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Original article

Efficacy of oleylphosphocholine (OlPC) *in vitro* and in a mouse model of invasive aspergillosis

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Short title: Oleylphosphocholine for invasive aspergillosis

Keywords: oleylphosphocholine; in vitro susceptibility; *Aspergillus fumigatus*; mouse model

Abstract

Background: Invasive aspergillosis has become increasingly common and is characterized by high morbidity and mortality. Upcoming resistance threatens treatment with azoles and highlights the need for novel therapeutics.

Objectives: This explorative laboratory study investigated the *in vitro* and *in vivo* potential of the alkylphospholipid oleylphosphocholine (OlPC) against *Aspergillus*.

Methods: *In vitro* activities of OlPC, miltefosine, posaconazole and voriconazole were determined for *A. fumigatus*, *A. niger*, *A. terreus* and *A. flavus*. *In vivo* efficacy was evaluated in a systemic *A. fumigatus* mouse model, adopting a short-term and long-term oral or intraperitoneal dosing regimen.

Results: OlPC showed potent and selective *in vitro* activity against *A. fumigatus* (IC_{50} = 1.04 µM). Intraperitoneal administration of 50 mg/kg/day OlPC significantly reduced the fungal organ burdens at 4 days post-infection (dpi). Although 5- and 10-day OlPC treatment improved survival, organ burdens were not reduced at 10 and 15 dpi.

Conclusions: While this study showed excellent *in vitro* activity of OlPC against *Aspergillus* spp., its therapeutic efficacy in an acute mouse model for invasive aspergillosis was less convincing. Given the limited therapeutic options in the current antifungal market for invasive infections, it may be useful to evaluate OlPC activity in a less stringent *in vivo* model, including its combination potential with other already marketed antifungal drugs.

1. Introduction

Invasive aspergillosis (IA) is caused by opportunistic *Aspergillus* species and associated with high morbidity and mortality [1, 2]. Due to a rise in immunocompromised patients, such as transplant and intensive care patients, IA has become increasingly common [3-5]. The primary causative agent of human aspergillosis is *A. fumigatus*, followed by *A. flavus*, *A. terreus* and *A. niger* [6]. The current antifungal therapy for IA is associated with limited efficacy, adverse effects and drug-drug interactions [7]. Coinciding with the rise of IA, resistance to itraconazole (ITR), posaconazole (POS) and voriconazole (VOR) emerges and encourages the development of alternative treatment strategies, such as prophylactic and combination therapy, or the discovery of novel antifungals [8-12].

Oleylphosphocholine (OlPC) belongs to the family of alkylphosphocholines and is structurally related to miltefosine (MIL), as they only differ in alkyl chain length and the presence of unsaturated bonds. Both compounds show antileishmanial activity [13] and most likely share a similar mode-of-action, i.e. influence on fungal phospholipase B1 and induction of apoptosis-like cell death by targeting mitochondrial function through cytochrome-C oxidase (subunit COX9) [14, 15]. Laboratory studies on *Leishmania* have indicated that OlPC shows an efficacy profile similar or superior to MIL and has the advantages of better tolerance and oral bioavailability.

The present study explored the antifungal potential of OlPC, since several studies demonstrated the *in vitro* potential of MIL against dermatophytes [16] and filamentous fungi [17]. In addition, MIL showed activity in a mouse model of cryptococcosis [14] and contributed to the successful management of an invasive *Scedosporium prolificans* infection [18]. This study is the first to evaluate the antifungal potential of OlPC in an *in vitro* panel of four *Aspergillus* spp, including *A. fumigatus*, *A. terreus*, *A. niger* and *A. flavus* in comparison to POS, VOR and MIL. A preliminary assessment of the *in vivo* efficacy was performed in an acute mouse model of disseminated *A. fumigatus* infection based on maximal drug exposure, i.e. high daily dose starting on the day of infection including intraperitoneal (IP) administration.

2. Materials and Methods

Fungal isolates The panel of clinical *Aspergillus* isolates was obtained from the Scientific Institute of Public Health (WIV, Brussels, Belgium) and Prof. J. Maertens (University Hospital Leuven, Belgium) and included *A. flavus* (B59000), *A. niger* (J930117), *A. terreus* (IHEM 5918) and *A. fumigatus* (B19119 and AF198-R [azole-resistant]). The isolates were cultivated on Sabouraud dextrose agar (SDA) (Lab M, U.K.) supplemented with 0.5% (w/v) chloramphenicol (Sigma-Aldrich, Diegem, Belgium). Stocks with a concentration of $5x10^6$ colony forming units (cfu)/ml were prepared in RPMI-MOPS with 10% glycerol and 0.01% Tween-80 and frozen in liquid nitrogen. For the *in vitro* experiments, pre-titrated cryopreserved vials were used. Fresh inocula of *A. fumigatus* B19119 were used for the *in vivo* studies.

Antifungal compounds MIL, VOR (Sigma-Aldrich) and POS (Merck, Noxafil® oral formulation 40 mg/ml) were used as reference drugs. Dafra Pharma Research and Development (Turnhout, Belgium) supplied liposomal OlPC (18 mg/ml or 41.5 µM) for *in vitro* and *in vivo* use.

***In vitro* susceptibility testing** The standard microdilution broth assay was performed according to the Clinical Laboratory Standards Institute (CLSI) [19, 20] with some minor adjustments. Plate production and endpoint determination were performed as previously described [21]. Briefly, 96-well U-bottom plates (Greiner Bio-One) were spotted with serially diluted compound solution covering a broad dose-range (64 µM as the highest in-test concentration for MIL, POS and VOR; 41.5 µM for OlPC). The inoculum (10^3 cfu in 200 µl per well) was prepared from pre-titrated cryopreserved stock solutions ($5x10^6$ cfu/ml) in RPMI-MOPS. Inhibition of fungal growth was determined by adding 10 µl of 0.005% (w/v) resazurin (Sigma-Aldrich) and fluorimetric reading (λ_{ex} 550 nm, λ_{em} 590 nm; Tecan Genios) after an additional incubation period, allowing calculation of IC_{50} (µM) values, i.e. the drug concentration that results in 50% growth inhibition compared to the untreated controls on the same plate. To determine the minimal fungicidal concentration (MFC), which is defined as the lowest concentration of antifungal compound at which no growth could be observed, the entire volume of the well was transferred to an SDA Petri dish for incubation. Three independent replicates were analyzed for each compound.

Animals Female Swiss mice weighing between 25 and 30 g (Janvier, France) were housed in groups of five and individually marked. Food pellets (Carfil Quality, Oud-Turnhout, Belgium) and water were available *ad libitum*. Husbandry conditions were 22°C, 60% RH and a 12h dark-light cycle. The animal experiments were approved by the Ethical Committee of the University of Antwerp and conducted in accordance with ethical standards (UA-ECD 2012-029).

***In vivo* efficacy testing** Systemic aspergillosis was established using fresh inocula of *A. fumigatus* B19119. Spores were collected in PBS-Tween-80 (0.01% v/v) and diluted to obtain a concentration of 10^8 cfu/ml. The concentration of the inoculum was confirmed by serial dilution on SDA before and after each experiment. On day 0 of the experiment, groups of 5 mice were intravenously injected with $1x10^7$ cfu in 100 µl and were given either oral (PO) or intraperitoneal (IP) treatment. Based on pilot dose-response and toxicity studies, POS was dosed at 10 mg/kg to obtain 100% survival and significant organ burden reduction, while OlPC could be dosed at 50 mg/kg without any toxic effect. Three different dosing regimens were used: (A) Short-term treatment regimen combined with survival in which the animals were treated daily for 5 days, starting on the day of infection. For the vehicle-treated controls (VIC), necropsies were performed at day 4 post-infection (dpi). At this time point, the VIC-animals were always in severe moribund state and showed maximal liver, spleen and kidney burdens. At 10 dpi, all survivors in the treated groups were sacrificed. (B) Long-term treatment regimen combined with survival in which the animals were treated daily for 10 days, starting on the day of infection. Necropsies for the determination of organ burdens were performed 4 dpi in the VIC and 15 dpi in the survivors of the different treatment groups; (C) Short-term treatment regimen in which all animals were sacrificed 4 dpi. All animals were weighed and observed daily for the occurrence of clinical signs during the course of the experiment. Moribund animals were euthanized by decapitation and recorded as dead on the following day. The organs of individual animals were weighed and homogenized in PBS-Tween-80. The homogenates were 10-fold serially diluted (up to 1/1000) and 200 µl of each dilution was spread onto an SDA plate for fungal enumeration after 48h.

Statistical analysis Statistical analyses were performed with GraphPad Prism, version 4.01 (Graph Pad Software, Inc., San Diego, CA). Kaplan-Meyer analysis compared survival in the

treatment groups. Fungal burden data were log-transformed before plotting and statistical analysis. Comparison of the fungal burdens between the treated groups and VIC was performed using Kruskal-Wallis or Mann-Whitney U tests. Mice that died before day 4 (VIC) or before day 10 or 15 (treated groups) were assumed to have organ counts at least as high as the highest counts in the VIC group. Statistical significance was defined as $P \leq 0.05$.

3. Results

3.1. OlPC showed promising *in vitro* activity against *A. fumigatus*

Table 1 shows the *in vitro* IC_{50}- and MFC-values for OlPC, MIL, POS and VOR against the different *Aspergillus* species. The repeatability of the susceptibility data was good (coefficients of variation (CV) below 10%, except for *A. flavus*). The reference drugs POS and VOR showed potent activity against *A. fumigatus* B19119 (IC_{50} 0.25 ± 0.04 µM and 0.53 ± 0.07 µM), which is in accordance with values described by CLSI [19]. The azole-resistant *A. fumigatus* AF198-R showed an average IC_{50}-value of 0.82 ± 0.32 µM, which exceeds the threshold for azole-resistance [22]. For MIL and OlPC, *A. flavus* appeared to be the least susceptible with IC_{50}-values of 9.46 µM and 14.02 µM. For the other *Aspergillus* spp., OlPC was more potent than MIL, as reflected by OlPC IC_{50}-values between 0.19 and 1.84 µM and MIL IC_{50}-values between 4.00 and 5.96 µM. For *A. fumigatus*, the MFC to IC_{50} ratio of OlPC was ≤4, suggesting a fungicidal mode-of-action [23].

3.2. IP administration of 50 mg/kg/day OlPC reduced organ burdens significantly at 4 dpi

In view of its promising *in vitro* activity, the *in vivo* potential of OlPC was explored in an acute mouse model of disseminated aspergillosis. The model had been optimized with respect to strain (*A. fumigatus* B19119) and inoculum concentration ($1x10^7$ cfu/animal) to obtain a reproducible infection. The vehicle-treated mice (VIC) were always moribund at 4 dpi and showed consistently high organ burdens. Three therapy regimens were evaluated (Table 2): (A) short-term (5-day) treatment combined with survival; (B) long-term (10-day) treatment combined with survival and (C) short-term (5-day) treatment with autopsy at 4 dpi. Mice treated with POS (10 mg/kg) showed a 100% survival in all treatment regimens and a major reduction of the fungal organ burden. For OlPC, 60% survival at 10 dpi was obtained under regimen A after oral administration of 50 mg/kg and 25 mg/kg, while less survival was seen

at 15 dpi under regiment B. Organ burdens only showed a significant reduction using 50 mg/kg/day IP in the short-term treatment regimen C (P = 0.0079) (Table 2).

4. Discussion

Compared to its structural analogue MIL, OlPC is characterized by a higher bioavailability and less side effects. Since several experimental studies [14, 16, 17] concluded that MIL shows potential as antifungal compound, we evaluated OlPC as a candidate drug for invasive aspergillosis. This study is the first to evaluate the antifungal potential of OlPC in an *in vitro* panel of *Aspergillus* spp. and in an acute mouse model of disseminated *A. fumigatus* infection.

Our protocol for *in vitro* susceptibility testing was based on the CLSI guidelines [19] and modified with regard to inoculum preparation and endpoint reading. Addition of 0.01% Tween-80 to the stock solutions and the use of exact counts to determine the inoculum size assured the repeatability of both the *in vitro* [24] and *in vivo* experiments. As dispersion of mycelia in culture medium may complicate conventional absorbance measurements, test reliability was improved by fluorimetric endpoint reading for resazurin-based assessment of metabolic activity [25]. In antifungal susceptibility testing, resazurin proved to have satisfactory agreement with the CLSI standards [26]. The *in vitro* antifungal activities were expressed as IC_{50} values in µM because this allows straightforward comparison of the different compounds. Using the modified CLSI protocol, the activities of POS and VOR against the different *Aspergillus* spp. were in agreement with data found in literature [27].

Our *in vitro* findings on MIL-activity against different *Aspergillus* species were within the range reported by Widmer *et al*. [14] and Biswas *et al*. [17]. The *in vitro* potency of OlPC against the *Aspergillus* panel was about five times higher than that of MIL. Both MIL and OlPC showed a lower activity on *A. flavus*, while POS-susceptibility was the same for all *Aspergillus* spp., except for the azole-resistant AF198-R strain. Widmer *et al.* have already described the lower susceptibility of *A. flavus* [14], but the variability of the IC_{50}-values (CV between 30-60%) may have contributed to this result.

For the exploratory *in vivo* study of OlPC, an acute mouse model of disseminated *A. fumigatus* infection was developed [28]. Three different dosing regimens were chosen using

survival and overall fungal burden in the target organs (liver, spleen and kidneys) as endpoints for efficacy. Although invasive aspergillosis mainly occurs in immunodeficient individuals, the animal model was based on immunocompetent mice [29]. In this model, a functional immune system supports drug activity, which may be important for the initial demonstration of the therapeutic potential of the candidate compound. In addition, our efforts to develop an invasive aspergillosis model using immunosuppressed mice were hampered by very high mortality rates and inconsistent clinical outcomes.

Because of the rapid clearance of VOR in mice, POS was chosen as reference drug [30]. The OlPC dosages of 25 and 50 mg/kg/day for 5 days were selected based on previous drug safety data in rodents (Dafra Pharma, unpublished). Next to oral dosing, IP treatment was included to maximize systemic availability. In all dosing regimens, POS at 10 mg/kg significantly improved survival and organ burden. In the short-term 5-day (A) and long-term 10-day (B) treatment schedules, OlPC enhanced survival in each treatment group, however, without affecting the organ burden. Only in the short-term 5-day treatment regimen with necropsy on 4 dpi (C), the organ burdens significantly decreased after IP dosing at 50 mg/kg/day. The mortality of OlPC-treated animals and the inability of OlPC to consistently reduce the organ burdens suggest its limited therapeutic potential in our mouse model of acute systemic aspergillosis. Although pharmacokinetic analyses were not included in this study, favorable pharmacokinetic properties have been established in rats and dogs, including good oral bioavailability, a long terminal half-life and extensive tissue distribution (Dafra, unpublished data). There is little doubt that the acute and virulent nature of our disseminated aspergillosis mouse model may have contributed to the limited *in vivo* activity. Attenuation of the infection through administration of a lower inoculum size or the use of immunosuppressed mouse models resulted in a less reproducible infection and an unpredictable clinical outcome. Currently, the use of less virulent *A. fumigatus* strains is being explored.

In conclusion, while OlPC shows promising *in vitro* activity against different *Aspergillus* species, our preliminary *in vivo* experiments could not entirely confirm this finding, despite different dosage strategies to maximize exposure. Given the limited amount of therapeutic options in the current antifungal market for invasive infections, the therapeutic potential of OlPC should be assessed in a less stringent animal model. Other options to explore include

the combined use of OlPC with other already marketed antifungal drugs. Recent data showing *in vitro* fungicidal MIL-activity against *Scedosporium, Fusarium* and mucormycetes [17] may argument for the exploration of OlPC-activity against these uncommon moulds.

Acknowledgments

Caroline Paulussen received a research grant of the Flemish Institute for Research and Technology (IWT-093449). The authors thank An Matheeussen for her help in performing the *in vitro* assays and Pim-Bart Feijens for his contribution in running the mouse model.

Author Contributions

CP and LM designed the study, developed the methodology, collected the data, performed the analysis and wrote the manuscript. GB performed the analysis and wrote the manuscript. TM, NA, PC reviewed the study design, methods, data and manuscript.

Conflicts of Interest

Anny Fortin and Tom Bosschaerts are employees of Dafra Pharma Research & Development. The other authors declare no conflict of interest.

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***Aspergillus* species / strain**	**OlPC**				**posaconazole**				**voriconazole**				**miltefosine**			
	IC_{50}	±	*sd*	MFC	IC_{50}	±	*sd*	MFC	IC_{50}	±	*sd*	MFC	IC_{50}	±	*sd*	MFC
A. fumigatus B19119 (*)	1.04	±	*0.00*	2.6	0.25	±	*0.04*	0.3	0.53	±	*0.07*	0.6	5.61	±	*0.06*	8.0
A. fumigatus 198-R	1.02	±	*0.01*	1.3	0.82	±	*0.32*	1.0	0.72	±	*0.28*	1.0	5.66	±	*0.00*	8.0
A. niger J930117	0.19	±	*0.03*	> 41.5	0.33	±	*0.02*	0.5	0.50	±	*0.23*	0.5	4.00	±	*0.45*	> 64
A. flavus B59000	14.02	±	*4.93*	> 41.5	0.26	±	*0.05*	0.2	1.02	±	*0.34*	2.0	9.46	±	*1.85*	> 64
A. terreus IHEM 5918	1.84	±	*0*	10.4	0.18	±	*0.05*	0.3	0.46	±	*0.16*	0.5	5.95	±	*0.3*	4

Table 1. *In vitro* activity (IC_{50}-values and MFC in µM) of OlPC, posaconazole, voriconazole and miltefosine against different *Aspergillus* spp

(*) strain used for *in vivo* evaluation

Table 2. Survival combined with *in vivo* efficacy of OlPC (organ burden reduction and log cfu/g organ) of mice infected with *Aspergillus fumigatus* B19119. Three different dosing regimens were evaluated: (A) Short-term treatment combined with survival; (B) Long-term treatment combined with survival and (C) Short-term treatment with necropsy 4 dpi.

	Short-term treatment combined with survival (10 dpi)[a]				Long-term treatment combined with survival (15 dpi)[b]				Short-term treatment (4dpi)[c]		
Treatment (No. Animals)	Survivors No.	Mean survival time ± SEM (days)	Total organ burden reduction (%)	Log CFU/g ± SEM	Survivors No.	Mean survival time ± SEM (days)	Total organ burden reduction (%)	Log CFU/g ± SEM	Survivors No.	Total organ burden reduction (%)	Log CFU/g ± SEM
VIC (5)	0	5.0 ± 0.0	-	5.3 ± 0.1	0	5.0 ± 0.0	-	5.9 ± 0.1	0	-	5.7 ± 0.0
POS 10 mg/kg PO (5)	5	11.0 ± 0.0	91.7	4.2 ± 0.1**	5	16.0 ± 0.0	99.4	3.0 ± 0.8**	5	75.3	5.1 ± 0.1**
OlPC 50 mg/kg PO (5)	3	8.0 ± 1.2	43.8	4.4 ± 0.6	2	8.8 ± 3.0	18.4	5.1 ± 0.7	2	36.9	5.3 ± 0.3
OlPC 25 mg/kg PO (5)	3	9.0 ± 1.4	43.7	4.6 ± 0.3	0	7.4 ± 2.0	0	ND	0	7.5	5.6 ± 0.1
OlPC 50 mg/kg IP (5)	ND[d]				2	10.0 ± 2.6	18.9	5.3 ± 0.5	2	67.4	5.2 ± 0.1**
OlPC 25 mg/kg IP (5)	ND				3	11.0 ± 3.1	18.4	5.1 ± 0.7	3	42	5.3 ± 0.3

[a] 5-day treatment was started immediately after infection, survivors were sacrificed at 10 dpi

[b] 10-day treatment was started immediately after infection, survivors were sacrificed at 15 dpi

[c] 5-day treatment was started immediately after infection, survivors were sacrificed at 4 dpi

[d] Not done

** Significant difference $P \leq 0.05$