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Synthesis, antifungal and haemolytic activity of a series of bis(pyridinium)alkanes

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Abstract—A series of bis(pyridinium)alkanes have been prepared and their antifungal activity, haemolytic activity and ability to inhibit fungal phospholipase B1 have been investigated, together with those of the commercially available antiseptics octenidine and dequalinium. Removal of the amino substituents from the pyridinium rings resulted in a significant decrease in antifungal activity. However, shortening or removing the alkyl chains attached to the amino groups had little effect on antifungal activity and significantly reduced haemolytic activity. Only octenidine was a strong inhibitor of fungal phospholipase B1.

1. Introduction

Invasive fungal infections (mycoses) are becoming increasingly implicated as a cause of serious and fatal diseases. This is especially the case in immunocompromised patients, who are prone to infections caused by opportunistic fungal pathogens that are normally kept in check by a functioning immune system.[1,2] Despite recent additions of new classes of antifungal agents, (e.g., caspofungin),[3] the number of currently available drugs for treatment of fungal infections is limited. Many of these are fungistatic (inhibit fungal growth) rather than fungicidal, whilst fungicidal drugs such as amphotericin B are toxic.[4] The emergence of fungi resistant to these drugs is also becoming problematic. It is now widely recognised that there is a need for the development of new antifungal drugs that have a different mode of action to those currently in use.[5]

We have been investigating inhibition of fungal phospholipase B1 (PLB1),[6,7] a multifunctional enzyme (Fig. 1) that was recently discovered to be a fungal virulence factor[8–12] and is therefore a novel target for antifungal drug discovery. Our studies have shown that fungal PLB1 is strongly inhibited by compounds in which two cationic headgroups are linked by an alkyl spacer. While a limited number of cationic headgroups have been investigated to date, we have found that there are specific structural

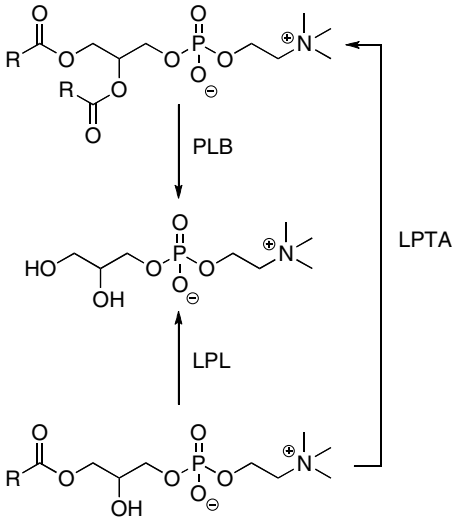

Figure 1. The three activities of cryptococcal phospholipase PLB1: phospholipase B (PLB), lysophospholipase (LPL) and lysophospholipase transacylase (LPTA).

Keywords: Antifungal activity; Bispyridinium; Haemolytic activity; Fungal phospholipase B1.
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requirements for maintenance of both antifungal activity and inhibition of fungal PLB1.[6,7] In a series of bisammonium compounds we found that antifungal activity and PLB1 inhibition were correlated, suggesting that inhibition of PLB1 may be a mode of action for these compounds.[7] In our search for inhibitors of fungal PLB1 as novel antifungal agents, we have now investigated a series of bispyridinium compounds, including the known antiseptics, octenidine and dequalinium.

2. Results

Our initial studies on this class of compounds focused on the commercially available antiseptic agents, octenidine (**1**) and dequalinium (**2**) (Fig. 2). Both octenidine and dequalinium have been used as antimicrobial agents for several decades and are active ingredients in a number of topical formulations including mouthwashes.[13,14] More recently, dequalinium has been investigated as an inhibitor of protein kinase C,[15] a K^+ channel blocker[16] and an anticancer agent.[17] Both **1** and **2** have been found to exhibit strong antifungal activity as well as antibacterial activity.[18,19] Given the structural similarities of these compounds to recently discovered inhibitors of PLB1 (two cationic headgroups linked by an alkyl chain),[6,7] we investigated whether inhibition of this multifunctional enzyme may contribute to the mode of antifungal action of these compounds.

Inhibition assays against the secreted cryptococcal activities of a crude supernatant were performed at pH 4, under conditions previously optimised to measure phospholipase B (PLB), lysophospholipase (LPL) and lysophospholipase transacylase (LPTA) activities of the secreted form of the PLB1 multifunctional enzyme (Fig. 1).[20] Octenidine strongly inhibits all three of the activities of PLB1 at 250 μM, maintaining significant inhibition of the PLB activity at 2.5 μM (Table 1). Significant inhibition of the LPL and LPTA activities of the enzyme remained at concentrations of 25 μM, but this decreased at lower concentrations of **1**. Conversely, dequalinium shows only moderate inhibition (30%) of the PLB activity of the enzyme at 250 μM, with no activity observed against the LPL or LPTA activities of the enzyme.

Octenidine is similar in structure to bolaform surfactants and has previously been reported to cause lysis of cells (both fungal and human).[18,21] This is not a desirable property if compounds such as octenidine are to be developed as treatments for systemic antifungal infections. Given the significant differences in inhibition of PLB1 displayed by the structurally similar compounds **1** and **2**, and in attempts to find inhibitors of fungal PLB1 that are less haemolytic than **1**, we synthesised a number of bispyridinium and bisquinolinium compounds and examined their antifungal, enzyme inhibitory and haemolytic activities.

2.1. Chemistry

The bispyridinium salts **3**–**16** (Fig. 2) were readily prepared by treatment of the appropriate dibromide with

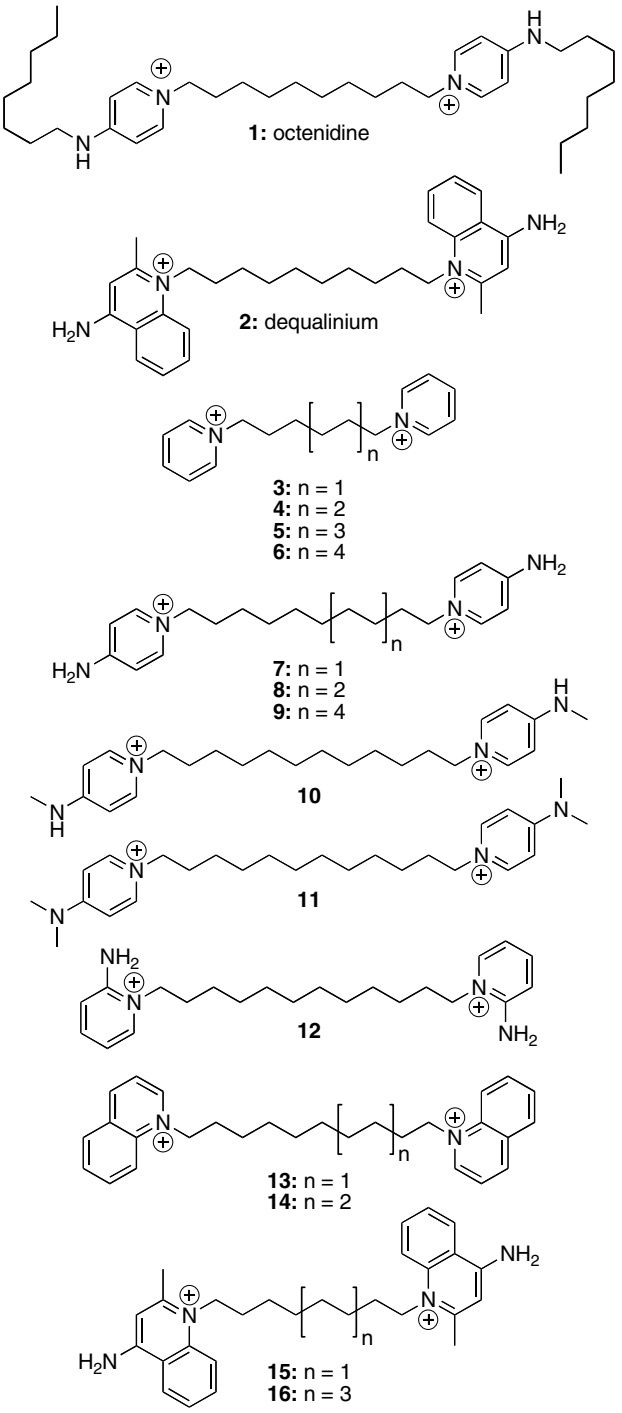
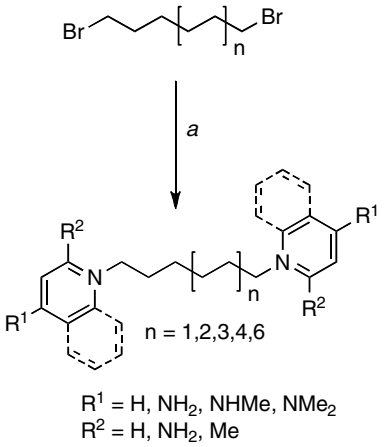


Figure 2. Structures of the bispyridinium and bisquinolinium compounds. (Counter ions are given in Table 2.)

Table 1. Inhibition of the activities of *Cryptococcus neoformans* (strain H99) secretory PLB1 by octenidine

	% Inhibition		
	PLB	LPL	LPTA
250 μM	100	97	97
25 μM	80	46	43
2.5 μM	50	16	12
0.25 μM	0	0	0



Scheme 1. Reagents and condition: (a) excess pyridine or quinoline derivative, methyl ethyl ketone or neat, reflux.

a slight excess of the appropriate pyridine derivative (Scheme 1) and, in most cases, were purified by recrystallisation as the dibromide salts. Where this did not give compounds of sufficient purity, the compounds were purified by column chromatography on silica gel, followed by ion exchange to give the dichloride salts (**14** and **16**).

2.2. Antifungal activity

Compounds **1**–**16** were assayed by a standardised serial dilution sensitivity test for their antifungal activities against reference strains of *Cryptococcus neoformans* and *Candida albicans* (Table 2). Both **1** and **2** are strongly antifungal, in accord with literature data.[18,19] Compounds **3**–**5** bearing unsubstituted pyridinium headgroups were not antifungal. Increasing the length of the alkyl spacer between the pyridinium headgroups to 12 carbons in length (**6**) resulted in a slight increase in antifungal activity. The incorporation of amino substituents on the pyridinium headgroups resulted in a significant increase in antifungal activity. For example, compound **7** comprising a 10-carbon spacer between 4-aminopyridinium headgroups is 10 and 35 times more active against *C. neoformans* and *C. albicans*, respectively, than compound **5**, which lacks the amino substituents. In the 4-aminopyridinium series of compounds (**7**–**9**), antifungal activity was found to increase as the distance between the headgroups was increased, with the most active compound against both *Cryptococcus* and *Candida* species having a 16-carbon chain between the two headgroups. The incorporation of one or two methyl groups (compounds **10** and **11**, respectively) onto the amino substituents had little effect on antifungal activity, while changing the position of the amino substituent to give the 2-aminopyridinium compound, **12**, resulted in a fivefold decrease in activity against *C. albicans* but no change in activity against *C. neoformans*, compared to that shown by **8**. Increasing the size of the aromatic headgroup from pyridinium to quinolinium (compounds **13** and **14**) resulted in a slight increase in antifungal activity. In contrast with all the other compound series examined, increasing the chain length between the aminoquinaldinium headgroups from 8- to 12-carbon atoms in the dequalinium series (**2**, **15** and **16**) resulted in a significant decrease in antifungal activity.

Compound **10** was chosen for further screening against a broad range of fungal pathogens (Table 3). Whilst **10** is less potent than the broad-spectrum antifungal drug amphotericin B against *Aspergillus* and *Fusarium* species, it shows activity comparable to amphotericin B against the *Scedosporium* species tested.

2.3. Inhibition of PLB1

Selected compounds were assayed against fungal PLB1, as described for **1** and **2** above. None strongly

Table 2. Effect of variation in headgroup and alkyl chain length on antifungal activity against *Cryptococcus neoformans* and *Candida albicans*

Compound	Headgroup	X^-	n[a]	MIC (μM)	
				Cryptococcus neoformans (ATCC 90112)	*Candida albicans* (ATCC 10231)
1[b]	4-(*N*-Octylamino)pyridinium	Cl	10	0.7	1.4
2[c]	4-Aminoquinaldinium	Cl	10	11	5.5
3	Pyridinium	Br	6	>350	175
4	Pyridinium	Br	8	>350	175
5	Pyridinium	Br	10	350	88
6	Pyridinium	Br	12	88	44
7	4-Aminopyridinium	Br	10	22	2.8
8	4-Aminopyridinium	Br	12	5.5	1.4
9	4-Aminopyridinium	Br	16	1.4	1.4
10	4-(*N*-Methylamino)pyridinium	Br	12	2.8	1.4
11	4-(*N*,*N*-Dimethylamino)pyridinium	Br	12	2.8	2.8
12	2-Aminopyridinium	Br	12	5.5	5.5
13	Quinolinium	Br	10	88	88
14	Quinolinium	Cl	12	11	22
15	4-Aminoquinaldinium	Br	8	5.5	5.5
16	4-Aminoquinaldinium	Cl	12	88	22

[a] n, number of methylene groups in alkyl chain.
[b] Octenidine.
[c] Dequalinium.

Table 3. In vitro antifungal activity spectrum of **10** compared with that of amphotericin B

	MIC (μM)	
	Compound **10**[a]	Amphotericin B[b]
Aspergillus fumigatus ATCC 204 305	44	0.55
Aspergillus terreus 03-232-378	11	1.1
Aspergillus flavus ATCC 204 304	44	0.55
Scedosporium prolificans 1-003-040	2.8	4.4
Scedosporium apiospermum 1-003-056	2.8	0.55
Fusarium solani 04-132-4207	44	0.28
Cryptococcus neoformans ATCC 90112	2.8	0.55
Candida albicans ATCC 10231	1.4	0.55

[a] Molecular weight 516.0.
[b] Molecular weight 924.1.

inhibited the LPL or LPTA activities of the enzyme at any concentration tested. Compounds **8**, **9**, **11** and **12** inhibited the PLB activity of the enzyme by 40–65% at 250 μM, but activity was not maintained at lower compound concentrations. This behaviour is similar to that observed for dequalinium, but in stark contrast to the strong inhibition of all three activities of the enzyme by octenidine.

2.4. Haemolytic activity

Since a number of the compounds exhibit strong antifungal activity, they have potential to be developed as antifungal therapeutics. However, given the reported ability of octenidine to lyse cells,[21] it was thought that these compounds might have a simple lytic mode of action and be broadly cytotoxic against human cells. We therefore determined the haemolytic activity of these compounds against human erythrocytes as a measure of their cytotoxicity and an initial test of their suitability for further development. Octenidine was found to be strongly haemolytic (43% cell lysis at a compound concentration of 50 μM), retaining 9% haemolytic activity at concentrations as low as 5 μM. None of the other compounds showed any cell lysis at concentrations up to 500 μM. Thus, the haemolytic activity of **1** can be attributed to the presence of the terminal octyl chains.

3. Conclusions

The antiseptics **1** and **2** are strongly antifungal, but despite their structural similarities, only compound **1** is an inhibitor of fungal PLB1. Compound **1** is also strongly haemolytic whilst **2** did not lyse human red blood cells at any of the concentrations examined. Analogues of **1** and **2** were synthesised and examined for antifungal activity, PLB1 inhibition and haemolytic activity. Removal of the amino substituents from the pyridine ring severely reduced the antifungal activity of these compounds. However, some activity was regained when the heterocyclic headgroup was increased in size from pyridinium to quinolinium. Antifungal activity was maintained when the amino substituent was unsubstituted or bore one or two methyl substituents, and changing the position of the amino substituent from C-4 to C-2 had little effect on antifungal activity. It is probable that the role of the amino substituent is electronic, resulting in delocalisation of the positive charge (Fig. 3). Increasing the distance between the two headgroups resulted in increased antifungal activity, except where the headgroup was 4-aminoquinaldinium. None of the compounds investigated (except **1**) showed any haemolytic activity at concentrations up to 500 μM, indicating that it is possible to maintain antifungal activity whilst reducing the haemolytic activity of this class of compounds.

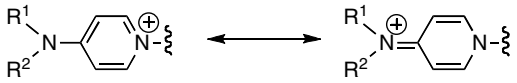


Figure 3. Delocalisation of the positive charge by the amino substituent.

The only compound that strongly inhibited fungal PLB1 was **1**, indicating that for this class of compounds the octyl chains are required for PLB1 inhibition, and that the antifungal activity observed for the other compounds must occur via an alternative mode of action. Similar bis-pyridiniumalkanes have been found to interfere with phospholipid metabolism and choline uptake in *Plasmodium* species[22] and this may also be the case in fungi. Further investigation into the mode of action of these compounds will allow their full potential as antifungal agents to be explored.

4. Experimental

4.1. Chemistry

Melting points were determined using a Gallenkamp melting point apparatus and are reported in degrees Celsius (uncorrected). NMR spectra were recorded on Bruker Avance DPX 200 and Bruker Avance DPX 300 spectrometers. The solvent ^{1}H and ^{13}C signals, δ_H 3.31 and δ_C 49.0 for MeOH-d_4; δ_H 2.50 and δ_C 40.5 for DMSO-d_6; and δ_H 7.26 and δ_C 77.0 for $CDCl_3$, were used as internal references. Low resolution mass spectra were recorded on a Finnigan LCQ ion trap mass spectrometer (ESI). High resolution mass spectra were recorded on a BioApex Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (ESI). Flash chromatography was performed on silica gel (Merck silica gel 60, 40–63 μm) at a pressure of 0.3–0.4 bar. Elemental analyses were performed by Campbell Microanalytical Laboratories. Octenidine was a gift from Dr. J. Siebert, Schulke & Mayr GmbH, Norderstedt, Germany. Dequalinium was purchased from Sigma.

4.1.1. 1,6-Bis(pyridinium)hexane dibromide (3). 1,6-Dibromohexane (0.50 g, 2.0 mmol) was dissolved in pyridine (3.3 mL, 40 mmol) and the resulting mixture was refluxed under a nitrogen atmosphere for 24 h. The mixture was cooled and the resulting precipitate was collected by filtration and triturated with diethyl ether (3× 20 mL) to give **3** (0.78 g, 98%) as a colourless solid; mp 242–245 °C (lit. mp[23] 238–241 °C). ^{1}H NMR (200 MHz, CD_3OD) δ 9.13 (4H, m), 8.65 (2H, app. t, J = 7.7 Hz), 8.18 (4H, app. t, J = 6.5 Hz), 4.75 (4H, t, J = 7.5 Hz,), 2.13 (4H, m), 1.56 (4H, br s,); MS (ESI) m/z 324 ([M−Br$^-$]$^+$ 100%), 322 ([M−Br$^-$]$^+$ 89); Found: C, 47.8; H, 5.4; N, 6.9%. $C_{16}H_{22}N_2Br_2$ requires C, 47.8; H, 5.5; N, 7.0%.

4.1.2. 1,8-Bis(pyridinium)octane dibromide (4). 1,8-Dibromooctane (0.50 g, 1.8 mmol) was dissolved in pyridine (2.9 mL, 36 mmol) and the resulting mixture was refluxed under a nitrogen atmosphere for 24 h. The mixture was cooled and the resulting precipitate was collected by filtration and triturated with diethyl ether (3× 20 mL) to give **4** (0.70 g, 89%) as a colourless solid; mp 189–191 °C (lit. mp[24] 194 °C). ^{1}H NMR (200 MHz, CD_3OD) δ 9.09 (4H, d, J = 5.9 Hz), 8.65 (2H, app. t, J = 7.4 Hz), 8.17 (4H, app. t, J = 6.8 Hz), 4.70 (4H, t, J = 7.5 Hz), 2.08 (4H, m), 1.47 (8H, br s); MS (ESI) m/z 351 ([M−Br$^-$]$^+$ 95%), 349 ([M−Br$^-$]$^+$ 100); Found: C, 50.15; H, 6.0; N, 6.4%. $C_{18}H_{26}N_2$ Br_2 requires C, 50.25; H, 6.1; N 6.5%.

4.1.3. 1,10-Bis(pyridinium)decane dibromide (5). 1,10-Dibromodecane (0.50 g, 1.7 mmol) was dissolved in pyridine (2.6 mL, 34 mmol) and the resulting mixture was refluxed under a nitrogen atmosphere for 24 h. The mixture was cooled and the resulting precipitate was collected by filtration and triturated with diethyl ether (3× 20 mL) to give **5** (0.75 g, 98%) as a colourless solid; mp 196–198 °C (lit. mp[24] 196 °C). ^{1}H NMR (200 MHz, CD_3OD) δ 9.12 (4H, m), 8.67 (2H, app. t, J = 7.7 Hz), 8.19 (4H, app. t, J = 6.8 Hz), 4.73 (4H, t, J = 7.3 Hz), 2.09 (4H, app t, J = 6.5 Hz), 1.42 (12H, m); Found: C, 52.6; H, 6.6; N, 6.1%. $C_{20}H_{30}N_2Br_2$ requires C, 52.4; H, 6.6; N, 6.1%.

4.1.4. 1,12-Bis(pyridinium)dodecane dibromide (6). 1,12-Dibromododecane (0.50 g, 1.5 mmol) was dissolved in pyridine (2.4 mL, 30 mmol) and the resulting mixture was refluxed under a nitrogen atmosphere for 24 h. The mixture was cooled and the resulting precipitate was collected by filtration and triturated with diethyl ether (3× 20 mL) to give **6** (0.71 g, 96%) as a colourless solid; mp 196–198 °C (lit. mp[24] 198–199 °C). ^{1}H NMR (200 MHz, CD_3OD) δ 9.07 (4H, d, J = 5.6 Hz), 8.65 (2H, app. t, J = 7.8 Hz), 8.17 (4H, app. t, J = 6.9 Hz), 4.69 (4H, t, J = 7.6 Hz), 2.07 (4H, app. t, J = 6.4 Hz), 1.43–1.36 (16H, m); MS (ESI) m/z 407 ([M−^{79}Br$^-$]$^+$ 100%), 405 ([M−^{81}Br$^-$]$^+$ 90); Found: C, 52.6; H, 7.25; N 5.5%. $C_{22}H_{34}N_2Br_2{\cdot}H_2O$ requires C, 52.4; H, 7.0; N, 5.55.

4.1.5. 1,10-Bis(4-aminopyridinium)decane dibromide (7). 1,10-Dibromododecane (0.50 g, 1.6 mmol) was dissolved in methyl isobutyl ketone (5 mL) and 4-aminopyridine (0.47 g, 4.9 mmol) was added. The mixture was stirred at reflux for 24 h, then cooled and the resulting precipitate was collected by filtration and triturated with anhydrous acetonitrile (3× 20 mL) to give **7** (0.80 g, 98%) as a colourless solid; mp 250–253 °C (lit. mp[25] 249–251 °C). ^{1}H NMR (200 MHz, DMSO-d_6) δ 8.25 (d, J = 7.0 Hz, 4H), 6.86 (d, J = 7.0 Hz, 4H), 4.13 (t, J = 6.9 Hz, 4H), 1.76 (m, 4H), 1.26 (m, 12 H); MS (ESI) m/z 409 ([M−^{79}Br$^-$]$^+$ 16%), 407 ([M−^{81}Br$^-$]$^+$ 7), 327 ([M−2Br$^-$−H$^+$]$^+$, 45), 164 ([M−2Br]$^{2+}$, 100); Found: C, 48.1; H, 6.4; N, 11.1%. $C_{20}H_{32}N_4Br_2{\cdot}0.5H_2O$ requires C, 48.3; H, 6.7; N 11.3%.

4.1.6. 1,12-Bis(4-aminopyridinium)dodecane dibromide (8). 1,12-Dibromododecane (0.50 g, 1.52 mmol) was dissolved in methyl isobutyl ketone (5 mL) and 4-aminopyridine (0.29 g, 3.05 mmol) was added. The mixture was stirred at reflux for 24 h, then cooled and the resulting precipitate was collected by filtration and recrystallised from MeOH/Et_2O to give **8** as a white solid (0.76 g, quant.); mp 215–218 °C (lit. mp[26] 216–217 °C). ^{1}H NMR (200 MHz, CD_3OD): δ 8.12 (4H, d, J = 7.5 Hz), 6.87 (4H, d, J = 7.5 Hz), 4.16 (4H, t, J = 7.3 Hz), 1.91–1.84 (4H, m), 1.34 (16H, br s), NH_2 not observed. ^{13}C NMR (75 MHz, CD_3OD): 144.4, 111.2, 59.7, 32.3, 31.0, 30.6, 27.6, 2 signals obscured or overlapping. MS: m/z ESI (positive ion) 178 [M−2Br$^-$]$^{2+}$ (100%), 355 [M−2Br$^-$−H$^-$]$^+$ (25). Anal. Calcd for $C_{22}H_{36}Br_2N_4{\cdot}0.5H_2O$: C, 50.3; H, 6.9; N, 10.7. Found: C, 50.1; H, 7.2; N, 10.5.

4.1.7. 1,16-Bis(4-aminopyridinium)hexadecane dibromide (9). 1,16-Dibromohexadecane (0.09 g, 0.22 mmol) was dissolved in methyl isobutyl ketone (3 mL) and 4-aminopyridine (0.04 g, 0.45 mmol) was added. The mixture was stirred at reflux for 48 h, then cooled and the resulting precipitate was collected by filtration and recrystallised from MeOH/Et_2O to give **9** as an off-white solid (0.05 g, 35%); mp 173–174 °C. ^{1}H NMR (200 MHz, CD_3OD): δ 7.98 (4H, d, J = 7.5 Hz), 6.73 (4H, d, J = 7.5 Hz), 4.02 (4H, t, J = 7.3, 8.0 Hz), 1.91–1.84 (4H, m), 1.34 (24H, m), NH_2 not observed. ^{13}C NMR (75 MHz, $CDCl_3$): δ 142.2, 110.7, 58.8, 50.0, 31.2, 29.7, 29.6, 29.2, 26.3, 2 signals obscured or overlapping. MS: m/z 206 [M−2Br$^-$]$^+$ (100%), 411 [M−2Br$^-$−H$^-$]$^+$ (25). Found: [M−2Br$^-$]$^{2+}$ 206.1777, [$C_{28}H_{44}N_4$]$^{2+}$ requires 206.1783.

4.1.8. 1,12-Bis(4-*N*-methylaminopyridinium)dodecane dibromide (10). 1,12-Dibromododecane (0.50 g, 1.52 mmol) was dissolved in methyl isobutyl ketone (5 mL) and 4-methylaminopyridine (0.29 g, 3.05 mmol) was added. The mixture was stirred at reflux for 24 h, then cooled and the resulting precipitate was collected by filtration and recrystallised from MeOH/Et_2O to give **10** as a colourless solid (0.76 g, quant.); mp 196–198 °C. ^{1}H NMR (200 MHz, CD_3OD): δ 8.19 (2H, d, J = 6.0 Hz), 8.03 (2H, d, J = 6.1 Hz), 6.86 (4H, d, J = 7.6 Hz), 4.15 (4H, t, J 7.3 Hz), 2.98 (6H, s), 1.91–1.84 (4H, m), 1.34 (16H, br s), NH not observed. ^{13}C NMR (75 MHz, CD_3OD): 143.7, 141.2, 110.7, 105.2, 58.0, 30.9, 29.5, 29.1, 28.9, 26.1. MS: m/z ESI (positive ion) 192 [M−2Br$^-$]$^{2+}$ (100%), 383 [M−2Br$^-$−H$^-$]$^+$

(50). Anal. Calcd for $C_{24}H_{40}Br_2N_4 \cdot 0.5H_2O$: C, 52.1; H, 7.3; N, 10.1. Found: C, 52.0; H, 7.5; N, 10.0.

4.1.9. 1,12-Bis(4-*N*,*N*-dimethylaminopyridinium)dodecane dibromide (11). 1,12-Dibromododecane (0.50 g, 1.52 mmol) was dissolved in methyl isobutyl ketone (5 mL) and 4-*N*,*N*-dimethylaminopyridine (0.37 g, 3.05 mmol) was added. The mixture was stirred at reflux for 24 h, then cooled and the resulting precipitate was collected by filtration and recrystallised from MeOH/Et_2O to give **11** as a colourless solid (0.53 g, 84%); mp 268–271 °C. ^{1}H NMR (200 MHz, CD_3OD): δ 8.19 (4H, d, J = 8.0 Hz), 6.86 (4H, d, J = 6.0 Hz), 4.07 (4H, t, J = 7.3 Hz), 3.15 (12H, s), 1.78–1.72 (4H, m), 1.08 (16H, br s, CH_2). ^{13}C NMR (75 MHz, CD_3OD): 148.4, 141.9, 107.8, 57.9, 47.1 39.2, 30.9, 29.6, 29.1, 26.1. MS: *m*/*z* ESI (positive ion) 206 $[M-2Br^-]^{2+}$ (100%), 411 $[M-2Br^- - H^+]^+$ (15). Anal. Calcd for $C_{26}H_{44}Br_2N_4$: C, 54.6; H, 7.8; N, 9.8. Found: C, 54.8; H, 7.9; N, 9.7.

4.1.10. 1,12-Bis(2-aminopyridinium)dodecane dibromide (12). 1,12-Dibromododecane (0.50 g, 1.52 mmol) was dissolved in methyl isobutyl ketone (5 mL) and 2-aminopyridine (0.29 g, 3.05 mmol) was added. The mixture was stirred at reflux for 24 h, then cooled and the resulting precipitate was collected by filtration. The crude precipitate was recrystallised from MeOH/Et_2O to afford **12** as a beige powder (0.22 g, 28%); mp 226–234 °C (lit. mp[27] 230–232 °C). ^{1}H NMR (200 MHz, CD_3OD): δ 8.00 (2H, d, J = 6.5 Hz), 7.89 (2H, dd, J = 8.4, 6.9 Hz), 7.12 (2H, d, J = 8.9 Hz), 6.95 (2H, dd, J = 6.9, 6.9 Hz), 4.21 (4H, t, J = 7.6 Hz), 1.87–1.82 (4H, m), 1.44–1.41 (16H, br s), NH_2 not observed. ^{13}C NMR (75 MHz, CD_3OD):154.5, 142.5, 139.8, 115.2, 113.4, 65.8, 54.0, 29.5, 29.3, 26.1, 14.4. MS: *m*/*z* ESI (positive ion) 178 $[M-2Br^-]^{2+}$ (100%), 355 $[M-2Br^- - H^-]^+$ (25). Anal. Calcd for $C_{22}H_{36}Br_2N_4$: C, 51.2; H, 7.0; N, 10.9. Found: C, 51.5; H, 7.3; N, 10.6.

4.1.11. 1,10-Bis(quinolinium)decane dibromide (13). To 1,10 dibromodecane (0.25 g, 0.83 mmol) in methyl isobutyl ketone (1 mL) was added quinoline (0.43 g, 3.33 mmol). The mixture was deoxygenated by freeze/thaw and refluxed for 18 h, then cooled to room temperature and the resulting precipitate was collected by filtration. The crude product was recrystallised from MeOH/Et_2O to yield **13** as a beige powder (0.16 g, 35%); mp 264–272 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 9.58 (2H, d, J = 5.8 Hz), 8.96 (2H, d, J = 8.4 Hz), 8.29–8.08 (6H, m), 8.08–7.82 (4H, m), 4.99 (4H, t, J = 7.6 Hz), 2.02–1.87 (4H, m), 1.33–1.08 (12H, m). ^{13}C NMR (75 MHz, $CDCl_3$): δ 153.7, 151.9, 142.2, 135.4, 134.8, 126.6, 122.6, 62.7, 34.4, 33.0, 32.9, 30.5, 2 signals obscured or overlapping. MS: *m*/*z* ESI (positive ion) 199 $[M-2Br^-]^{2+}$ (100%), 477 $[M-2Br^- - H^+]^+$ (87). Found: $[M-2Br^-]^{2+}$ 199.1357, $[C_{28}H_{34}N_2]^{2+}$ requires 199.1355 Anal. Calcd for $C_{28}H_{34}Br_2N_2 \cdot 2H_2O$: C, 56.6; H, 6.4; N, 4.7. Found: C, 56.7; H, 6.4; N, 4.8.

4.1.12. 1,12-Bis(quinolinium)dodecane dichloride (14). 1,12-Dibromododecane (0.50 g, 1.52 mmol) was dissolved in methyl isobutyl ketone (5 mL) and quinoline (0.39 g, 3.05 mmol) was added. The mixture was stirred at reflux for 24 h and the solvent was removed in vacuo. The crude mixture was purified by flash chromatography, eluting with 90:30:1 CH_2Cl_2/MeOH/$NH_{3(aq)}$ (R_f 0.63), and the combined fractions were concentrated under reduced pressure. The residue was then passed through a column of Lewatit MP-64 anion resin (Cl^-), eluting with H_2O. The resulting fractions were combined and the solvent removed in vacuo to give **14** as an off-white solid (0.05 g, 8%). ^{1}H NMR (200 MHz, CD_3OD): δ 9.37 (2H, d, J = 5.7 Hz), 9.15 (2H, d, J = 8.3 Hz), 8.42 (2H, d, J = 9.0 Hz), 8.38 (2H, d, J = 8.2 Hz), 8.22 (2H, dt, J = 8.0, 1.3 Hz), 8.08–7.91 (4H, m), 5.03 (4H, t, J = 7.5 Hz), 2.05–1.91 (4H, m), 1.32–1.05 (16H, m). MS: *m*/*z* ESI (positive ion) 213 $[M-2Cl^-]^{2+}$ (100%), 461 $[M-2Cl^- - H^+]^+$ (87). Found: $[M-2Cl^-]^{2+}$ 213.1511, $[C_{30}H_{38}N_2]^{2+}$ requires 213.1535.

4.1.13. 1,8-Bis(4-aminoquinaldinium)octane dibromide (15). 1,8-Dibromooctane (0.50 g, 1.84 mmol) was dissolved in methyl isobutyl ketone (5 mL) and 4-aminoquinaldine (0.58 g, 3.67 mmol) was added. The mixture was stirred at reflux for 24 h, at which time precipitate was formed. The crude mixture was cooled, the precipitate was filtered off and recrystallised from MeOH/Et_2O to give **15** as an off-white solid (0.21 g, 19%); mp 302–313 °C. ^{1}H NMR (200 MHz, CD_3OD): δ 8.36–8.23 (2H, m), 8.22–7.88 (4H, m), 7.76–7.56 (2H, m), 6.55 (2H, s), 4.43 (4H, t, J 8.1 Hz), 2.75 (6H, s), 1.78–1.74 (4H, m), 1.51–1.41 (8H, m, CH_2), NH_2 not observed. ^{13}C NMR (75 MHz, CD_3OD): δ 157.9, 156.2, 139.8, 134.6, 126.3, 124.2, 118.3, 104.4, 48.8, 29.3, 29.1, 26.3, 21.5, 21.1. MS: *m*/*z* ESI (positive ion) 214 $[M-2Br^-]^{2+}$ (100%), 427 $[M-2Br^- - H^+]^+$ (55). Anal. Calcd for $C_{28}H_{36}Cl_2N_4 \cdot 1H_2O$: C, 55.5; H, 6.0; N, 8.7. Found: C, 55.2; H, 6.5; N, 8.7.

4.1.14. 1,12-Bis(4-aminoquinaldinium)dodecane dichloride (16). 1,12-Dibromododecane (0.50 g, 1.52 mmol) was dissolved in methyl isobutyl ketone (5 mL) and 4-aminoquinaldine (0.48 g, 3.05 mmol) was added. The mixture was stirred at reflux for 24 h and the solvent was removed in vacuo. The crude mixture was purified by flash chromatography, eluting with 90:30:1 CH_2Cl_2/MeOH/$NH_{3(aq)}$ (R_f 0.68), and the combined fractions were concentrated under reduced pressure. The residue was then passed through a column of Lewatit MP-64 anion resin (Cl^-), eluting with H_2O. The resulting fractions were combined and the solvent removed under reduced pressure to give **16** as an off-white solid (0.04 g, 4%); mp: 159–165 °C. ^{1}H NMR (200 MHz, CD_3OD): δ 8.45–8.33 (2H, m), 8.12–7.97 (4H, m), 7.80–7.66 (2H, m), 6.78 (2H, s), 4.52 (4H, t, J = 7.5 Hz), 2.78 (6H, s), 1.87–1.79 (4H, m), 1.32–1.05 (16H, m), NH_2 not observed. ^{13}C NMR (75 MHz, CD_3OD): δ 156.9, 141.3, 136.1, 128.0, 127.8, 125.7, 119.7, 105.8, 50.3, 31.1, 30.8, 30.1, 29.7, 28.5, 27.3, 22.5. MS: *m*/*z* ESI (positive ion) 242 $[M-2Cl^-]^{2+}$ (100%). Found: $[M-2Cl^-]^{2+}$ 242.1778, $[C_{32}H_{44}N_4]^{2+}$ requires 242.1778.

4.2. Antifungal susceptibility testing

The antifungal activity of the compounds was measured by standard broth microdilution methods of the US National Committee for Clinical Laboratory Standards for

yeasts[28] and filamentous fungi.[29] The minimal inhibitory concentration (MIC) was defined as that which produced no visible growth after 48, or 72 h, of culture at 35 °C.[28,29] All tests were performed in duplicate.

4.3. Fungal isolates and media

A virulent clinical isolate of *C. neoformans* var. *grubii* (serotype A), H99, which produces high levels of secreted phospholipase B activity was used for cell-associated phospholipase characterisation and inhibition of phospholipase activities. Isolate H99 was kindly supplied by Dr. Gary Cox (Duke University Medical Center, Durham, NC, USA) and subcultured onto Sabouraud dextrose agar (SDA) at 30 °C.

4.4. Preparation of supernatants containing secreted phospholipase activities

Isolate H99 was grown to confluence on SDA in 16 cm diameter Petri dishes for 72 h at 30 °C in air. Cells scraped from 10–20 dishes were washed sequentially with isotonic saline and imidazole buffer (10 mM imidazole, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 56 mM D-glucose, made up in isotonic saline, pH 5.5), resuspended in a volume of this buffer of about 10% of the cell volume and incubated for 24 h at 37 °C. The cell-free supernatant was separated by centrifugation as previously described[30] and stored at −70 °C.

4.5. Radiometric assay method for fungal phospholipases

Enzyme activities were measured as described previously[10,20,30] in a final volume of 125 μL at 37 °C. For the determination of secreted PLB activity, carrier dipalmitoyl phosphatidylcholine (DPPC, final concentration 800 μM) and 1,2-di[1-^{14}C] palmitoyl phosphatidylcholine (20,000 dpm) were dried under nitrogen and suspended in 125 mM imidazole acetate buffer (assay buffer, pH 4.0) by sonication using a Branson 450 sonifier. The reaction time was 22 min, using 1 μg total protein, and PLB activity was determined by the rate of decrease of the radiolabelled PC substrate, with appearance of the label in free fatty acid. Secreted LPL and LPTA activities were measured simultaneously in a reaction mixture containing 1-[^{14}C]palmitoyl lyso-PC (25,000 dpm) and carrier lyso-PC (final concentration 200 μM) in assay buffer. The reaction time was 15 s with 1 μg of total protein and LPL activity was measured by the rate of loss of 1-[^{14}C]palmitoyl lyso-PC with release of radiolabelled fatty acids. LPTA activity was estimated from the rate of formation of radiolabelled PC. All reactions were terminated by adding 0.5 mL of chloroform/methanol (2:1 v/v). The reaction products were extracted by the method of Bligh and Dyer,[31] separated by TLC and quantified as previously described.[30] The TLC plates were developed in chloroform/methanol/water (65:25:4; v/v/v).

4.6. Protein assays

Total protein estimations were performed using a Coomassie blue binding assay with BSA as standard (Pierce Chemical Co., IL, USA).

4.7. Testing of bis-pyridinium salts as inhibitors

Solutions of bis-pyridinium compounds were prepared as stock solutions of 700 μM in assay buffer containing 5 mM EDTA, which was then diluted serially with buffer to give solutions with concentrations of 70, 7 and 0.7 μM. In each assay, 45 μL of the stock or diluted solutions was used, and the final volume of 125 μL was made up of substrate, enzyme and buffer. This gave solutions with compound concentrations of 250, 25, 2.5 and 0.25 μM. The radiometric assay was carried out as above. Inhibition was calculated as the percent of substrates (DPPC or Lyso-PC) remaining in the case of PLB and LPL activities, or of DPPC produced, in the case of the LPTA activity. The amounts converted, or produced, in the inhibitor-free control were normalised to 100%, and the inhibition calculated against it. All assays were performed in triplicate, with a difference between runs of <10% in all cases.

4.8. Haemolytic activity assay

Human blood was collected in 10 mL Vacutainer tubes containing potassium-EDTA as anticoagulant. The blood from each Vacutainer was transferred to a 50 mL centrifuge tube and the cells washed three times with 30 mL of calcium- and magnesium-free phosphate-buffered saline (PBS; Gibco). Cells were collected by centrifugation at 2000*g* for 10 mins in a Beckman TJ-6 centrifuge. The third supernatant was clear and colourless. Cells were stored in PBS (20 mL) for up to 2 weeks. Then 0.5 mL cell suspension in PBS was mixed with 0.5 mL of test substance using stock solutions of concentrations 700, 350, 175, 70 and 7 μM (final erythrocyte concentration approx. 0.5×10^9 per mL). The mixtures were incubated at 37 °C for 1 h with gentle shaking, centrifuged at 2000*g* for 10 min, the supernatant diluted 10-fold with PBS, and optical density measured at 540 nm. The values for 0% and 100% lysis were determined by incubating cells with PBS or 0.1% (w/v) Triton X-100 (in water), respectively. Assays were carried out in triplicate and the difference between runs was <5% in all cases. The concentration of test compounds in the assays was 500, 250, 50, 25 and 5 μM.

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