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Synthesis and antifungal activities of miltefosine analogs

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Abstract

Miltefosine is an alkylphosphocholine that shows broad-spectrum *in vitro* antifungal activities and limited *in vivo* efficacy in mouse models of cryptococcosis. To further explore the potential of this class of compounds for the treatment of systemic mycoses, nine analogs (**3a–3i**) were synthesized by modifying the choline structural moiety and the alkyl chain length of miltefosine. *In vitro* testing of these compounds against the opportunistic fungal pathogens *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Aspergillus fumigatus*, and *Cryptococcus neoformans* revealed that *N*-benzyl-*N,N*-dimethyl-2-[[hexadecyloxy]hydroxyphosphinyl]oxy}ethanaminium inner salt (**3a**), *N,N*-dimethyl-*N*-(4-nitrobenzyl)-2-[[hexadecyloxy]hydroxyphosphinyl]oxy}ethanaminium inner salt (**3d**), and *N*-(4-methoxybenzyl)-*N,N*-dimethyl-2-[[hexadecyloxy]hydroxyphosphinyl]oxy}ethanaminium inner salt (**3e**) exhibited minimum inhibitory concentrations (MIC) of 2.5–5.0 µg/mL against all tested pathogens, when compared to miltefosine with MICs of 2.5–3.3 µg/mL. Compound **3a** showed low *in vitro* cytotoxicity against three mammalian cell lines similar to miltefosine. *In vivo* testing of **3a** and miltefosine against *C. albicans* in a mouse model of systemic infection did not demonstrate efficacy. The results of this study indicate that further investigation will be required to determine the potential usefulness of the alkylphosphocholines in the treatment of invasive fungal infections.

Keywords

alkylphosphocholine; miltefosine; antifungal; cryptococcosis; candidiasis

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Supplementary data Experimental procedures and spectroscopic data for compounds **3a–3i**. This material is available free of charge via the Internet.

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Miltefosine (hexadecylphosphocholine) is a synthetic alkylphosphocholine that belongs to the class of phospholipids. It was initially developed as an antineoplastic agent,^{1,2} and was later discovered to possess antileishmanial properties and registered as the first oral drug for the treatment of visceral leishmaniasis in India and Germany, and for the treatment of cutaneous leishmaniasis in Colombia.³ Pharmacokinetic studies indicate that miltefosine has good bioavailability and a long half-life in patients with leishmania (7 days for the first elimination and 31 days for the terminal elimination).⁴ This may be attributable to its improved *in vivo* antileishmanial activity relative to analogs with even more potent *in vitro* activities.³ Miltefosine also possesses antibacterial,⁵ antiprotozoal,⁶ and antiviral activities.⁷

Miltefosine was demonstrated to be active against *Candida albicans* and *Cryptococcus neoformans* in 1999.⁸ In recent years, miltefosine was found to exhibit broad-spectrum antifungal activities against clinically important fungal pathogens⁹ and dermatophytes¹⁰ in addition to inhibiting *Candida albicans* biofilm formation and maturation.¹¹ Mechanistic studies indicated that miltefosine inhibits cytochrome c oxidase in the model organism *Saccharomyces cerevisiae* and phospholipase B in the fungal pathogen *C. neoformans*,^{9,12} while in human cells it inhibits activation of the protein kinase B pathway as well as phosphatidylcholine synthesis.¹³ However, none of these targets is essential for the survival of fungal cells according to what is known in *S. cerevisiae*. Therefore, miltefosine and analogs remain to be an intriguing class of compounds in terms of their precise antifungal target.

Miltefosine gained particular interest in antifungal therapy due to the reported *in vivo* efficacy in a mouse model of cryptococcosis.⁹ In a more recent study aimed at further evaluating its *in vivo* efficacy in mouse models of cryptococcal meningoencephalitis and disseminated cryptococcosis, miltefosine demonstrated limited effects in mice that were challenged with a low infecting inoculum.¹⁴ Meningoencephalitis requires the drug to cross the blood brain barrier to exert its action. Given that miltefosine has a higher distribution in the lung and kidney of mice than in brain,¹⁵ we hypothesized that it might be more active *in vivo* against systemic mycoses rather than infections in the brain. With chemical synthesis, new analogs could be prepared and included for testing this hypothesis. Therefore, we designed and synthesized several new miltefosine analogs and evaluated their antifungal activities *in vitro* and *in vivo* in a candidiasis mouse model.

The available structure–antifungal activity relationship (SAR) information on alkylphosphocholines was the basis for designing new compounds in this study. A hydrophobic chain in the miltefosine analogs with 16 to 18 carbon atoms is necessary for antifungal activity.¹⁶ Reduction of the alkyl chain length to 12 carbon atoms,^{16,17} increasing the chain length to 22 carbon atoms,⁸ or insertion of ester/amide functionalities in the middle of this chain¹⁶ significantly reduces the antifungal activity. Structurally more complex alkylglycerophosphocholines exhibit moderate activities against *C. albicans* and *C. neoformans*, when compared with alkylphosphocholine derivatives.¹⁶ Extensive modification of the *N*-substitution and the C2 unit of the choline moiety (head group) resulted in a large number of compounds,¹⁷⁻¹⁹ some of which showed activities more potent than erucylphosphocholine¹⁹ that is 8-fold less potent than miltefosine.¹⁶ Within this class, octadecylphosphocholine demonstrates as much as a 4-fold increase in *in vitro* potency against *C. albicans* when compared to miltefosine.¹⁶ It appears that the intact head group or the presence of at least two small *N*-methyl groups plays a key role for antifungal activity. Based on the above SAR information, we decided to synthesize compounds by slightly modifying the structure of miltefosine (Figure 1).

We first designed compound **3a** with a benzyl group replacing one methyl group of the choline structural moiety in miltefosine, taking into consideration of the strong antimicrobial

activity of benzalkonium chloride that possesses the benzyldimethylammonio structural moiety.²⁰ However, the zwitterion nature of **3a** makes it distinctly different from the cationic surface-acting benzalkonium chloride. While this design allows the compound to retain most of the structural features required for antifungal activity within the class, it also increases lipophilicity due to the introduction of an aromatic ring, as indicated by the calculated octane-water partition coefficient (cLog P) from 1.80 for miltefosine to 3.80 for **3a**,²¹ which may improve antifungal properties. The synthetic method for the preparation of **3a** is an adaption of the reported procedures^{18,22} and is depicted in Scheme 1. Quaternization of *N,N*-dimethylaminoethanol (**1**) with benzyl bromide afforded the quaternary ammonium salt **2**, which was subject to phosphorylation reaction of *n*-hexadecanol with POCl₃ followed by hydrolysis to afford the target compound.²³

As shown in Table 1, *in vitro* antifungal testing by the method described previously²⁴ indicated that compound **3a** showed potent activities with minimum inhibitory concentrations (MICs) ranging from 2.5 to 5.0 $\mu\text{g/mL}$ against the opportunistic fungal pathogens *C. albicans*, *Candida glabrata*, *Candida krusei*, *Aspergillus fumigatus*, and *C. neoformans*. The compound was also fungicidal against all tested fungal pathogens with minimum fungicidal concentrations (MFCs)²⁵ from 2.5 to 15.0 $\mu\text{g/mL}$. Its antifungal potency is similar to that of miltefosine with MICs and MFCs of 2.1–3.3 and 2.1–9.2 g/mL , respectively, against the aforementioned pathogens.

To investigate the influence of the chain length to the antifungal activity within this series, analogs **3b** and **3c** with the same head group but an alkyl chain length of C14 and C18, respectively, were prepared by a synthetic method similar for **3a**. However, compound **3b** showed decreased activity against *C. albicans*, *C. glabrata*, *C. krusei*, and *A. fumigatus* in terms of MICs and MFCs when compared with **3a** (Table 1), and compound **3c** was only active against *C. glabrata* with an MIC/MFC of 4.2/4.2 $\mu\text{g/mL}$, indicating that C16 is an optimal alkyl chain length.

Keeping a constant C16 alkyl chain, we next synthesized six analogs (**3d–3i**) with different head groups by replacing one methyl group of the choline moiety in miltefosine with *p*-nitrobenzyl, *p*-chlorobenzyl, *p*-bromobenzyl, *p*-methoxybenzyl, cinnamyl, and allyl groups. Among these, compound **3d** with an *N*-4-nitrobenzyl substitution produced the best *in vitro* activity profiles, exhibiting slightly improved potency against *C. glabrata* and *A. fumigatus* when compared to miltefosine (Table 1). Compound **3e** with an *N*-4-methoxybenzyl substitution also showed good activities similar to **3a** and **3d** (Table 1). Compounds **3f** and **3g** with a halogen-substituted aromatic ring were only active against *C. glabrata* and *A. fumigatus*, and **3i** with an *N*-allylic substitution, the only compound without an aromatic ring in this series, was only active against *C. glabrata* with an MIC/MFC of 16.6/16.6 $\mu\text{g/mL}$. It appears that among the five tested fungal species, *C. glabrata* is most susceptible to this series of compounds. Evidently, the minor structural differences of these compounds, especially for compounds **3a** and **3d–3g**, have a significant effect on their activity profiles. In addition, the permeability of the compounds towards different fungal cells, which may be associated with their lipophilicities, may play a role in the observed activities. Coincidentally, the three compounds **3a**, **3d**, and **3e** with close chemical structures that showed excellent activity profiles have similar calculated Log P values ranging from 3.69 to 3.80 (Scheme 1).

The *in vitro* antifungal activity data of miltefosine obtained in this study (Table 1) are similar to those reported in the literature.⁹ The potent activities of the three synthetic analogs (**3a**, **3d**, and **3e**) are further evident by comparison with the ‘gold standard’ clinical drug amphotericin B. Compounds **3a** and **3d** that showed strong activity against *C. albicans* with

the same MIC/MFC of 2.5/2.5 $\mu\text{g}/\text{mL}$ are equally potent as miltefosine, and are about 2- to 3-fold less potent than amphotericin B which has an MIC/MFC of 0.9/1.0 $\mu\text{g}/\text{mL}$ (Table 1).

The *in vitro* cytotoxicity testing of compounds **3a–3i** and miltefosine were performed by the method described previously²⁶ against HepG2 (human hepatic carcinoma), Vero (African green monkey kidney fibroblast) and LLC-PK₁₁ (pig kidney epithelial) cells in comparison with the cytotoxic drug doxorubicin. These compounds were not cytotoxic against Vero cells up to the highest tested concentration of 25 $\mu\text{g}/\text{mL}$. However, they showed cytotoxicity towards HepG2 cells with IC₅₀ values in the range of 11.5 to >25 $\mu\text{g}/\text{mL}$ and LLC-PK₁₁ cells with IC₅₀ values ranging from 1.6 to 10.8 $\mu\text{g}/\text{mL}$ (Table 1). It is important to note that the *in vitro* antifungal activity of these compounds was not correlated with the *in vitro* cytotoxicity. For example, the antifungal activity of compounds **3c** and **3h** was less potent than compound **3a** but they were more cytotoxic than **3a** (Table 1). Among the three potent antifungal compounds (**3a**, **3d**, and **3e**), **3a** was least cytotoxic, which was similar to miltefosine. Therefore, compound **3a** was selected, along with miltefosine, for *in vivo* efficacy studies in a mouse model of candidiasis.

Taking advantage of the reported dosing values of miltefosine used in the mouse model of cryptococcosis (1.8 to 45 mg/kg orally)^{9,14} and the 50% effective dose of miltefosine produced in the mouse model of leishmaniasis (7.72 mg/kg intraperitoneally),³ the mice were treated with **3a** and miltefosine at 5 and 10 mg/kg via intraperitoneal administration at 4, 24, 48, 72, and 96 hours post inoculation with 1×10^6 cells of *C. albicans* SC5314 via tail-vein injection. The results showed that all compound-treated mice died on or before day 12, which is similar to the control mice treated with phosphate buffered saline (PBS) (Figure 2A). In a separate experiment with reduced dosing for **3a** at 1 and 5 mg/kg and miltefosine at 5 mg/kg via the same treatment, the mice died on or before day 8, same as the control group treated with PBS. Oral administration of compound **3a** at 5 and 25 mg/kg also did not enhance the survival rate of mice when compared with the control mice treated with PBS (Figure 2B). This indicated that miltefosine and compound **3a** did not exhibit *in vivo* efficacy against *C. albicans* in a mouse model of systemic infection.

Since miltefosine as an antileishmanial drug has proven favorable pharmacokinetic profiles in mice¹⁵ and human,⁴ the failure of demonstrating *in vivo* antifungal efficacy in our candidiasis mouse model cannot be attributed to its chemical instability within bloodstream and infection sites. The previous study by Wiederhold et al¹⁴ suggested that protein binding could be associated with the ineffectiveness of miltefosine in the mouse model of disseminated cryptococcosis. While the reasons for the lack of the *in vivo* antifungal activity of miltefosine and analog **3a** may be worth investigating, we should not exclude the possibility that chemical optimization may afford novel miltefosine-based derivatives that are highly selective and permeable to fungal cells and ultimately exert *in vivo* efficacy. Another potential therapeutic area for this class of compounds is the treatment of fungal biofilm-related infections on medical devices such as catheters, given that miltefosine can inhibit *C. albicans* biofilm formation and maturation.¹¹ Thus, further work will be required to determine the potential usefulness of miltefosine and its analogs in the treatment of invasive or device-related fungal infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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23. Spectroscopic data for *N*-benzyl-*N,N*-dimethyl-2-[[hexadecyloxy]hydroxyphosphinyl]oxy}-ethanaminium inner salt (**3a**): ¹H NMR (400 MHz, CD₃OD) δ 7.59 (d, 2H, *J* = 7.2 Hz, aromatic H), 7.51 (m, 3H, aromatic H), 4.62 (br s, 2H, *N*-CH₂-Ph), 4.30 (br s, 2H, *N*-CH₂-CH₂-O), 3.85 (q, 2H, *J* = 6.4 Hz, -OCH₂-), 3.62 (m, 2H, *N*-CH₂-CH₂-O), 3.11 (s, 6H, 2×*N*-CH₃), 1.57 (m, 2H, -CH₂), 1.39–1.20 (m, 26H, 13×CH₂), 0.86 (t, 3H, *J* = 6.7 Hz, CH₃) (the assignment of the ¹H NMR resonances was facilitated by 2D NMR experiments of HMQC and HMBC); ¹³C NMR (100 MHz, CD₃OD) δ 134.4 (2C), 131.8, 130.2 (2C), 128.8, 70.2, 66.9 (d), 65.3 (m), 60.1 (d), 51.2 (t, 2C), 33.1, 31.9 (d), 30.8 (6C), 30.8 (2C), 30.5 (2C), 26.9, 23.7, 14.6; HRESIMS *m/z* 482.3395 (calcd for [C₂₇H₅₀NO₄P-H]⁺, 482.3405).
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25. Minimum fungicidal concentration (MFC) was determined by removing 5 μL from each clear well, transferring to agar, and incubating at 35°C for 48 h for *Candida* spp. and *Aspergillus fumigatus* and 72 h for *Cryptococcus neoformans*. The MFC was defined as the lowest test concentration that allows no growth of the organism on agar.
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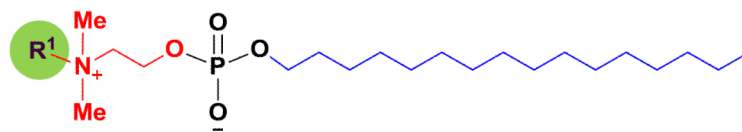


Figure 1.
Miltefosine (R¹ = Me) based synthetic template.

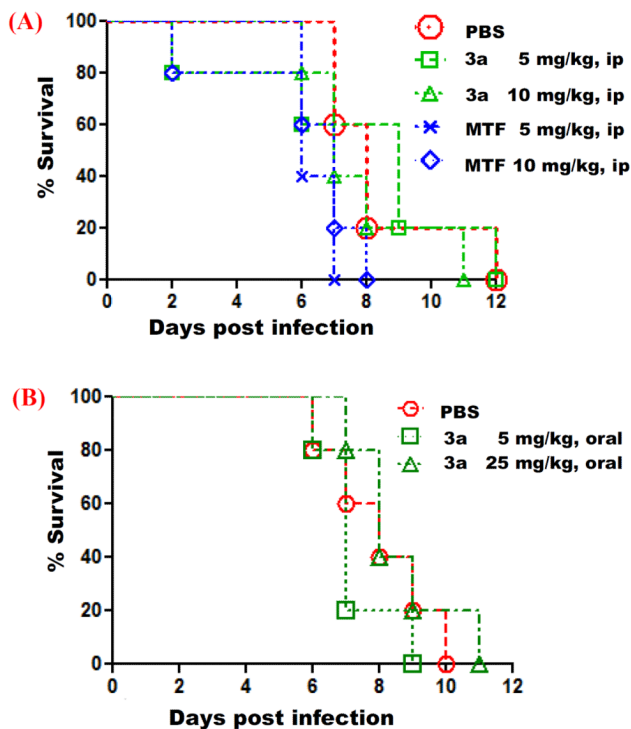
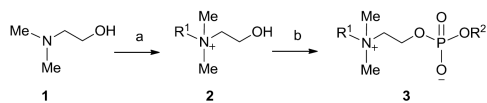


Figure 2.

In vivo antifungal efficacy of compound **3a** and miltefosine (MTF) against *Candida albicans* infection. Five male CD1 mice (6-7 weeks old) per group were infected with 1×10^6 cells of *C. albicans* SC5314, followed by treatment with phosphate buffered saline (PBS) or compound by intraperitoneal injection (panel A) and by oral route (panel B) at 4, 24, 48, 72, and 96 hours post inoculation. Survival of the animals was monitored for 12 days. For the intraperitoneal administration experiment, *P* values evaluated by a Log-rank test are as follows: 0.8758 for PBS vs **3a** 5 mg/kg, 0.0179 for PBS vs MTF 5 mg/kg, 0.1021 for PBS vs MTF 10 mg/kg, and 0.1822 for **3a** 5 mg/kg vs MTF 5 mg/kg.



	R ¹	R ²	c LogP ^a
3a		<i>n</i> -C ₁₆ H ₃₃	3.80
3b		<i>n</i> -C ₁₄ H ₂₉	2.97
3c		<i>n</i> -C ₁₈ H ₃₇	4.49
3d		<i>n</i> -C ₁₆ H ₃₃	3.69
3e		<i>n</i> -C ₁₆ H ₃₃	3.77
3f		<i>n</i> -C ₁₆ H ₃₃	4.29
3g		<i>n</i> -C ₁₆ H ₃₃	4.39
3h		<i>n</i> -C ₁₆ H ₃₃	4.21
3i		<i>n</i> -C ₁₆ H ₃₃	2.38

^a calculated by ACD/Structure Designer Suite 12.0 – ADME Suite 5.0.

Scheme 1.

Reagents and conditions: (a) R₁X, CH₃CN, room temperature, 1-3 h; (b) (1) R₂OH, POCl₃, Et₃N, CHCl₃, 0°C, room temperature, 2 h; (2) pyridine, **2a-2i**, 0°C, room temperature, 12 h; (3) H₂O, room temperature, 1 h.

Table 1

In vitro antifungal activity and cytotoxicity of miltefosine and compounds **3a-3i**^a

	Antifungal activity (MIC/MFC, $\mu\text{g/mL}$) ^b					Cytotoxicity (IC ₅₀ , $\mu\text{g/mL}$) ^c		
	<i>C. albicans</i> ATCC 90028	<i>C. glabrata</i> ATCC 90030	<i>C. krusei</i> ATCC 6258	<i>A. fumigatus</i> ATCC 90906	<i>C. neoformans</i> ATCC 90113	Vero	HepG2	LLC-PK ₁₁
Miltefosine	2.5/2.5	3.3/4.2	2.5/3.3	2.9/9.2	2.1/2.1	>25	>25	2.7
3a	2.5/2.5	3.3/3.3	5.0/15.0	2.5/2.5	3.3/3.3	>25	>25	4.8
3b	6.7/8.3	8.3/8.3	10.0/10.0	10.0/–	3.3/3.3	>25	>25	10.8
3c	–/– ^d	4.2/4.2	–/–	–/–	–/–	>25	13	4.7
3d	2.5/2.5	2.5/2.5	2.5/3.3	2.5/7.5	2.5/2.5	>25	11.5	1.6
3e	4.2/4.2	2.5/3.3	2.0/3.3	2.5/7.5	4.2/4.2	>25	12.9	1.9
3f	–/–	11.2/11.2	–/–	12.5/12.5	–/–	>25	21	3.2
3g	–/–	10.8/10.8	–/–	20.0/20.0	–/–	>25	21.3	5.5
3h	–/–	2.5/3.3	3.3/3.3	2.5/5.0	–/–	>25	19.6	2.1
3i	–/–	16.6/16.6	–/–	–/–	–/–	>25	13.2	4.1
Amphotericin B	0.9/1.0	1.3/1.5	1.7/1.7	2.5/5.0	0.5/0.5			
Doxorubicin						>5	0.9	0.7

^aBoth antifungal and cytotoxicity data are mean values based on three independent experiments except for the antifungal data of compound **3f** with mean values from two independent experiments.

^bMIC: minimum inhibitory concentration (lowest concentration that allows no detectable growth). MFC: minimum fungicidal concentration (the lowest concentration that kills the fungus), which was determined by removing 5 μL from each assay well with no visible growth, transferring to fresh media and incubating at the appropriate temperature for 2-3 days. The highest test concentration for compounds **3a-3i** and miltefosine was 20 $\mu\text{g/mL}$; the highest test concentration for amphotericin B was 5 $\mu\text{g/mL}$.

^cIC₅₀: 50% growth inhibition. The highest test concentration for compounds **3a-3i** and miltefosine was 25 $\mu\text{g/mL}$; the highest test concentration for doxorubicin was 5 $\mu\text{g/mL}$.

^dNot active at 20 $\mu\text{g/mL}$.