cm^{-1} 1600, 1560, 1480, 1425, 1335, 1310, 1220, 1190, 1160, 1100, 1085, 1015, 980, 950, 845, 810, 810, 760, 720, 650. MS(CI) *m*/*z* 241 (MH^+, 100), 243 (62). HRMS calcd for $C_{10}H_7Cl_2N_2O$, 240.9935; found 240.9910.

2,2′-N,N′-(hexane-1,6-diyl)bis[4-(4-chlorophenoxy)2-aminopyrimidinium] Dihydrochloride ***10***

A solution of **13** (3.62 g, 15 mmol) and 1,6-diaminohexane (1.31 g, 11.3 mmol) in dioxane (36 mL) containing triethylamine (2.91 mL,

21 mmol) was heated under reflux for 10 h. The cooled mixture was evaporated in vacuo, mixed with water, and the solid collected and washed well with water. Crystallization from ethanol (activated carbon) afforded the free base as an off-white powder (1.55 g, 39%), mp 158–159°C. δ_H ($CDCl_3$) 1.05–1.4 (4H, br s, 2 × CH_2). 1.4–1.74 (4H, br s 2 × CH_2), 3.1–3.5 (4H, br s, 2 × $NHCH_2$), 4.8–5.7 (2H, br s, 2 × NH), 6.08 (2H, d, *J* 5, pyrimid H-5), 7.09 (4H, d, *J* 8, phenyl), 7.37 (4H, d, *J* 8, phenyl), 8.10 (2H, d, *J* 8, pyrimid H-6). MS(CI) *m*/*z* 525 (MH^+, 100), 527(70).

The free base (787 mg, 1.5 mmol) as a suspension in warm methanol (5 mL) was treated with concentrated hydrochloric acid (0.6 mL, 7 mmol) and the resulting solution treated with activated charcoal. Ethyl ether was added until precipitation commenced. Crystallization at ice-bath temperature afforded **10** as an off-white powder (653 mg, 73%), mp 214.5–216°C. δ_H ([D_6]DMSO) 0.8–1.6 (8H, m, 4 × CH_2), 2.9–3.15 (2H, br s, $NHCH_2$), 3.15–3.45 (2H, br s, $NHCH_2$), 6.45–6.71 (2H, br d, pyrimid H-5), 7.30 (4H, d, *J* 8, phenyl), 7.42–7.62 (4H, br d, phenyl), 8.10–8.45 (2H, m, pyrimid H-6), 8.7–9.1 (2H, bm, 2 × NH). ν_{max} (KBr disc)/cm^{-1} 3600–2200, 1630, 1550, 1480, 1440, 1420, 1380, 1340, 1320, 1290, 1270, 1210, 1100, 1080, 1010, 970, 860, 800, 700, 610. MS(CI) *m*/*z* 525 (MH^+ – 2HCl, 100), 527(70).

6-Chloro-2-methylthio-N-*(4-chlorophenyl)-4-pyrimidineamine* ***14***

A mixture of 4,6-dichloro-2-methylthiopyrimidine (21.60 g, 0.11 mol) and 4-chloroaniline (12.80 g, 0.10 mol) in glacial acetic acid was treated with concentrated hydrochloric acid (5 mL). The stirred mixture was heated to 100°C for 3 h. and during this time a thick, white precipitate formed. After cooling the reaction, the solid was collected and washed well with glacial acetic acid. The solid was suspended in ethanol (200 mL) and made alkaline (pH 8–9) with ammonium hydroxide. Sufficient water was added to completely precipitate the crude compound that was collected, which was washed well with water. Crystallization from a mixture of ethanol and water (1 : 1) afforded **14** as colourless crystals (18.94 g, 66%), mp 157–159°C. δ_H ($CDCl_3$) 2.52 (3H, s, CH_3S), 6.31 (1H, s, pyrimid H-5), 6.8–7.0 (1H, bs, NH), 7.29 (2H, d, *J* 9.0, phenyl), 7.35 (2H, d, *J* 9.0, phenyl). ^{13}C NMR (250 MHz; $CDCl_3$) 14.17, 98.49, 123.92, 129.58, 130.70, 135.94, 160.02, 161.07. ν_{max} (KBr disc)/cm^{-1} 3280, 3190, 3140, 3070, 3000, 2920, 1610, 1565, 1535, 1480, 1400, 1350, 1285, 1230, 1200, 1120, 1090, 1010, 970, 860, 820, 730, 690, 660. MS(CI) *m*/*z* 286 (MH^+, 100), 288 (67), 290 (14), 250 (36), 177 (44).

6-Chloro-2-methylsulfonyl-N-*(4-chlorophenyl)-4-pyrimidineamine* ***15***

To a stirred suspension of **14** (3.43 g, 0.012 mol) in glacial acetic acid (12 mL) was added hydrogen peroxide (30% w/v)(4.0 mL, 0.035 mol) dropwise. The mixture was stirred at room temperature for 3 d, after which time, an additional aliquot of hydrogen peroxide (30% w/v, 4.0 mL) was added. The mixture was stirred at room temperature for an additional 24 h. Water (30–40 mL) was added and the resulting precipitate that formed was collected, washed with water, and dissolved in ethyl acetate (50–60 mL). The solution was washed with 1M sodium carbonate (3 ×), water (3 ×), dried (Na_2SO_4), and the solvent removed to yield a white solid. Crystallization from ethanol afforded the sulfone **15** as colourless needles (1.84 g, 54%), mp 173–174°C. δ_H ($CDCl_3$/[D_6]DMSO) 3.29 (3H, s, CH_3SO_2) 6.92 (1H, s, pyrimid H-5), 7.35 (2H, d, *J* 8.8, phenyl), 7.60 (2H, d, *J* 8.8, phenyl), 10.53 (1H, s, NH). ν_{max} (mull)/cm^{-1} 3390, 1610, 1565, 1300, 1230, 1140, 1100, 1010, 970, 960, 825, 760. MS(CI) *m*/*z* 318 (MH^+, 100), 320 (67), 322 (14).

2-Methylsulfonyl-N-*(4-chlorophenyl)-4-pyrimidineamine* ***16***

A mixture of **15** (1.91 g, 6 mmol) in methanol was treated with magnesium oxide (2.40 g, 40 mmol) and Pd/charcoal catalyst (10%) (800 mg). The vigorously stirred mixture was hydrogenated at atmospheric pressure and temperature for 6.5 h. when HPLC indicated that no starting material remained. The mixture was filtered through a celite pad and the filtrate evaporated in vacuo to yield a white powder. Crystallization from methanol afforded the pyrimidinesulfone **16** as a white powder (0.750 g, 44%), mp 190–191°C. δ_H ([D_6]DMSO) 3.31 (3H, s, CH_3SO_2), 7.44 (1H, s, *J* 6.0, pyrimid H-5), 7.44 (2H, d, *J* 9.0, phenyl), 7.71 (2H, d, *J* 9.0, phenyl), 8.45 (1H, s, pyrimid H-6), 10.40 (1H, s, NH). ν_{max} (KBr disc)/cm^{-1} 3330, 1620, 1580, 1500, 1400, 1375, 1350, 1290, 1210, 1125, 1010, 990, 970, 935, 825, 780, 720. MS(CI) *m*/*z* 284 (MH^+, 100), 286(35). HRMS: calcd for $C_{11}H_{10}ClN_3SO_2$ 284.0260, found 284.0270.

2,2′-N,N′*-(hexane-1,6-diyl)bis[4-(4-chloroanilino)2-aminopyrimidinium Dihydrochloride* ***11***

A solution of 1,6-diaminohexane (244 mg, 2.1 mmol) and **16** (57 mg, 2 mmol) in DMSO (3 mL) was heated to 100–105°C for 5 h. The mixture was maintained at room temperature overnight and water was added dropwise to the stirred solution until precipitation was complete. The precipitate was well washed with water (decanting) and dried in vacuo to yield a sticky white solid. The solid was purified by column chromatography on alumina (60 g) eluting with methanol to yield the free base as white crystals (160 mg, 31%), mp 90–93°C. δ_H (60 MHz; $CDCl_3$) 1.2–1.8 (8H, br s, 4 × CH_2), 3.15–3.55 (4H, br m, 2 × CH_2, 2 × NH), 5.2–5.6 (2H, br s, 2 × NH), 6.02 (2H, d, *J* 6, pyrimid H-5), 7.04–7.64 (8H, br s, phenyl), 8.02 (2H, d, *J* 6, pyrimid H-6). MS(CI) *m*/*z* 523 (MH^+), 525 (70), 527 (14), 489 (14). HRMS: calcd for $C_{26}H_{29}Cl_2N_8$ 523.1892, found 523.1861.

A warm solution of the free base (170 mg, 0.325 mmol) in methanol (6 mL) was treated dropwise with concentrated hydrochloric acid (0.1 mL). The solution was filtered and the filtrate treated with ethyl ether (approx. 25 mL) until precipitation commenced. The mixture was cooled in the refrigerator overnight and the precipitate collected to afford the dihydrochloride **11** as off-white beads (185 mg, 95%), mp 234–236.5°C. δ_H ([D_6]DMSO) 1.18–1.51 (4H, br s, 4 × CH_2), 1.51–1.71 (4H, br s, 4 × CH_2), 3.1–3.8 (8H, m, 2 × CH_2, H_2O), 6.42 (2H, d, *J* 6.5, pyrimid H-5), 7.18–7.49 (4H, br s, phenyl), 7.49–7.84 (4H, br s, phenyl), 7.88 (2H, d, pyrimid H-6). 8.4–8.7 (2H, bs, 2 × NH), 11.0–11.4 (2H, bs, 2 × NH), 12.2–12.8 (2H, bs, 2 × NH^+). ν_{max} (KBr disc)/cm^{-1} 3550–2300, 1645, 1550, 1520, 1485, 1450, 1380, 1220, 1080, 1010, 830, 780. MS(CI) *m*/*z* 523 (MH^+ – 2HCl, 100), 525 (70), 527 (14) 489 (14).

Bis-N-*(4-chlorophenyl)-2,4-pyrimidineamine hydrochloride* ***12***

This compound was prepared by the method of Gosh.[28] The crude salt was crystallized twice from ethanol to afford **12** as fluffy needles (2.30 g, 35%), mp 225–227°C (lit[39] mp 225°C). The literature melting point was incorrectly reported as that for the free base. δ_H ([D_6]DMSO) 2.9–4.2 (2H, bs, $2H_2O$), 6.58 (1H, d, *J* 7, pyrimid H-5), 7.39–7.54 (6H, m, phenyl), 7.64 (2H, d, *J* 8.5, phenyl), 8.03 (1H, d, *J* 7, pyrimid H-6), 10.90 (1H, s, NH), 11.32 (1H, s, NH), NH^+ not seen. δ_C (250 MHz; [D_6]DMSO) 99.78, 123.46, 124.02, 128.59, 128.76, 128.89, 135.61, 136.33, 143.50, 152.47, 160.92. ν_{max} (KBr disc)/cm^{-1} 3400, 3200, 3120, 3090, 1660, 1605, 1585, 1550, 1525, 1490, 1450, 1380, 1210, 1100, 1010, 820, 780, 740. MS(CI) *m*/*z* 331(MH^+-HCl, 100). HRMS: calcd for $C_{16}H_{14}Cl_3N_4$·HCl 331.0501, found 331.0517.

Microbiology

Stock solutions of chlorhexidine diacetate (Sigma) and test compounds were prepared in water or DMSO at 10 000 μg mL^{-1}. Compounds were screened against *Staphylococcus aureus* (NCTC 4163), a clinical isolate of methicillin resistant *S. aureus* (MRSA), *Escherichia coli* (NCTC 8196), *Pseudomonas aeruginosa* (NCTC 6749), and *Candida albicans* (RMIT, QAP 1987). Susceptibility testing was by macro broth dilution (1 mL volumes) in log_2 dilution from 512 to 0.25 μg mL^{-1} in Oxoid Tryptone Soya Broth, inoculum density of 2–5 × 10^6 cfu mL^{-1} and incubation at 35°C to 24 h. MIC were expressed as $-\log_{10}$MIC [μM] and are the results of at least two replicates.

QSAR Analyses

Multiple regression analyses were run on a personal computer using the statistical analysis package Statview (ver. 4.02). The log *P* (octanol/water) values were calculated by the CLogP (ver. 1.0) program[40] although this was missing a parameter for the aryl guanide moiety. We found this could be overcome by using a urea moiety in place of the guanide by comparisons with experimental log *P* values. The log *P*

values were also checked against those provided by the HINT! program of Abraham and Kellogg.[41] These programs calculated log *P* values for chlorhexidine very close to the experimental log *P* value of 4.87.[37] Partition coefficients were not corrected for ionization due to pK_a as these authors have shown the average difference between the log *P* of biguanide free bases and their salts was 2.35 ± 0.07 log units. An indicator variable was used to discriminate between bisbiguanides and other compounds, as this was found to be important in QSAR studies of related compounds.[11] We omitted compounds with unquantified, low activity. We also attempted statistical analyses with the inactive compounds set to values four times their reported 'greater than' MIC values to explore the importance of basicity of the molecules on activity. Due to the small number of data points care was taken to ensure that a minimum of parameters were screened in the QSAR analyses in order to reduce the risk of chance correlations.[42] The ionic speciation calculations and conjugate acid p*K* calculations were performed using the University of Georgia SPARC Calculator.[43] Calculated p*K* values were consistent to those of similar compounds (where available) from Perrin's compilations.[44]

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[31] Figure courtesy of Avecia Inc (http://www.avecia.com).

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