

In Vitro Antifungal Activity of Cysteine Derivatives and their Combinations with Antifungal Agents Against Clinically Relevant *Scedosporium* species

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Abstract

Background: Members of the genus *Scedosporium* are emerging human pathogens, causing a wide range of infections. Their treatment means a challenge for clinicians, mostly because of the low susceptibility of these causative agents to conventional antifungal drugs. New alternative therapeutic approaches are required for better clinical outcomes. Cysteine and its derivatives alone or in combinations with antifungal drugs might have improved antifungal effects against *Scedosporium* species.

Methods: The *in vitro* antifungal effects of two cysteine forms and three of their derivatives (i.e., D-cysteine, L-cysteine, L-cysteine-methyl ester hydrochloride, N-isobutyryl-D-cysteine, and N-isobutyryl-L-cysteine) were determined using the CLSI M38-A2 broth microdilution method. The *in vitro* drug interactions between the most effective cysteine derivatives and some conventional antifungal drugs were also investigated using checkerboard assays in case of four clinical isolates.

Results: L-cysteine-methyl ester hydrochloride proved to be the most effective among the investigated compounds. N-isobutyryl-L-cysteine exhibited antifungal activity against one *Scedosporium aurantiacum* strain only. D-cysteine, L-cysteine and N-isobutyryl-D-cysteine proved to be ineffective against *Scedosporium* spp. in the investigated concentration range. No significant differences were found in the susceptibilities between environmental and clinical isolates. L-cysteine-methyl ester hydrochloride could interact synergistically with amphotericin B, caspofungin, terbinafine and voriconazole. Antagonistic interactions were not observed between the investigated compounds.

Conclusions: The present study provides an evidence for the *in vitro* antifungal activity of L-cysteine-methyl ester hydrochloride and the synergistic interactions between this cysteine derivative and conventional antifungal agents. Based on our results, L-cysteine-methyl ester hydrochloride in combination with antifungal drugs could be applicable in the treatment of human *Scedosporium* infections in the future. However, further studies are required to clarify the antifungal mechanism of L-cysteine-methyl ester hydrochloride and to investigate its activity *in vivo*.

Introduction

Scedosporium species are emerging human pathogens, responsible for a broad spectrum of infections, including superficial or deep localized diseases in immunocompetent hosts, and disseminated infections in immunocompromised or in near-drowning patients [1]. *Scedosporium boydii* and *Scedosporium apiospermium* are mainly associated with infections in temperate climates (e.g., Central Europe), while *Scedosporium aurantiacum* and *Scedosporium prolificans* infections are predominant in hot and arid countries (e.g., Spain, Australia) [2]. According to a recent clinical study in Spain, the members of this genus are the second most frequently isolated filamentous fungi from human infections after *Aspergillus* species [3]. The clinical manifestations of *Scedosporium* infections are very similar to those of aspergillosis, fusariosis and other hyalohyphomycosis [4]. The proper diagnosis is complicated by the low interspecies diversity and high intraspecies variability, especially within the *S. boydii* species complex. However, the accurate identification of the causative agent is crucial to find the most effective therapeutic approach, since the antifungal susceptibility profile of *Scedosporium* species is different from those of hyaline filamentous fungi, and also varies within the genus itself [2]. Another challenge in the treatment is the frequently observed antifungal resistance of the *Scedosporium* isolates to conventional antifungal agents [1]. In a consequence of these, clinicians have limited options to treat *Scedosporium*

infections. Therefore, new therapeutic strategies are required beside of the currently available ones.

Cysteine and their derivatives have been previously reported to have antifungal activity against different filamentous fungi (Table 1) [5-11]. Previously we proved the antifungal activity of N-acetyl-L-cysteine against *Scedosporium* species [12]. D-cysteine (DC), L-cysteine (LC), L-cysteine-methyl ester (LCME), N-isobutyryl-D-cysteine (NIDC), and N-isobutyryl-L-cysteine (NILC) showed antifungal effect against species belonging to Mucorales [6]. LC was reported to inhibit spore germination and to cause reduced hyphal growth in different filamentous fungi (e.g. dermatohyetes, *Aspergillus* spp., *Fusarium* spp.) [7,8,11].

The aims of the present study were (i) to determine the *in vitro* susceptibility of clinical and environmental *Scedosporium* isolates

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Citation: Galgóczy L, Homa M, Papp T, Manikandan P, Vágvölgyi C (2016) *In Vitro* Antifungal Activity of Cysteine Derivatives and their Combinations with Antifungal Agents Against Clinically Relevant *Scedosporium* species. Int J Clin Med Microbiol 1: 111. doi: <https://doi.org/10.15344/2456-4028/2016/111>

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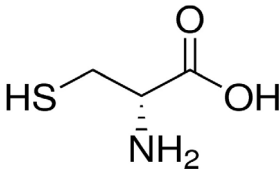
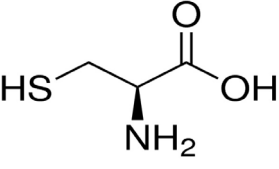
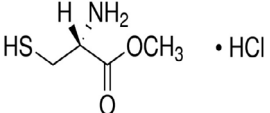
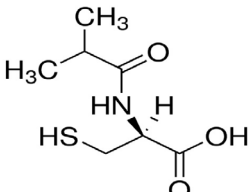
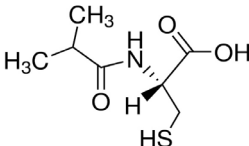
Cysteine/derivatives ^a	Chemical structure ^b	Investigated organisms	Antifungal effect/mode of action	References
DC		<i>Eutypa lata</i>	50% growth inhibition	[5]
		Mucorales spp.	Reduced hyphal growth, MEC: 0.625-10 mM	[6]
LC		Dermatohyetes	Poor growth Change in colony morphology	[7]
		<i>Microsporium gypseum</i> , <i>Trichophyton mentagrophytes</i>	MIC: 0.5-0.4% (w/v) MFC: 0.8-0.9% (w/v)	[8]
		<i>Alternaria</i> spp.	Inhibited spore germination	[9]
		<i>Inonotus obliquus</i>	Inhibited mycelial growth Reduced ergosterol production	[9]
		<i>Aspergillus</i> spp. <i>Fusarium</i> spp.	MIC: 0.078-0.625% (w/v) Inhibited conidial germination May reduce conidial wall permeability to nutrients	[10]
LCME		<i>Eutypa lata</i>	Inhibited growth, MIC: 10 mM Modified structural organization of the mycelium (complete disorganization of the cell content, nuclear degeneration)	[5]
		Mucorales spp.	Reduced hyphal growth, MEC: 0.625-10 mM Change in colony morphology Inhibited sporangiospore germination	[6]
NIDC		Mucorales spp.	Reduced hyphal growth, MEC: 0.625-5 mM	[6]
NILC		Mucorales spp.	Reduced hyphal growth, MEC: 0.625-10 mM Change in colony morphology	[6]

Table 1: Overview of the antifungal activity of the tested cysteine forms and their derivatives.

^aDC, D-cysteine; LC, L-cysteine; LCME, L-cysteine-methyl ester hydrochloride; NIDC, N-isobutyryl-D-cysteine; NILC, N-isobutyryl-L-cysteine.

^bSource: <http://www.sigmaaldrich.com/>

to DC, LC and their derivatives, such as LCME, NIDC, and NILC; and (ii) to investigate the in vitro combinations of the most effective cysteine compound with conventional antifungal agents against clinical *Scedosporium* isolates. developed for the diagnosis of UTIs. Rapid biochemical dipstick tests are available and currently used as predictors of bacterial UTI, but must often be correlated with other testing and clinical information. In many clinical settings, in fact, it has been proved that the dipstick urinalysis leads to many false positive and negative results when compared with the gold standard culture method, demonstrating the low sensitivity and positive predictive value [10]. The Micro Biological Survey method (MBS) is an alternative method for bacterial counting developed and patented by Roma Tre University [11,12]. It is based on a colorimetric survey performed in mono-use disposable reaction

vials in which samples can be inoculated without any preliminary treatment. The analysis can be carried out using a thermostatic optical reader that automatically detects the color change. The ease of use of the MBS method has been evaluated in a previous study on food samples demonstrating the possibility to use it anywhere and without the need of an equipped laboratory and specialized personnel [13]. In previous studies carried out on artificially contaminated urine samples, this method has already been proven to be suitable for the evaluation of the bacterial load and the assessment of the susceptibility to a panel of antibiotics [14,15]. The present study has been undertaken to clinically evaluate the performance and operational characteristics of the MBS POCT for the diagnosis and the antibiotic treatment of UTIs. The MBS method was also compared with another biochemical rapid test for UTI diagnosis, namely the urine dipstick assay [16].

Materials and Methods

Strains and culture conditions

Nine *Scedosporium* isolates derived from different environmental and clinical sources were involved in this study (Table 2). Prior to the tests, to get the required amount of conidia all the strains were grown on malt extract slants (MEA, Biolab, Hungary) for 2 weeks at 30°C. Susceptibility tests were performed in RPMI 1640 medium (Sigma-Aldrich, USA) supplemented with 0.3 g/l L-glutamine and buffered to pH 7.0 with 0.165 M 4-morpholinopropanesulfonic acid (Sigma-Aldrich, USA) and were incubated at 37°C for 72 hours.

Results and Discussion

We evaluated the antifungal effect of two cysteine forms, LC and DC, and their derivatives against nine *Scedosporium* isolates. The MICs were in the range of 512 - >1024 µg/ml (Table 2). LCME proved to be most effective among the investigated compounds with a MIC range of 512 - >1024 µg/ml. It demonstrated similar antifungal activity against the tested isolates (512 - 1024 µg/ml) except for *P. ellipsoidea* CBS 301.79 strain where the MIC was >1024 µg/ml. DC, LC and NIDC were ineffective against the *Scedosporium* isolates in the investigated concentration range (MIC: >1024 µg/ml). While NILC proved to be active against one strain only, *S. aurantiacum* CBS

Species (Strain number ^a)	Source	Mean MICs of cysteine and its derivatives ^b (µg/ml)				
		DC	LC	LCME	NIDC	NILC
<i>S. aurantiacum</i> (CBS 116910)	Wound exudate/Spain	>1024	>1024	512	>1024	1024
<i>S. aurantiacum</i> (CBS 136046)	Human lung/Australia	>1024	>1024	1024	>1024	>1024
<i>S. aurantiacum</i> (CBS 136047)	Soil/Australia	>1024	>1024	1024	>1024	>1024
<i>S. aurantiacum</i> (CBS 136049)	Soil/Austria	>1024	>1024	1024	>1024	>1024
<i>S. boydii</i> (CBS 117410)	Soil/Spain	>1024	>1024	1024	>1024	>1024
<i>S. boydii</i> (CBS 117432)	Sputum/France	>1024	>1024	1024	>1024	>1024
<i>S. boydii</i> (CBS 120157)	Human lung/France	>1024	>1024	1024	>1024	>1024
<i>P. angusta</i> (CBS 254.72)	Sewage/USA	>1024	>1024	1024	>1024	>1024
<i>P. ellipsoidea</i> (CBS 301.79)	Dung/The Netherlands	>1024	>1024	>1024	>1024	>1024
MIC range		>1024	>1024	512 - >1024	>1024	1024 - >1024

Table 2 The antifungal activity of cysteine and its derivatives against *Scedosporium* isolates.

^aCBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

^bDC, D-cysteine; LC, L-cysteine; LCME, L-cysteine-methyl ester hydrochloride; NIDC, N-isobutyryl-D-cysteine; NILC, N-isobutyryl-L-cysteine.

Microdilution tests

The susceptibility of *Scedosporium* isolates to cysteine forms and their derivatives were determined following the slightly modified instructions of the Clinical and Laboratory Standards Institute's M38-A2 broth microdilution method [13]. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of a drug which was required for the total growth inhibition of a certain isolate after 72 hours-long incubation. Modifications connected to stock solution and inoculum preparation were detailed previously [14]. We evaluated the antifungal effect of five compounds: DC, LC, LCME, NIDC, and NILC (Sigma-Aldrich, USA). The final drug concentrations in the tests ranged from 64 to 1024 µg/ml.

Drug interactions between LCME and conventional antifungal agents (i.e., amphotericin B, AMB; caspofungin, CSP; terbinafine, TRB; and voriconazole, VRC) were investigated against the four clinical isolates using the checkerboard microdilution method [15]. The final LCME concentrations ranged from 64 to 2048 µg/ml. The final concentrations of the antifungal drugs were between 0.25 and 128 µg/ml. Fractional inhibitory concentration indexes (FICIs) were calculated to demonstrate the type of interaction between the paired combinations of compounds [16]. For these calculations, the MICs of antifungals have been already determined in a recent study of our research group [14]. Synergism was defined as $FICI \leq 0.5$, indifference as $0.5 < FICI \leq 4$ and antagonism was defined when $FICI > 4$ [17].

Both single and combination tests were performed in three replicates.

116910 (MIC: 1024 µg/ml). Differences between the susceptibilities of environmental and clinical isolates to the investigated compounds were not observed.

The antifungal activity of cysteine and its derivatives against *Scedosporium* species has not been investigated yet. The MICs observed in this study are comparable to previously reported data against other fungal species. The *in vitro* inhibitory effect of DC was proved previously against *Eutypa lata* and different Mucorales species. It caused reduced hyphal growth in a concentration of 0.625 - 10 mM (~ 75 - 1200 µg/ml) [5,6]. The antifungal effect of LC was reported against different filamentous fungi: dermatophytes, *Alternaria* spp., *Aspergillus* spp., *Eutypa lata*, *Fusarium* spp., and *Inonotus obliquus* [5-11]. Our previous publication showed the antifungal effect of LCME, NIDC, and NILC against Mucorales species: the MEC values were between 0.625 and 10 mM (~ 100 - 1700 µg/ml at LCME; ~ 60 - 1000 µg/ml at NIDC and NILC) [6]. Beside the reduced hyphal growth, LCME inhibited the germination of sporangiospores, while the presence of NILC in the culture media led to changes in the colony morphology [6]. The mode of action of cysteine and their derivatives has not been clarified yet. Kahlos and Tikka [10] observed that LC reduced the ergosterol production of *Inonotus obliquus*. Octave et al. [5] reported the complete structural disorganization of the mycelia of *Eutypa lata*. According to Daigle and Cotty [9] the mercaptan and amino groups of LC were essential to the inhibition of conidial germination.

In the combination tests, the *in vitro* interactions between the most effective non-antifungal agent, LCME and four conventional

antifungal drugs were evaluated. These results are summarized in Table 3. We revealed no antagonistic interaction for LCME with any of the antifungal drugs. Between LCME and AMB, and LCME and TRB only synergistic interactions were observed (FICI_{LCME+AMB}: 0.06 - 0.13, FICI_{LCME+TRB}: 0.09 - 0.16). For *S. aurantiacum* CBS 116910 strain, the drug interaction between LCME and CSP and between LCME and VRC proved to be indifferent. However, we found synergistic interaction in all other cases between these compounds (FICI_{LCME+CSP}: 0.13 - 0.63, FICI_{LCME+VRC}: 0.13 - 1.00) (Table 3). Previously, we investigated the *in vitro* combinations of another L-cysteine derivative, N-acetyl-cysteine with AMB, CSP, TRB, and VRC [12]. Similarly, synergistic interactions were revealed predominantly between the investigated agents and antagonistic interactions were not registered.

Conclusions

This is the first study investigating the activity of cysteine derivatives against *Scedosporium* species and the first report of the *in vitro* antifungal effect of LCME and its synergistic interactions with conventional antifungal agents against *Scedosporium* isolates. According to our results, LCME alone or in combination with other drugs might be useful for the treatment of human *Scedosporium* infections. However, further studies are required to investigate the antifungal mechanism of LCME and its pharmacokinetic and pharmacodynamic properties.

Competing Interests

The authors declare that they have no competing interests.

Isolate ^a	Mean MIC (µg/ml) ^b		Interaction ^d		FICI ^c	
	LCME _{alone}	LCME _{comb}	AMB _{alone} [*]	AMB _{comb}		
<i>S. aurantiacum</i> (CBS 116910)	512	64	128	1	0.13	S
<i>S. aurantiacum</i> (CBS 136046)	1024	64	128	4	0.09	S
<i>S. boydii</i> (CBS 117432)	1024	64	128	0.25	0.06	S
<i>S. boydii</i> (CBS 120157)	1024	64	64	2	0.09	S
	LCME _{alone}	LCME _{comb}	CSP _{alone} [*]	CSP _{comb}		
<i>S. aurantiacum</i> (CBS 116910)	512	64	32	16	0.63	NI
<i>S. aurantiacum</i> (CBS 136046)	1024	128	64	0.125	0.13	S
<i>S. boydii</i> (CBS 117432)	1024	64	64	8	0.19	S
<i>S. boydii</i> (CBS 120157)	1024	128	32	0.125	0.13	S
	LCME _{alone}	LCME _{comb}	TRB _{alone} [*]	TRB _{comb}		
<i>S. aurantiacum</i> (CBS 116910)	512	64	128	4	0.16	S
<i>S. aurantiacum</i> (CBS 136046)	1024	64	128	4	0.09	S
<i>S. boydii</i> (CBS 117432)	1024	64	128	4	0.09	S
<i>S. boydii</i> (CBS 120157)	1024	64	128	8	0.13	S
	LCME _{alone}	LCME _{comb}	VRC _{alone} [*]	VRC _{comb}		
<i>S. aurantiacum</i> (CBS 116910)	512	256	8	4	1.00	NI
<i>S. aurantiacum</i> (CBS 136046)	1024	128	32	0.125	0.13	S
<i>S. boydii</i> (CBS 117432)	1024	64	16	4	0.31	S
<i>S. boydii</i> (CBS 120157)	1024	64	16	1	0.13	S

Table 3: The combination test results of LCME and conventional antifungal drugs against clinical *Scedosporium* isolates.

^aCBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

^bLCME_{alone}, AMB_{alone}, CSP_{alone}, TRB_{alone} and VRC_{alone}, mean MICs of L-cysteine methyl ester hydrochloride, amphotericin B, caspofungin, terbinafine and voriconazole, respectively, when applied alone; LCME_{comb}, AMB_{comb}, CSP_{comb}, TRB_{comb} and VRC_{comb}, mean MICs of L-cysteine methyl ester hydrochloride, amphotericin B, caspofungin, terbinafine and voriconazole, respectively, when applied in combination.

^cFICI, fractional inhibitory concentration index value

^dNI, no interaction (0.5 < FICI ≤ 4); S, synergism (FICI ≤ 0.5) [17].

^{*}The MICs of AMB, CSP, TRB and VRC were determined in a previous study of our research group [14].

When used alone, the MIC range of LCME were 512 - 1024 µg/ml, but in combination with AMB, CSP, TRB, and VRC, these values reduced to 64 µg/ml, 64 - 128 µg/ml, 64 µg/ml, and 64 - 256 µg/ml, respectively. Compared to the single use, the MICs of antifungal agents were also decreased in the combination tests. The previously determined MIC ranges of AMB, CSP, TRB, and VRC were 8 - 128 µg/ml, 32 - 64 µg/ml, 128 µg/ml and 8 - 64 µg/ml, respectively [14]. In combination with LCME, the MIC ranges of AMB, CSP, TRB, and VRC were decreased to 0.25 - 4 µg/ml, 0.125 - 16 µg/ml, 4 - 8 µg/ml, and 0.125 - 4 µg/ml, respectively (Table 3). Compared to the single use, the MIC values of antifungal drugs in the combination tests could be decreased to their achievable therapeutic plasma concentrations in several cases [18-21].

Author Contributions

László Galgóczy, Tamás Papp, Palanisamy Manikandan, and Csaba Vágvolgyi took part in the experimental design, the evaluation of the results and the final preparation of the manuscript. The single and combination tests for the fungal isolates were carried out by László Galgóczy and Mónika Homa.

Acknowledgements

L.G. holds a Lise Meitner-Position (M 1776-B20) from the Austrian Science Fund (FWF). T.P. is a grantee of J. Bolyai Scientific Scholarship of the Hungarian Academy of Sciences. The study was supported by the European Union co-financed by the European Social

Fund (TÁMOP-4.2.2.B-15/1/KONV-2015-0006). This work was also funded by the grant LP2016-8/2016.

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