Bioorganic & Medicinal Chemistry Letters 23 (2013) 4828–4831

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis and antifungal activities of miltefosine analogs

Ranga Rao Ravu [a], Ying-Lien Chen [b], Melissa R. Jacob [a], Xuewen Pan [c], Ameeta K. Agarwal [a], Shabana I. Khan [a,d], Joseph Heitman [b], Alice M. Clark [a,d], Xing-Cong Li [a,d,*]

[a] National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences, The University of Mississippi, MS 38677, USA
[b] Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC, USA
[c] Verna and Marrs McLean Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, TX, USA
[d] Department of Pharmacognosy, School of Pharmacy, The University of Mississippi, MS 38677, USA

ARTICLE INFO

Article history:
Received 24 May 2013
Revised 19 June 2013
Accepted 27 June 2013
Available online 6 July 2013

Keywords:
Alkylphosphocholine
Miltefosine
Antifungal
Cryptococcosis
Candidiasis

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.

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 pathogens[9] and dermatophytes[10] in addition to inhibiting *C. 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

* Corresponding author. Address: National Center for Natural Products Research, School of Pharmacy, The University of Mississippi, University, MS 38677, USA. Tel.: +1 662 915 6742; fax: +1 662 915 7989.
E-mail address: xcli7@olemiss.edu (X.-C. Li).

0960-894X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmcl.2013.06.096

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 16–18 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 chain[16] 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 erucylphosphocholine[19] that is eight-fold less potent than miltefosine.[16] Within this class, octadecylphosphocholine demonstrates as much as a four-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 (Fig. 1).

We first designed compound **3a** with a benzyl group replacing one methyl group of the choline structural moiety in miltefosine, taking into consideration 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 (c log *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 $POCl_3$ followed by hydrolysis to afford the target compound.[23]

As shown in Table 1, in vitro antifungal testing by the method described previously[24] indicated that compound **3a** showed potent activities with minimum inhibitory concentrations (MICs) ranging from 2.5 to 5.0 μg/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 μg/mL. Its antifungal potency is similar to that of miltefosine with MICs and MFCs of 2.1–3.3 and 2.1–9.2 μg/mL, respectively, against the aforementioned pathogens.

To investigate the influence of the chain length on 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

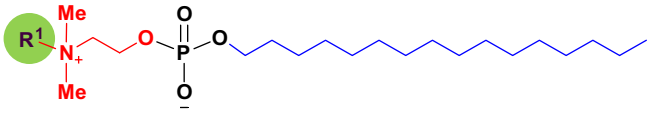
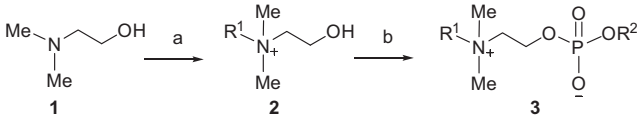
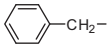
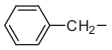
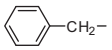
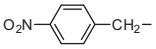
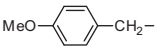
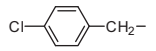
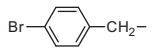
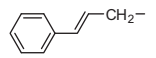
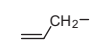


Figure 1. Miltefosine (R^1 = Me) based synthetic template.



	R^1	R^2	c Log*P*
3a		*n*-$C_{16}H_{33}$	3.80
3b		*n*-$C_{14}H_{29}$	2.97
3c		*n*-$C_{18}H_{37}$	4.49
3d		*n*-$C_{16}H_{33}$	3.69
3e		*n*-$C_{16}H_{33}$	3.77
3f		*n*-$C_{16}H_{33}$	4.29
3g		*n*-$C_{16}H_{33}$	4.39
3h		*n*-$C_{16}H_{33}$	4.21
3i		*n*-$C_{16}H_{33}$	2.38

Scheme 1. Reagents and conditions: (a) R_1X, CH_3CN, room temperature, 1–3 h; (b) (1) R_2OH, $POCl_3$, Et_3N, $CHCl_3$, 0 °C, room temperature, 2 h, (2) pyridine, **2a**–**2i**, 0 °C, room temperature, 12 h, (3) H_2O, room temperature, 1 h.

when compared with **3a** (Table 1), and compound **3c** was only active against *C. glabrata* with an MIC/MFC of 4.2/4.2 μg/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 μg/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