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# Synthesis, antifungal and antimicrobial activity of alkylphospholipids

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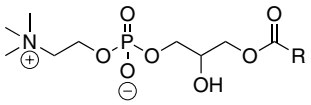
**Abstract**—The antifungal, antibacterial and haemolytic activity of a series of alkylphosphocholines (e.g., miltefosine) and alkylglycerophosphocholines (e.g., edelfosine) has been investigated. These compound classes exhibit significant antifungal and moderate antibacterial activities. Several new alkylphosphocholine derivatives with amide or ester bonds in the alkyl chain have been synthesised. These compounds show much lower haemolytic activity than miltefosine. Alkylphosphocholines and alkylglycerophosphocholines show significant promise as novel orally available antifungal and antibacterial therapeutics.

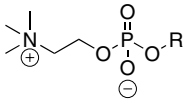

## 1. Introduction

Metabolically stable lysophospholipid analogues in the form of alkylphosphocholines (APCs) and alkylglycerophosphocholines (AGPCs) (Fig. 1) were originally developed as novel anticancer agents with antineoplastic activity.[1] More recently, these compounds have shown great promise in the treatment of parasitic infections, in particular those caused by *Leishmania* spp., *Trypanosoma brucei* and *Trypanosoma cruzi*,[2] and *n*-hexadecylphosphocholine (miltefosine) **1** (Fig. 2) has been approved in several countries for clinical use in the treatment of leishmaniasis.[3]

Lysophospholipids are one of the substrates used by the multifunctional fungal enzyme, PLB1, a recently discovered fungal virulence factor (Fig. 3).[4,5] We were therefore interested in investigating whether APCs and/or AGPCs would act as inhibitors of fungal PLB1, with potential to be developed as antifungal agents with a novel mode of action. We recently discovered that miltefosine exhibits broad spectrum antifungal activity in vitro, and in vivo activity in a mouse model of disseminated cryptococcosis.[6] The naturally occurring alkyl bisphosphocholine derivative, irbacholine, has previously been reported to exhibit antifungal activity,[7] further indicating that APCs and AGPCs show promise as a new class of antifungal drugs.

Although miltefosine is approved for clinical treatment of leishmaniasis, there are undesirable gastrointestinal



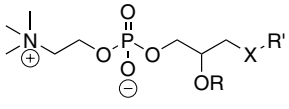




**Figure 1.** General structures of a lysophospholipid, an APC and an AGPC, illustrating the structural differences between these general classes of compounds.

*Keywords*: Alkylphospholipids; Hexadecylphosphocholine; Antifungal activity; Antibacterial activity; Haemolytic activity.
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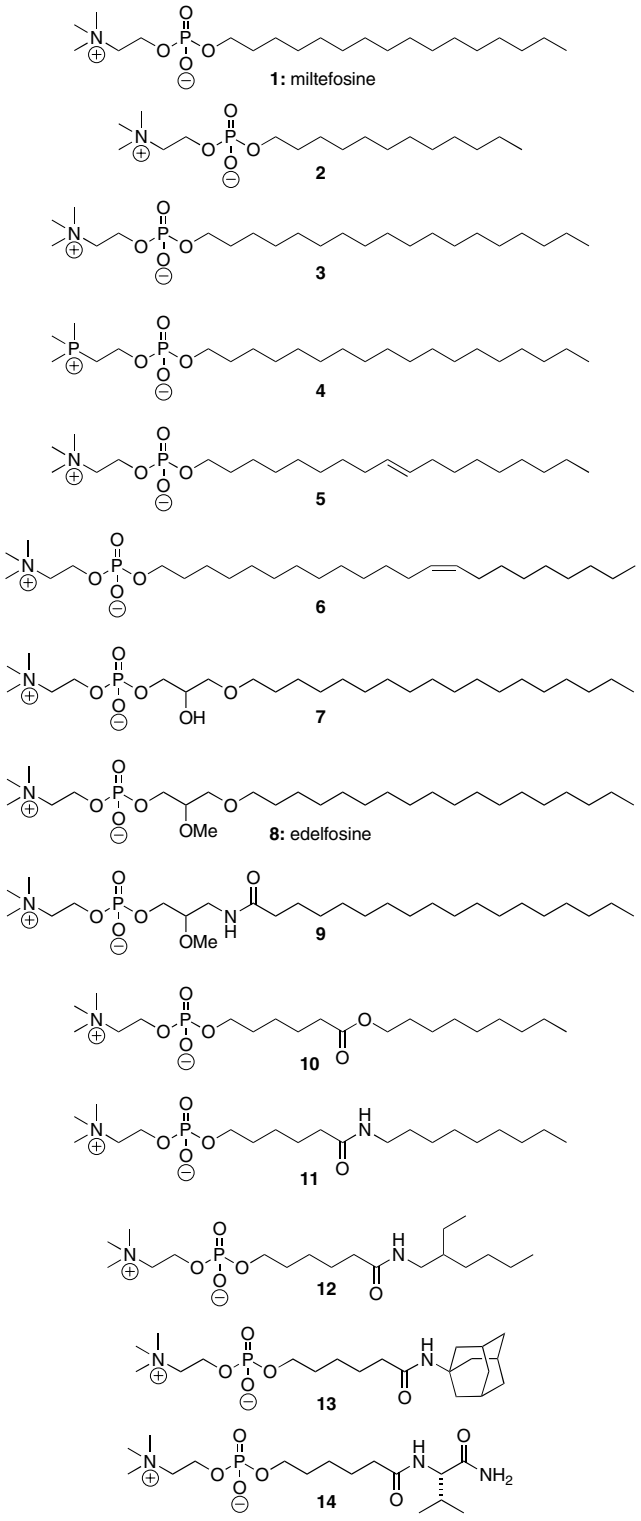



**Figure 2.** Structures of the APCs and AGPCs assayed.

side effects (vomiting and diarrhoea) associated with oral treatment[8] and the high haemolytic activity of miltefosine prevents intravenous delivery.[9] Several studies have been aimed at lowering the toxicity of miltefosine derivatives while maintaining antiproliferative[9,10] or antileishmanial activity.[11,12] However, apart from an early study on the antifungal activity of alkyl lysophospholipid ether derivatives,[13] there has been no investigation of the structure–antifungal activity relationships of APCs and AGCPs. For clinical use it is highly desirable to obtain miltefosine analogues in which the haemolytic activity is decreased, to allow intravenous delivery, while antifungal activity is maintained. We have therefore examined the antifungal and haemolytic activity of a number of miltefosine analogues in order to determine which structural features could be altered to achieve this goal. Since structure–antileishmanial activity studies have previously shown that modifications of the alkyl chain are effective in reducing cytotoxicity[12] we focussed our study on modifying this portion of the molecule while keeping the phosphocholine headgroup constant. We report here the first structure–antifungal activity study of a series of APCs and AGPCs, together with the synthesis of a number of new miltefosine analogues in which amide or ester linkages have been introduced into the alkyl chain to reduce the haemolytic activity of the compounds. Since these compounds are structural analogues of lysophosphocholine, we have investigated whether the inhibition of fungal PLB1 might be a mode of antifungal action for these compounds. Given the broad spectrum of activity of APCs and AGPCs against both protozoa and fungi, we also investigated these compounds for antibacterial activity.

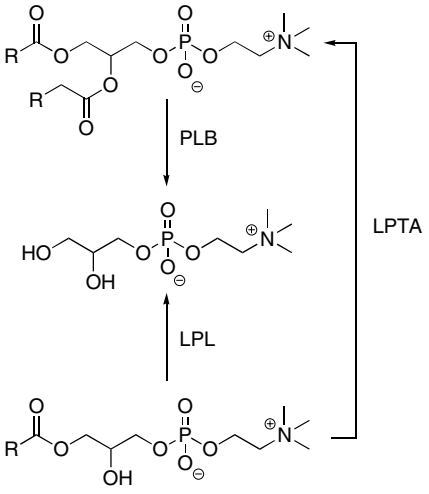
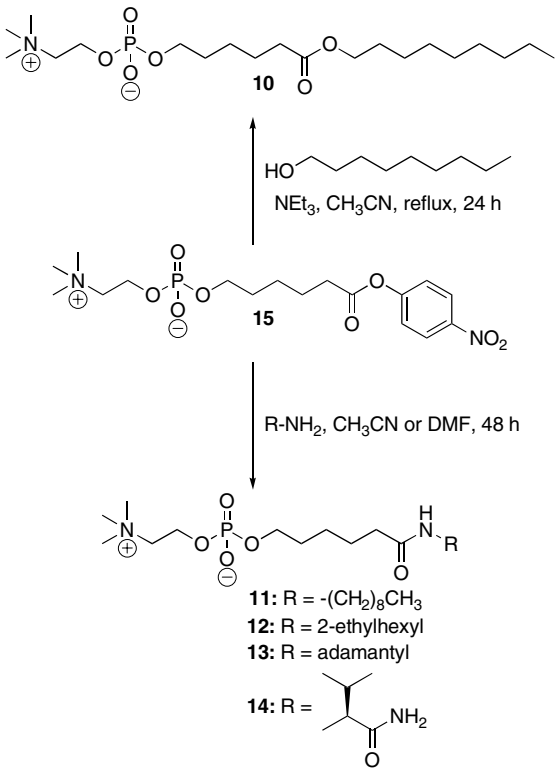



**Figure 3.** The three activities of cryptococcal phospholipase PLB1.

## 2. Results and discussion

### 2.1. Chemistry

In order to determine the effect that groups introduced to decrease the haemolytic activity of miltefosine would have on antifungal activity, we assembled thirteen analogues of the parent compound miltefosine (**1**) that varied in chain length, incorporated alkenes and/or contained hydrogen bond donors and acceptors either immediately adjacent to the PC headgroup or in the centre of the alkyl chain. Compounds **2**–**9** were selected from the pool of commercially available APC and AGPC derivatives, while the new phosphocholines **10**–**14** were synthesised for this study. These were readily prepared upon treatment of the known active ester




**Scheme 1.** Synthesis of compounds **10**–**14**.

**15**[14] with the appropriate amine or alcohol (Scheme 1). In all cases, thin layer chromatography indicated that the reactions proceeded to completion, but in some cases (compounds **10** and **13**) low yields were obtained as a result of the difficulties inherent in purifying these zwitterionic compounds. In compounds **10**–**14**, a polar group is introduced at a position six carbon atoms away from the phosphocholine (PC) headgroup, with the structural bulk of the substituent at the end of the alkyl chain furthest from the headgroup being varied. These new compounds, together with compounds **2**–**6**, are classified as APCs since the PC group is directly attached to an alkyl chain. Compounds with polar groups positioned adjacent to the PC headgroup (e.g., **7**–**9**) are classified as AGPCs as they contain a (sometimes modified) glycerol unit between the phosphocholine headgroup and the alkyl chain.

## 2.2. Antifungal activity

Compounds **1**–**14** were assayed for their antifungal activities against reference strains of *Cryptococcus neoformans* and *Candida albicans* using a standardised serial dilution sensitivity test (Table 1). For the APC compound series, reduction of the length of the alkyl chain to 12 carbon atoms (**2**) significantly decreased antifungal activity, whilst increasing the length of this chain to 18 carbon atoms (**3**) resulted in slightly better activity against both *Cryptococcus* and *Candida* species than with miltefosine (**1**). Substitution of the trimethylammonium group in the phosphocholine headgroup of miltefosine with a trimethylphosphonium group (**4**) had no effect on antifungal activity. Activity was retained upon introduction of a *trans*-alkene into the alkyl chain (**5**), but compound **6** which contains a slightly longer alkyl chain (22 carbon atoms) incorporating a *cis*-alkene showed reduced antifungal activity. The introduction of amide and ester bonds at a site six carbons removed from the phosphocholine headgroup (compounds **10**–**14**) completely abolished antifungal activity. This was not improved by increasing the bulk of the hydrophobic tail (**12**–**14**). In the AGPC compound series, the glycero-derivative bearing a free hydroxy group at C2 (**7**) was ineffective. However, replacement of this free hydroxy group with the corresponding methyl ether (edelfosine, **8**) resulted in substantial antifungal activity against *C. albicans*, but not *C. neoformans*. Compound **9** in which the C3 ether linkage in edelfosine has been replaced with an amide linkage showed moderate antifungal activity against both *Candida* and *Cryptococcus* species.

**Table 1.** Antifungal potency in vitro of compounds **1**–**14**

| Compound | MIC (μM) | |
|---|---|---|
| | *C. neoformans* (ATCC 90112) | *C. albicans* (ATCC 10231) |
| **1** | 2.75 | 5.5 |
| **2** | 175 | 350 |
| **3** | 1.4 | 1.4 |
| **4** | 2.75 | 2.75 |
| **5** | 2.75 | 2.75 |
| **6** | 22 | 44 |
| **7** | 88 | >350 |
| **8** | >350 | 5.5 |
| **9** | 22 | 5.5 |
| **10** | 175 | >350 |
| **11** | >350 | >350 |
| **12** | >350 | >350 |
| **13** | >350 | >350 |
| **14** | >350 | >350 |
| AmB[a] | 0.54 | 0.54 |

[a] Amphotericin B.

## 2.3. Inhibition of fungal PLB

Selected compounds were assayed against fungal PLB1, to investigate whether inhibition of this enzyme is a mode of action for this class of compounds. The inhibition assays against the secreted cryptococcal activities of a crude supernatant were performed at pH 4, under conditions previously optimised to measure PLB, LPL and LPTA activities against the secreted form of the PLB1 multifunctional enzyme.[15] Compounds were initially tested at concentrations of 250, 25 and 2.5 μM (Fig. 4). None strongly inhibited the PLB activity of the enzyme at any concentration, but compounds **1**, **3** and**4** significantly inhibited the LPL/LPTA activities at 250 μM. None of these compounds inhibited the LPL activity of the enzyme at 25 μM and only miltefosine (**1**) retained significant inhibition of LPTA activity at 25 μM. Notably, the compounds that showed some LPL/LPTA activity exhibited the strongest antifungal activity, whilst compounds **2** and **11**, which were not antifungal, did not inhibit PLB1 activity. However, since even the most active compounds are not strong inhibitors of PLB1, it is unlikely that this is their primary biochemical target.

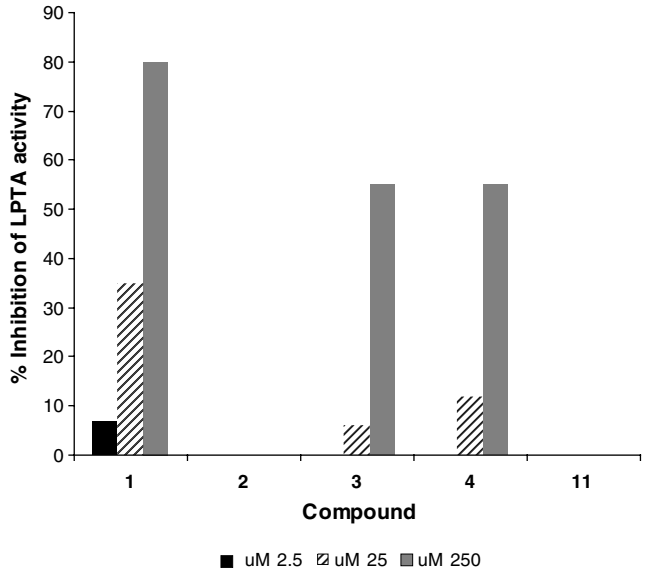



**Figure 4.** Inhibition of secreted cryptococcal PLB1 (LPTA) activity by selected compounds.

## 2.4. Haemolytic assays

To investigate whether changes in the alkyl chains would reduce toxicity, the haemolytic activity of the compounds against human erythrocytes was determined (Table 2). APCs bearing ester or amide bonds in the alkyl chain (**10**–**12**, **14**) did not lyse erythrocytes at any concentration, nor did compound **2** in which the alkyl chain was reduced to 12 carbon atoms in length. All other compounds exhibited haemolytic activity similar to or greater than that of miltefosine, with the C18-phosphocholine analogue (**3**) exhibiting the highest haemolytic activity of the compounds tested. Notably, compound **6** containing a *cis*-alkene had lower haemolytic activity than compound **5**, containing a *trans*-alkene, even though **6** contains a slightly longer alkyl chain (22 carbon atoms in **6** compared to 18 carbon atoms in **5**), indicating that the introduction of *cis*-alkenes is a useful feature for reduction of haemolytic activity. The AGPC compounds (**7**–**9**) with polar groups close to the PC head group showed haemolytic activity greater than that of miltefosine, while moving the polar group further along the alkyl chain (compounds **10**–**14**) significantly reduced haemolytic activity. Previous reports have indicated that the incorporation of ester linkages can reduce haemolytic activity, provided the terminal alkyl chain length is relatively short.[9]

**Table 2.** Haemolytic activity of selected compounds

| Compound | Percentage lysis of human red blood cells as a function of compound concentration (%) | | | | | |
|---|---|---|---|---|---|---|
| | 350 μM | 175 μM | 88 μM | 44 μM | 17.5 μM | 3.5 μM |
| **1** | 100 | 95 | 25 | 5 | 0 | 0 |
| **2** | nd[a] | 0 | 0 | 0 | 0 | 0 |
| **3** | 100 | 100 | 92 | 60 | 0 | 0 |
| **4** | nd[a] | 100 | 97 | 40 | 2 | 0 |
| **5** | 100 | 100 | 50 | 0 | 0 | 0 |
| **6** | 100 | 90 | 15 | 0 | 0 | 0 |
| **7** | 100 | 100 | 86 | 10 | 0 | 0 |
| **8** | 100 | 100 | 83 | 13 | 0 | 0 |
| **9** | 100 | 100 | 56 | 5 | 0 | 0 |
| **10** | nd[a] | 0 | 0 | 0 | 0 | 0 |
| **11** | nd[a] | 0 | 0 | 0 | 0 | 0 |
| **12** | nd[a] | 0 | 0 | 0 | 0 | 0 |
| **14** | 0 | 0 | 0 | 0 | 0 | 0 |

[a] Not determined.

## 2.5. Antibacterial activity

Since APCs and AGPCs are broadly active against both protozoa and fungi, we investigated whether these compounds also have antibacterial activity. Selected compounds were assayed by a standardised serial dilution sensitivity test against reference strains of *Escherichia coli* (as an example of gram-negative bacteria) and gram-positive bacteria *Staphylococcus aureus*, methicillin resistant *S. aureus* (MRSA) and vancomycin resistant *Enterococcus* (VRE) (Table 3). None of the compounds were active against *E. coli*. Compounds **11** and **12**, bearing amide linkages in the alkyl chains, had no antibacterial effect. Compounds **1**, **3**, **5** and **7**–**9** exhibited moderate activity against *S. aureus* and MRSA, with the activity of all compounds against the MRSA strain being better than that against the reference methicillin susceptible strain of *S. aureus*. Compounds **5**–**9** showed moderate activity against VRE. Surprisingly, compound **6** exhibited moderate activity against MRSA and VRE but none against the reference strain of *S. aureus*.

## 3. Conclusions

The broad spectrum antifungal activity of miltefosine has been previously reported.[6] We have now shown that a range of APCs and AGPCs exhibit significant antifungal activity. In this study, we focussed on modifying the alkyl tail of miltefosine in an attempt to reduce the haemolytic activity of this class of compounds. Our preliminary structure–activity relationship (SAR) data show that a hydrophobic chain, at least 16 carbon atoms long, is crucial if antifungal activity is to be maintained. Increasing the length of the alkyl chain provided a slight improvement in antifungal activity, but also increased haemolytic activity. Insertion of ester and amide bonds in the middle of this chain significantly decreased the haemolytic activity of these compounds but abolished antifungal activity. However, there is some scope for modification of the chain to improve haemolytic properties through the introduction of multiple bonds. A recent report indicating that 16-mercaptomiltefosine maintains antileishmanial activity[12] suggests that the introduction of modifications at the terminus of the alkyl chain might also be possible and compounds of this type are under investigation. Notably, the structurally more complex AGPCs appear to be less active against fungi than the APC derivatives but the two compound classes have similar antibacterial properties. An investigation of the effect of modifying the PC headgroup on antifungal SAR is currently underway.

**Table 3.** Antibacterial activity in vitro of selected compounds

| Compound | MIC (μM) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **1**[a] | **3** | **5** | **6** | **7** | **8** | **9** | **11** | **12** |
| *E. coli* (ATCC 25922)[b] | >350 | >350 | >350 | >350 | >350 | >350 | >350 | >350 | >350 |
| *S. aureus* (ATCC 25923)[c] | 44 | 22 | 22 | >350 | 22 | 44 | 44 | >350 | >350 |
| MRSA[d] | 22 | 22 | 22 | 22 | 11 | 11 | 11 | >350 | >350 |
| VRE (ATCC 51299)[e] | 44 | nd[f] | 22 | 11 | 11 | nd[f] | 22 | >350 | >350 |

[a] Miltefosine **(1)** was also tested against *Streptococcus pneumonia* (ATCC 46919) (MIC 11 μM) and *Pseudomonas aeruginosa* (ATCC 27853) (MIC >600 μM).
[b] Amoxicillin MIC 22 μM.
[c] Amoxicillin MIC 0.7 μM.
[d] Vancomycin MIC 0.7 μM.
[e] No positive control available.
[f] Not determined.

We have found that several APCs and AGPCs exhibit moderate antibacterial activity against a range of gram-positive bacteria, including the clinically problematic pathogens, MRSA and VRE. Notably, the compounds with the strongest antifungal activity also exhibited the strongest antibacterial activity. The mode(s) of action of these compounds is unclear. Inhibition of the LPTA activity of fungal PLB1 may contribute to the mode of antifungal action, but is unlikely to be the major mechanism operating, since 50% inhibition of enzyme activity required compound concentrations of >25 μM, while corresponding MICs ranged from 1.4 to 5.5 μM. Effects of APCs and AGPCs on alternative biochemical pathways have been proposed in tumour cells and leishmania, including inhibition of sphingomyelin synthesis,[15] inhibition of phosphatidylcholine synthesis[16] or transport[17] and reduction of choline uptake.[18] From the evidence available it is clear that APCs and AGPCs interfere with lipid and membrane homeostasis in a number of cell types and via a number of modes of action. In the present investigation, it is noteworthy that compounds which do not interfere with the cell membranes of erythrocytes have no antimicrobial activity, while compounds capable of causing haemolysis display significant antimicrobial activity, providing further evidence that biologically active APCs and AGPCs influence membrane physiology. Investigations into the mode(s) of action of these compounds in fungi are ongoing.

In conclusion, this first structure–activity study indicates that APCs and AGPCs have significant promise as new orally available antifungal and antibacterial therapeutics. We have elucidated some of the structural features required to maintain antifungal activity and shown that the haemolytic activity of this class of compounds can be substantially decreased by introducing structural modifications into the alkyl chain.

## 4. Experimental

### 4.1. Chemistry

Melting points were determined using a Gallenkamp melting point apparatus and are reported in degree Celsius (uncorrected). $^{1}H$ and $^{13}C$ NMR spectra were recorded on a Bruker Avance DPX 200 spectrometer. The solvent $^{1}H$ and $^{13}C$ signals, $\delta_H$ 3.31 and $\delta_C$ 49.0 for $d_4$-MeOH, were used as internal references. $^{31}P$ NMR spectra were recorded on a Bruker Avance DPX 400 spectrometer using trimethylphosphite ($\delta_P$ 140.9 ppm) as a reference. Infrared absorption spectra were obtained using a Shimadzu FTIR—8400S spectrometer as a thin film between sodium chloride plates. 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. Compound **15** was prepared from 6-(*O*-phosphorylcholine)hydroxyhexanoic acid[19] according to the method of Spande.[14] Compounds **1**, **7** and **8** were purchased from Cayman Chemicals; **2** was purchased from Avanti Polar Lipids; **3** and **4** were purchased from Bachem AG; **5** and **6** were purchased from AG Scientific and **9** was purchased from Sigma.

**4.1.1. Nonyl 6-(*O*-phosphorylcholine)hydroxyhexanoate (10).** A solution of **15** (0.140 g, 0.33 mmol) in anhydrous acetonitrile (4 mL) was treated with nonanol (2.850 g, 1.98 mmol) and triethylamine (0.066 g, 0.66 mmol), then the mixture was heated at reflux for 24 h under an atmosphere of nitrogen. The solvent was removed under reduced pressure and the residue was purified by chromatography in methanol on Sephadex LH-20 to give **10** (0.007 g, 5%) as a yellow oil. $^{1}H$ NMR (200 MHz, $CD_3CN$) $\delta$ 4.27 (m, 2H), 4.09 (t, $J$ = 6.5 Hz, 2H), 3.83 (m, 2H), 3.65 (m, 2H), 3.23 (s, 9H), 2.36 (t, $J$ = 7.2 Hz, 2H), 1.95 (m, 2H), 1.66–1.59 (m, 6H), 1.49–1.34 (m, 12H) 0.94 (m, 3H); $^{13}C$ NMR (50 MHz, $CD_3OD$) $\delta$ 175.4, 68.3, 66.8, 65.9, 60.7, 55.9, 35.7, 33.5, 32.2, 31.2, 30.9, 30.4, 27.6, 27.1, 26.5, 24.3, 15.3, one signal obscured or overlapping; $^{31}P$ NMR (162 MHz) $\delta$ 1.1 (s). IR $\upsilon_{max}$/$cm^{-1}$ 3346, 1735, 1576, 1232, 1087, 1062, 970; *m*/*z* 446 [$(M+Na)^+$, 100%], 424 [$(M+H)^+$, 30]; HRMS *m*/*z* calcd for $C_{20}H_{42}O_6NPNa$ $(M+Na)^+$: 446.2642, found: 446.2639.

**4.1.2. *N,N,N*-Trimethyl-2-{[(nonylamino-6′-oxohexyl)oxy]-hydroxyphosphinyloxy}ethanaminium inner salt (11).** A solution of **15** (0.164 g, 0.39 mmol) in anhydrous acetonitrile (2 mL) was treated with nonylamine (0.143 g, 0.58 mmol)

under an atmosphere of nitrogen. The resulting mixture was stirred at room temperature for 48 h. The solvent was removed under reduced pressure and the residue was subjected to column chromatography (6:4:1:0.2 chloroform/methanol/water/acetic acid). Concentration of the appropriate fractions ($R_f$ 0.65) gave a yellow oil which was azeotroped with toluene (5× 20 mL) to give **11** (0.148 g, 90%). $^1$H NMR (200 MHz, $CD_3OD$) $\delta$ 4.25 (m, 2H), 3.88 (q, $J$ = 6.5 Hz, 2H), 3.62 (m, 2H), 3.20 (s, 9H), 3.13 (t, $J$ = 6.9 Hz, 2H), 2.17 (t, $J$ = 7.2 Hz, 2H), 1.65 (m, 4H), 1.46 (m, 4H), 1.29 (s, 12H), 0.89 (t, $J$ = 6.3 Hz, 3H), NH not observed; $^{13}$C NMR (50 MHz, $CD_3OD$) $\delta$ 175.2, 66.7, 65.8, 59.4, 53.9, 39.6, 36.2, 32.2, 30.7, 30.6, 29.8, 29.6, 27.2, 25.9, 25.7, 22.9, 13.6, one signal obscured or overlapping; $^{31}$P NMR (162 MHz) $\delta$ 1.1 (s); IR $\upsilon_{max}$/cm$^{-1}$ 3299, 2926, 2854, 1643, 1569, 1234, 1088, 1065, 970, 750; *m/z* 867 [(2M+Na)$^+$, 100 %], 445 [(M+Na)$^+$, 80]; HRMS *m/z* calcd for $C_{20}H_{43}O_5N_2PNa$ (M+Na)$^+$: 445.2802, found: 445.2805.

**4.1.3. *N,N,N*-Trimethyl-2-{[(2″-ethylhexyl-6′-oxohexyl)oxy]-hydroxyphosphinyloxy}ethanaminium inner salt (12).** A solution of **15** (0.146 g, 0.35 mmol) in anhydrous acetonitrile (3 mL) was treated with 2-ethylhexylamine (0.054 g, 0.42 mmol) under an atmosphere of nitrogen. The mixture was stirred at room temperature for 48 h. The solvent was removed under reduced pressure and the residue was subjected to column chromatography (6:4:1:0.2 chloroform/methanol/water/acetic acid). Concentration of the appropriate fractions ($R_f$ 0.60) followed by azeotroping with toluene (5× 20 mL) to remove excess water and acetic acid gave **12** (0.079 g, 55%) as a yellow oil. $^1$H NMR (200 MHz, $CD_3OD$) $\delta$ 4.30 (m, 2H), 3.91 (q, $J$ = 6.5 Hz, 2H), 3.69 (m, 2H), 3.27 (s, 9H), 3.13 (d, $J$ = 6.1 Hz, 2H), 2.27 (t, $J$ = 7.2 Hz, 2H), 2.20 (m, 1H), 1.68 (m, 4H), 1.48 (m, 10H) 0.95 (m, 6H), NH not observed; $^{13}$C NMR (50 MHz, $CD_3OD$) $\delta$ 176.5, 66.6, 65.9, 59.6, 53.9, 42.6, 39.8, 36.2, 31.2, 30.6, 29.2, 26.0, 25.7, 24.4, 23.3, 13.6, 10.4; $^{31}$P NMR (162 MHz) $\delta$ 0.8 (s); IR $\upsilon_{max}$/cm$^{-1}$ 3336, 2958, 2931, 1701, 1550, 1415, 1206, 1089, 970; *m/z* 839 [(2M+Na)$^+$, 25%], 431 [(M+Na)$^+$, 100]; HRMS *m/z* calcd for $C_{19}H_{41}O_5N_2PNa$ (M+Na)$^+$: 431.2646, found: 431.2636.

**4.1.4. *N,N,N*-Trimethyl-2-{[(adamantylamino-6′-oxohexyl)oxy]-hydroxyphosphinyloxy}-ethanaminium inner salt (13).** A solution of **15** (0.140 g, 0.33 mmol) in a mixture of 1:1 anhydrous acetonitrile/DMF (3 mL) was treated with 1-adamantanamine (0.061 g, 0.40 mmol) under an atmosphere of nitrogen. The mixture was stirred at 40 °C for 48 h. The solvent was removed under reduced pressure and the residue was subjected to column chromatography (6:4:1:0.2 chloroform/methanol/water/acetic acid). Concentration of the appropriate fractions ($R_f$ 0.60) followed by azeotroping with toluene (5× 20 mL) to remove excess water and acetic acid gave **13** (0.047 g, 34%) as a yellow oil. $^1$H NMR (200 MHz, $CD_3OD$) $\delta$ 4.30 (m, 2H), 3.91 (q, $J$ = 6.5 Hz, 2H), 3.69 (m, 2H), 3.27 (s, 9H), 2.27 (m, 2H), 2.19–1.75 (m, 18H), 1.64–1.09 (m, 3H), NH not observed; $^{31}$P NMR (162 MHz) $\delta$ 1.1 (s); IR $\upsilon_{max}$/cm$^{-1}$ 3423, 3284, 2911, 1639, 1554, 1412, 1234, 1091,1018; *m/z* 883 [(2M+Na)$^+$, 25%], 453 [(M+Na)$^+$, 100], 431 [(M+H)$^+$, 25], 324 (55), 152 (75); HRMS *m/z* calcd for $C_{21}H_{39}O_5N_2PNa$ (M+Na)$^+$: 453.2489, found: 453.2506.

**4.1.5. *N,N,N*-Trimethyl-2-{[((1″-(*S*)-isopropylacetamido)-amino-6′-oxohexyl)oxy]-hydroxyphosphinyloxy}ethanaminium inner salt (14).** A solution of **15** (0.145 g, 0.35 mmol) in DMF (3 mL) was treated with valinamide (0.067 g, 0.42 mmol) and triethylamine (0.035 g, 0.35 mmol) under an atmosphere of nitrogen. The mixture was stirred for 48 h at 40 °C. The solvent was removed under reduced pressure and the residue was purified by chromatography on Sephadex LH-20 (methanol elution) to give **14** (0.028 g, 19%) as a yellow oil. $^1$H NMR (200 MHz, $CD_3OD$) $\delta$ 4.29 (m, 2H), 4.20 (d, $J$ = 7.0 Hz, 1H), 3.92 (q, $J$ = 6.5 Hz, 2H), 3.67 (m, 2H), 3.26 (s, 9H), 2.31 (t, $J$ = 7.2 Hz, 2H), 2.20 (m, 1H), 1.78–1.48 (m, 6H), 1.02 (d, $J$ = 6.7 Hz, 3H), 0.99 (d, $J$ = 6.7 Hz, 3H), NH and $NH_2$ not observed; $^{13}$C NMR (50 MHz, $CD_3OD$) $\delta$ 175.7, 175.2, 66.7, 65.8, 59.5, 59.1, 53.9, 35.9, 30.6, 25.7, 18.9, 17.7, one signal obscured or overlapping; IR $\upsilon_{max}$/cm$^{-1}$ 3369, 3303, 2966, 1702, 1228, 1085, 1057, 1012, 970; *m/z* 813 [(2M+Na)$^+$,10%], 418 [(M+Na)$^+$, 100]; HRMS *m/z* calcd for $C_{16}H_{34}O_6N_3PNa$ (M+Na)$^+$: 418.2077, found: 418.2077.

### 4.2. Fungal and bacterial isolates

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. The MRSA reference strain was obtained as a clinical isolate obtained from the microbiology laboratory at Westmead Hospital, Sydney, Australia. All other fungal and bacterial strains were obtained from the American Tissue Culture Collection (ATCC).

### 4.3. 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[20] and stored at −70 °C.

### 4.4. Radiometric assay method for fungal phospholipases

Enzyme activities were measured as described previously[4,20,21] 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,[22] separated by TLC and quantified as previously described.[21] The TLC plates were developed in chloroform/methanol/water (65:25:4; v/v/v).

### 4.5. Protein assays

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

### 4.6. Testing of alkylphosphocholines as inhibitors

Solutions of alkylphosphocholine 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.

### 4.7. 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[23] and filamentous fungi.[24] The minimal inhibitory concentration (MIC) was defined as that which produced no visible growth after 48, or 72 h, of culture at 35 °C.[23,24] All tests were performed in triplicate and results were reproducible between runs.

### 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 min in a Beckman TJ-6 centrifuge. The third supernatant was clear and colourless. Cells were stored in PBS (20 mL) for up to two 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 around $0.5 \times 10^9$ 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 350, 175, 88, 44, 17.5 and 3.5 μM.

### 4.9. Antibacterial susceptibility assays

The antibacterial activity of the compounds was measured by standard microdilution methods of the US National Committee for Clinical Laboratory Standards for bacteria that grow aerobically.[25] The minimal inhibitory concentration (MIC) was defined as that which produced no visible growth after 24 h of culture in Mueller–Hinton broth at 35 °C. All tests were performed in triplicate and results were reproducible between runs.

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