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ASP2397: A Novel Natural Product with Potent Fungicidal Activity against *Aspergillus* spp. (1) - A New Mode of Action and *In Vitro* Activity

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Abstract

Background: Anti-*Aspergillus* efficacy of existing antifungal agents for invasive pulmonary aspergillosis (IPA) is still insufficient. Aiming to identify a new anti-*Aspergillus* compound from our natural product library, we discovered ASP2397 (ASP) which is a novel antibiotic isolated from the fungus *Acremonium* sp. MF-347833. Here we report on its mechanism of action and the antifungal activity of ASP as a potential clinical candidate against IPA.
Methods: Susceptibility of medically important *Aspergillus* spp. to ASP was determined in human serum and RPMI in accordance with CLSI M38-A2 as broth media. Fungicidal activity was examined for growth from germinated conidia by in vitro time-kill assay and kinetic imaging for living cells. The mechanism of action was investigated using UV-induced *A. fumigatus* resistant mutant (RSV-1) and wild-type gene transfer to RSV-1. Uptake of ASP into germinated conidia was measured by LC/MS/MS.
Results: ASP had antifungal activities against *A. fumigatus*, azole-resistant *A. fumigatus*, *A. terreus*, *A. flavus*, and *A. nidulans* with an MIC range of 1 to 4 μg/mL in human serum. The in vitro antifungal activity of ASP against the most prevalent *Aspergillus* spp., *A. fumigatus* was examined. ASP showed a more rapid onset of inhibition of hyphal elongation from germinated conidia of *A. fumigatus* than voriconazole (VRCZ). ASP had steep time-kill curves against *A. fumigatus* with over 1 $\log_{10}$ CFU reduction compared with VRCZ. RSV-1 had a point mutation in a membrane transporter gene which is absent from mammalian cells. Introduction of the wild-type transporter gene into RSV-1 recovered sensitivity for ASP. In an uptake assay, ASP was actively and rapidly incorporated into wild-type *A. fumigatus* strains.
Conclusions: ASP was effective in vitro against a range of frequently occurring *Aspergillus* spp including azole-resistant *A. fumigatus* and was actively transported into *A. fumigatus* hyphae through a transporter. Furthermore, ASP showed a more rapid onset and potent fungicidal effect against *A. fumigatus* than VRCZ. These results suggest ASP is a potential candidate for anti-*Aspergillus* therapy.

Introduction

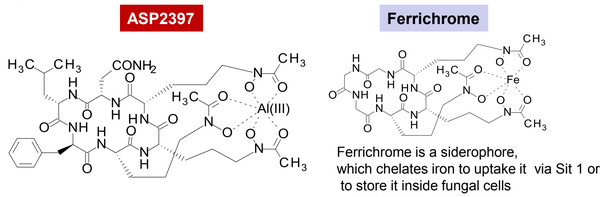
- *Aspergillus* species, *A. fumigatus*, *A. flavus*, *A. niger*, and *A. terreus,* cause invasive pulmonary aspergillosis (IPA) severely in immunocompromised hosts with *A. fumigatus* the most common.
- However, efficacy of the existing antifungal agents which have MOAs such as inhibition of cellular membrane or wall functions remains insufficient for IPA.
- Conventional in vitro assays are usually conducted using resting conidia as inoculum. In this study, germinated conidia as inoculum were used to reflect advanced IPA.
- We discovered a natural product, ASP2397 with a novel MOA and potent fungicidal actions against *Aspergillus* spp.

Methods

Resistant strain: ASP2397-resistant mutant (RSV-1) was generated by a UV irradiated method. The resistant clones were isolated on agar including 20 μg/mL of ASP2397.
MIC: The tests were conducted in heat-inactivated human serum and RPMI1640 (CLSI M38-A2). In the human serum assay containing 20 mmol /L HEPES (pH 7.4) incubation was under 5% CO_2 for 48 hours at 37 ℃. Both MICs were read as the endpoint that prevented discernible growth.
Take up assay into fungal cells: 1 μg /mL of ASP2397 was added to germinated conidia of *A. fumigatus* FP1305 at 0 time grown on membrane filter. The culture was separated into medium and cell fraction over 4 hr. by centrifugation. Distribution of ASP2397 in the fractions were determined by LC/MS/MS.
Rapid inhibition against hyphal elongation: Fungal images were taken as photographs after agent treatment to germinated *A. fumigatus* conidia grown in human serum. Fungal area as growth measure was quantified in a single image using IncuCyte Zoom (Essen Bioscience, Ann Arbor, MI, USA). n=4.
Time-kill curve assay: After germination of conidia of the *A. fumigatus* 20025 inoculated at ca. 1 ×10^4 CFU/mL in the human serum in 96 well microplate, increasing doses of agents were added. Viable counts at appropriate time were determined on Sabouraud dextrose agar.

Results

Fig.1. Structure of ASP2397 and Ferrichrome



Ferrichrome is a siderophore, which chelates iron to uptake it via Sit 1 or to store it inside fungal cells

Fig.2. Summary of Antifungal mechanism of ASP2397

- Active transport of ASP2397 into *A. fumigatus* cells via siderophore transporter Sit1 triggers antifungal effects (Table1, Fig.3)
- An unidentified intracellular antifungal target for ASP2397 is presumed to exist (Table2)
- Lack of Sit 1 in mammalian cells may be responsible for higher selectivity

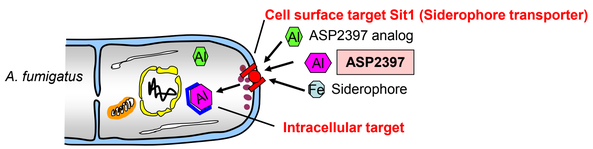
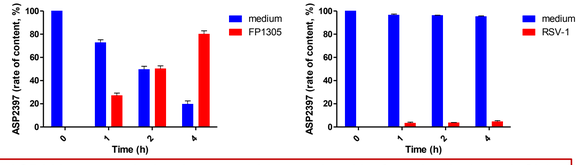


Table1. Sit1 mutation strongly affects the susceptibility of *A. fumigatus* to ASP2397

		MIC in RPMI: μg/mL	
		ASP2397	VRCZ
A. fumigatus FP1305	parent WT strain	**0.25**	0.25
A. fumigatus RSV-1*	UV induced **ASP-resistant strain**	**>16**	0.25
A. fumigatus RSS	**introduced with Sit1 gene**	**0.25**	0.25
A. fumigatus RSP	introduced with empty vector	**>16**	0.25

*RSV-1:a mutant of siderophore transporter Sit1 (C1437A)

Fig.3. ASP2397 is rapidly taken up and accumulated by *A. fumigatus*



- ASP2397 was actively taken up and accumulated by fungal cells through Sit1 which is absent in mammalian cells

Table2. Uptake into fungi is necessary but NOT sufficient to exhibit antifungal activity

	A. fumigatus				*Candida albicans*		*Candida glabrata*	
	FP1305		RSV-1					
	uptake	activity	uptake	activity	uptake	activity	uptake	activity
ASP2397	A) **Yes**	**Yes**	**No**	**No**	**No**	**No**	**Yes**	**Yes**
ASP2397 analogs*	B) **Yes**	**No**	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.

* ASP2397 analogs: a skeletal structure with different amino acid constitution N.T.: not tested

A) Uptake of ASP2397 into the pathogen determines antifungal spectrum
B) There appears to be an intracellular target for ASP2397 to express antifungal activity

Fig.4. In vitro assay model relevant to clinical setting of antifungal treatment

Hypothesis: After germination of *A. fumigatus* conidia in patients, antifungal treatment may be less effective
Concept: Antifungal activities against ***Germinated conidia*** as inoculum

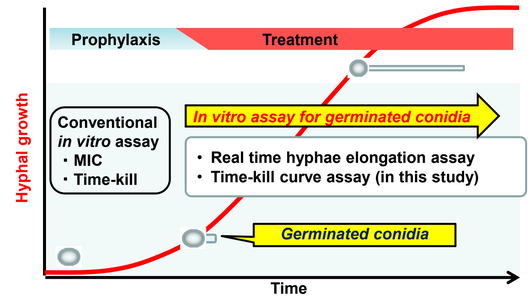
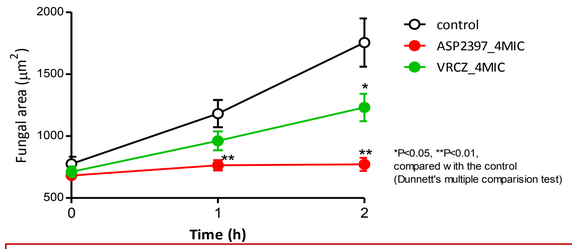
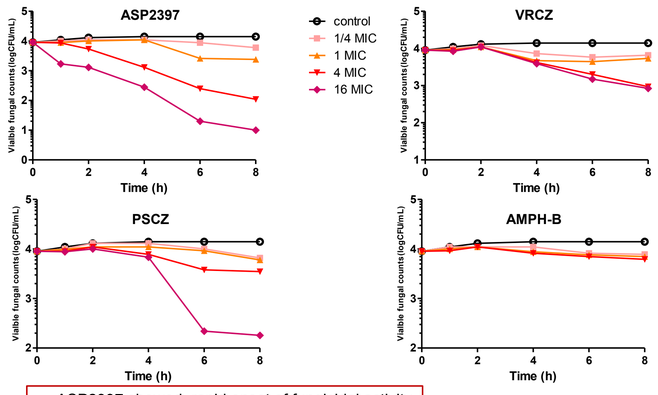


Fig.5. ASP2397 completely halts the hyphal elongation from germinated conidia revealed by live cell imaging



- ASP2397 stopped hyphal elongation as soon as treatment was initiated
- Rapid uptake and accumulation may exert this rapid action (Fig. 3)
- VRCZ displayed partial inhibition

Fig.6. ASP2397 showed a rapid and potent fungicidal activity against *A. fumigatus*



- ASP2397 showed rapid onset of fungicidal activity

Table 3. Antifungal spectrum in inactivated human serum

Susceptible MIC ≦ 2	*Aspergillus*	*A. fumigatus*, *A. terreus*, *A. flavus*, *A. nidulans*
	Yeast	*Candida glabrata, Candida kefyr, Cryptococcus neoformans, Trichosporon asahii*
Intermediate susceptible MIC ≦ 8	Molds	*Fusarium solani*
	Aspergillus	*A. niger*
	Candida	*C. albicans, C. tropicalis, C. parapsilosis, C. krusei, C. guilliermondii*
Less susceptible MIC >16	Mucorales	*Rhizopus oryzae, Cunninghamella elegans, Absidia corymbifera*
	Other molds	*Scedosporium apiospermum , Fonsecaea pedrosoi*

Table 4. Antifungal activities against clinical isolates of *Aspergillus* strains

Organism	No. of isolates	Drug	MIC in human serum:μg/ml Range			90%	MIC in RPMI: μg/ml Range			90%
A. fumigatus	49	**ASP2397**	**1**	**–**	**4**	**2**	**0.06**	**–**	**0.5**	**0.5**
		VRCZ	0.12	–	1	0.5	0.12	–	1	0.5
		ITCZ	0.5	–	8	4	0.12	–	0.5	0.5
		PSCZ	0.06	–	0.5	0.25	0.03	–	0.25	0.12
		AMPH-B	0.5	–	2	2	0.25	–	1	0.5
Azole-resistant *A. fumigatus*	4	**ASP2397**	**1**	**–**	**2**		**0.06**	**–**	**1**	
		VRCZ	0.12	–	8		0.25	–	4	
		ITCZ		>8				>8		
		PSCZ	1	–	8		1	–	>8	
		AMPH-B	0.5	–	2		0.25	–	1	
A. flavus	17	**ASP2397**	**2**	**–**	**4**	**2**	**2**	**–**	**>16**	**>16**
		VRCZ	0.25	–	1	1	0.25	–	1	1
		PSCZ	0.25	–	0.5	0.5	0.03	–	0.25	0.25
		AMPH-B	1	–	8	8	0.5	–	2	2
A. terreus	20	**ASP2397**	**2**	**–**	**4**	**4**	**0.25**	**–**	**8**	**1**
		VRCZ	0.12	–	1	1	0.25	–	0.5	0.5
		PSCZ	0.06	–	0.5	0.5	0.06	–	0.12	0.12
		AMPH-B	1	–	8	4	0.25	–	2	1
A. nidulans	5	**ASP2397**	**2**	**–**	**4**		**16**	**–**	**>16**	
		VRCZ	0.12	–	0.25		0.12	–	0.25	
		PSCZ	0.12	–	0.25		0.06	–	0.12	
		AMPH-B		4			0.5	–	2	

- ASP2397 was effective against major *Aspergillus* species including azole-resistant *A. fumigatus*

Conclusions

ASP2397

- Is an anti-*Aspergillus* agent with a novel structure and mechanism from our natural product library
- Is actively incorporated into fungal cells through a putative siderophore transporter, Sit1, which is absent in mammalian cells
- Has potent fungicidal activity following rapid inhibition against hyphal elongation
- Is effective in vitro against a range of frequently occurring *Aspergillus* species including azole-resistant organisms and *C. glabrata*
- Displays rapid onset of inhibition and potent fungicidal action against hyphal elongation compared to existing drugs
- Is a potential candidate for IPA which is refractory to existing drugs

Abbreviation

VRCZ, Voriconazole; PSCZ, Posaconazole; ITCZ,Itraconazole; AMPH-B, Amphotericin B