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In Vitro Activity of ASP2397 against *Aspergillus* Isolates with or without Acquired Azole Resistance Mechanisms

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ASP2397 is a new compound with a novel and as-yet-unknown target different from that of licensed antifungal agents. It has activity against *Aspergillus* and *Candida glabrata*. We compared its *in vitro* activity against wild-type and azole-resistant *A. fumigatus* and *A. terreus* isolates with that of amphotericin B, itraconazole, posaconazole, and voriconazole. Thirty-four isolates, including 4 wild-type *A. fumigatus* isolates, 24 *A. fumigatus* isolates with alterations in CYP51A TR/L98H (5 isolates), M220 (9 isolates), G54 (9 isolates), and HapE (1 isolate), and *A. terreus* isolates (2 wild-type isolates and 1 isolate with an M217I CYP51A alteration), were analyzed. EUCAST E.Def 9.2 and CLSI M38-A2 MIC susceptibility testing was performed. ASP2397 MIC_{50} values (in milligrams per liter, with MIC ranges in parentheses) determined by EUCAST and CLSI were 0.5 (0.25 to 1) and 0.25 (0.06 to 0.25) against *A. fumigatus* CYP51A wild-type isolates and were similarly 0.5 (0.125 to >4) and 0.125 (0.06 to >4) against azole-resistant *A. fumigatus* isolates, respectively. These values were comparable to those for amphotericin B, which were 0.25 (0.125 to 0.5) and 0.25 (0.125 to 0.25) against wild-type isolates and 0.25 (0.125 to 1) and 0.25 (0.125 to 1) against isolates with azole resistance mechanisms, respectively. In contrast, MICs for the azole compounds were elevated and highest for itraconazole: >4 (1 to >4) and 4 (0.5 to >4) against isolates with azole resistance mechanisms compared to 0.125 (0.125 to 0.25) and 0.125 (0.06 to 0.25) against wild-type isolates, respectively. ASP2397 was active against *A. terreus* CYP51A wild-type isolates (MIC 0.5 to 1), whereas MICs of both azole and ASP2397 were elevated for the mutant isolate. ASP2397 displayed *in vitro* activity against *A. fumigatus* and *A. terreus* isolates which was independent of the presence or absence of azole target gene resistance mutations in *A. fumigatus*. The findings are promising at a time when azole-resistant *A. fumigatus* is emerging globally.

Aspergillus causes invasive aspergillosis, which is a life-threatening infection, as well as a variety of less acute though still devastating chronic infections. The cornerstone in management of the disease is azole treatment due to the superiority of voriconazole in a randomized clinical trial and in postmarketing studies for treatment of invasive aspergillosis and the oral availability of azole drugs for outpatient treatment of chronic aspergillosis (1–8). However, increasing numbers of reports document emerging azole resistance in Europe, Africa, and the Asia-Pacific region, which highlights the importance of safe and efficacious alternatives (9–15). Amphotericin B is active but is associated with significant toxicity even in its lipid formulations, and echinocandins have only static activity against *Aspergillus*.

ASP2397 is a new compound with activity against *Aspergillus* species, including *A. fumigatus*, *A. terreus*, *A. flavus*, and *A. nidulans*, with an MIC range of 1 to 4 mg/liter in human serum (16). It is actively incorporated into *A. fumigatus* through the membrane siderophore transporter Sit1. The mode of action is novel and is as yet unknown but is different from that of the azoles and amphotericin. Hence, it may be a promising alternative option for the treatment of azole-resistant *Aspergillus* infections.

The purpose of this study was to evaluate the *in vitro* activity of ASP2397 in comparison with that of itraconazole, posaconazole, voriconazole, and amphotericin against a well-characterized panel of clinical *Aspergillus* species isolates with or without azole resistance mechanisms. Preliminary studies performed elsewhere had raised a potential concern that full inhibition might not be achieved if the higher inoculum and glucose concentration recommended by EUCAST were used. Therefore, the *in vitro* activity was examined using both the EUCAST and CLSI reference methodologies and modified EUCAST (mod-EUCAST) methods with either a 10-fold-lower inoculum or a spectrophotometer 50% growth inhibition endpoint.

MATERIALS AND METHODS

Thirty-four *Aspergillus* isolates and reference strains were included, specifically, (i) 24 *A. fumigatus* isolates with well-known azole resistance CYP51A target protein alterations (involving TR/L98H [5 isolates], alterations at the M220 codon [9 isolates] and at the G54 codon [9 isolates], and an HapE mutation [1 isolate]), (ii) 4 CYP51A wild-type *A. fumigatus* isolates, (iii) 1 *A. terreus* isolate with an M217I Cyp51Ap alteration, and (iv) 2 CYP51A wild-type *A. terreus* isolates. Finally, one EUCAST *Aspergillus* reference strain (*A. fumigatus* ATCC 204305), one CLSI *Aspergillus* reference strain (*A. fumigatus* ATCC MYA-2636), and the *Candida krusei* ATCC 6258 control strain recommended by both EUCAST and CLSI were included as controls.

CLSI and EUCAST testing was performed according to the EUCAST E.Def 9.2 and CLSI (M38-A2) methodologies (17, 18). In addition, a modified EUCAST method that uses a 10-fold lower fungal inoculum concentration (final inoculum concentration, 1×10^4 to 2.5×10^4 CFU/ml) was included. All isolates were cultured twice on Sabouraud agar (SSI

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Diagnostika, Hillerød, Denmark) before susceptibility testing was performed to ensure viability. The antifungal agents (manufacturers) used in stock solutions (5,000 mg/liter) in dimethyl sulfoxide (DMSO) (D8779; Sigma-Aldrich, Vallensbæk Strand, Denmark) were the following: ASP2397 (Astellas Pharma Inc., Tokyo, Japan), amphotericin B (Sigma-Aldrich), posaconazole (Merck, Ballerup, Denmark), and voriconazole (Pfizer A/S, Ballerup, Denmark). The drug concentration range studied was 0.03 to 4 mg/liter for all compounds. For both methods, plates were made in one batch, immediately frozen (−80°C), and used as soon as they were thawed. Inoculated plates were incubated at 35°C and read visually (blind to the species identity) at day 2. The primary endpoint was complete inhibition (MIC) for all drugs and methods as specified by CLSI and EUCAST. In addition, a spectrophotometer reading of the EUCAST plates using a 50% endpoint was performed. Finally, an additional visual endpoint reading for ASP2397 was performed for the EUCAST plates with a partial-inhibition endpoint (allowing weak growth) due to an initial concern that full inhibition would not be obtained for this agent in performing EUCAST susceptibility testing. The EUCAST MICs for the *A. fumigatus* ATCC 204305 and *C. krusei* ATCC 6258 reference strains were all within the recommended ranges for the licensed compounds (see Table S1 in the supplemental material). The CLSI MICs for ATCC MYA-2636 were also within the limits for voriconazole but were 1 dilution step lower than the recommended range for amphotericin B and itraconazole (ranges are not established for posaconazole). The quality control (QC) results confirmed the good performance of the susceptibility tests and the characteristic low-end MIC results as expected with freshly made trays.

RESULTS

ASP2397 and amphotericin B were equally efficacious *in vitro* against *A. fumigatus* isolates with or without *Cyp51A* mutations independently of the method or endpoint used (Fig. 1 and Table 1). The MICs obtained with the EUCAST method were in general 1 dilution step higher than those obtained with the other methods, and those obtained using a spectrophotometer 50% endpoint for the EUCAST plates were slightly lower (Table 1). The endpoints for ASP2397 were clear, with full inhibition at the high end concentrations even for the EUCAST method (see Fig. S1 in the supplemental material). A single *A. fumigatus* isolate with a G54E alteration was found to be consistently resistant to ASP2397 across all methods used, and one *A. fumigatus* isolate with a M220K alteration was found to be resistant by the EUCAST method due to only partial inhibition in the 1-to-4-mg/liter concentration range (Table 1 and Fig. 2).

The activities of the three azoles are also summarized in Table 1. All wild-type isolates were classified as susceptible (S), adopting the EUCAST clinical breakpoints for the EUCAST results (itraconazole, ≤1 mg/liter; posaconazole, ≤0.125 mg/liter; voriconazole, ≤1 mg/liter). The most notable MIC increase against *Cyp51A* mutants was observed for itraconazole (Table 1). For example, the EUCAST itraconazole MIC_{50} increased from 0.125 to >4 mg/liter, and all mutant isolates except one (an M220T isolate for which the MIC was 1 mg/liter) were classified as resistant (Fig. 1). Similarly, all isolates, with the exception of the two isolates harboring the M220T alteration (drug MICs of 0.06 mg/liter), were classified as intermediate or resistant to posaconazole. Finally, voriconazole retained activity against isolates with alterations at the G54 codon and slightly reduced activity against isolates with alterations at the M220 codon, whereas the TR_{34}/L98H and the HapE mutant isolates were classified as resistant. Again, the EUCAST MICs were slightly higher than the MICs obtained by the other methods (≤1 dilution step), but an overall excellent agreement was found between the MICs obtained by the different susceptibility methods.

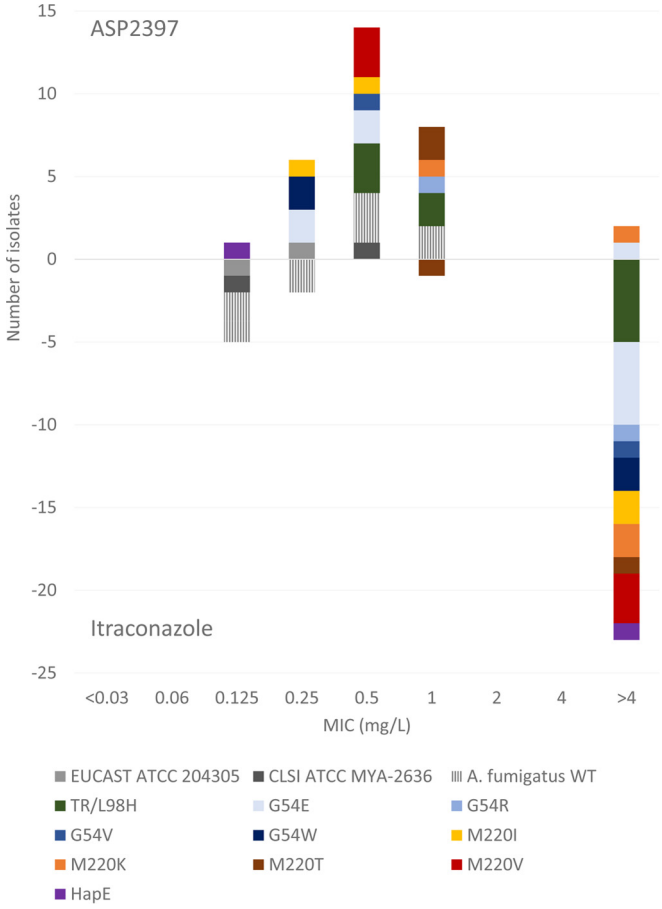

FIG 1 Histogram illustrating the uniform activity of ASP2397 (EUCAST MICs are shown above the *x* axis) across wild-type and azole-resistant *A. fumigatus* isolates compared to that of itraconazole (shown below the *x* axis). The strains are named according to the genotype, with "wt" indicating the wild-type strain versus the strains harboring different CYP51A alterations at position G54, M220, or TR/L98H. Reference strains are indicated with official strain numbers preceded by "EUCAST" or "CLSI."

Three *A. terreus* isolates, namely, two wild-type isolates and one isolate with a M217I alteration (corresponding to the M220I alteration in *A. fumigatus*), were included. The antifungal activity of ASP2397 against *A. terreus* appeared lower than against *A. fumigatus*, with CLSI MICs of 0.5 to 2 mg/liter, EUCAST MICs of 0.5 to >4 mg/liter, and mod-EUCAST MICs of 0.5 to 4 mg/liter (Table 1 and 2). Noticeably, the MICs were slightly higher against the M217I mutant isolate than against the wild-type isolates. This was also the case when either a less stringent visual minimum effective concentration (MEC) endpoint or a spectrophotometer reading with a 50% endpoint was used for the EUCAST tray (0.5 to 4 mg/liter) (Table 2). The amphotericin B MICs were within the expected range (1 to 4 mg/liter) for this species (Table 2). The efficacy of itraconazole and voriconazole against the M217I mutant was reduced, with MICs that were approximately 2 dilution steps higher than those against the wild-type isolates (the exact MIC could not be determined for posaconazole, as all endpoints were ≤0.03 mg/liter) (Table 2).

TABLE 1 MIC_{50} and MIC ranges for ASP2397 in comparison with amphotericin B, itraconazole, posaconazole, and voriconazole against *A. fumigatus* with or without *CYP51A* mutations[a]

Antifungal agent and isolate category	MIC_{50} (range) (mg/liter) obtained by the specified method				
	CLSI	EUCAST	EUCAST—partial inhib.	Modified EUCAST	EUCAST spec-50%
ASP2397					
Wild-type isolates	0.25 (0.06 to 0.25)	0.5 (0.25 to 1)	0.25 (0.25 to 0.5)	0.25 (0.125 to 0.5)	0.25 (0.25)
Cyp51A mutants	0.125 (0.06 to >4)[b]	0.5 (0.125 to >4)[c]	0.25 (0.15 to >4)[b]	0.25 (0.06 to >4)[b]	0.25 (0.06 to >4)[b,d]
Amphotericin B					
Wild-type isolates	0.25 (0.125 to 0.25)	0.25 (0.125 to 0.5)	ND	0.25 (0.125 to 0.25)	0.25 (0.125 to 0.5)
Cyp51A mutants	0.25 (0.125 to 1)	0.25 (0.125 to 1)	ND	0.25 (0.125 to 1)	0.25 (0.125 to 1)
Itraconazole					
Wild-type isolates	0.125 (0.06 to 0.25)	0.125 (0.125 to 0.25)	ND	0.125 (0.06 to 0.25)	0.06 (≤0.03 to 0.125)
Cyp51A mutants	4 (0.5 to >4)	>4 (1 to >4)	ND	>4 (0.5 to >4)	>4 (0.5 to >4)
Posaconazole					
Wild-type isolates	≤0.03 (≤0.03 to 0.06)	≤0.03 (≤0.03 to 0.06)	ND	≤0.03 (≤0.03)	≤0.03 (≤0.03)
Cyp51A mutants	0.5 (≤0.06 to 4)	0.5 (0.06 to >4)	ND	0.5 (0.125 to >4)	0.25 (≤0.03 to >4)
Voriconazole					
Wild-type isolates	0.25 (0.25 to 0.5)	0.5 (0.5 to 1)	ND	0.25 (0.25 to 0.5)	0.25 (0.125 to 0.5)
Cyp51A mutants	0.5 (0.125 to 4)	0.5 (0.25 to >4)	ND	0.5 (0.125 to 4)	0.25 (0.125 to 4)

[a] partial inhib., partial-inhibition endpoint; spec-50%, spectrophotometer reading using a 50% growth inhibition endpoint; ND, not done.
[b] One G54E isolate was found to be resistant (drug MIC of >4 mg/liter) to Asp2397 by all susceptibility tests methods used.
[c] One G54E isolate and one M220K isolate had drug MICs of >4 mg/liter using the full-inhibition endpoint—the drug MIC for the M220K isolate was >4 only using the full-inhibition endpoint (a week of growth at concentrations of 1 to 4 mg/liter) and not using the other methods.
[d] For one isolate, the growth curve crossed the 50% endpoint several times (trailing); thus, an automated endpoint could not be determined.

DISCUSSION

The *in vitro* testing of ASP2397 showed excellent *in vitro* efficacy against wild-type strains as well as azole-resistant mutants of *A. fumigatus*. Acknowledging the caveat that *in vitro* efficacy should always be confirmed *in vivo*, this is obviously a finding with promising implications at a time when azole-resistant *A. fumigatus* strains are emerging and the only fungicidal alternative is amphotericin B, with infusion-related side effects and renal toxicity (9–15). In this context, it is noteworthy that trailing growth was not a common phenomenon at the higher concentrations such as is found for the *Aspergillus* fungistatic echinocandins.

Overall, the efficacy of ASP2397 was method independent in the sense that clear endpoints were observed for the CLSI method, the EUCAST method, and the modified EUCAST method using a reduced inoculum concentration. However, the MICs were 1 dilution step higher for the EUCAST method than for the CLSI method. EUCAST has adopted a higher glucose and inoculum concentration, which facilitate increased growth, and higher endpoints have also been seen for other substances, including the new compound isavuconazole, when *A. fumigatus* isolates have been susceptibility tested using both methods (19, 20). Similarly, MICs obtained using a 50% growth inhibition endpoint read by a spectrophotometer yielded lower MIC values, as expected when a less stringent endpoint criterion is adopted. Therefore, clinical breakpoints would need to be method specific in order to obtain a reproducible classification of isolates as susceptible, intermediate, or resistant across the two reference methods.

One *A. fumigatus* isolate harboring the M220K mutation was categorized as ASP2397 resistant by the EUCAST method. This was due to weak growth in the EUCAST wells containing 1 to 4 mg/liter ASP2397. This isolate was, however, susceptible in testing using the partial-inhibition endpoint or 50% spectrophotometer endpoint as well as by the CLSI and the modified EUCAST methodology. The clinical significance of this finding remains unclear. Notably, however, another *A. fumigatus* isolate harboring the G54E alteration was found to be resistant to ASP2397 by all methods and using all endpoints. Some cryptic species of *A. fumigatus* have differential levels of susceptibility to azoles. Although the susceptibility of such species to ASP2397 has not been studied, *CYP51A* as well as β-tubulin sequencing was undertaken and confirmed the species identification as *A. fumigatus sensu stricto* (data not shown). ASP2397 was originally isolated from an isolate of *Acremonium* (16). *Acremonium* and *A. fumigatus* are both ubiquitous in the environment, and, hypothetically, a resistance selection could take place in the environment if these fungi were found in close proximity of each other and ASP2397 was secreted extracellularly. Future studies of ASP2397 resistance epidemiology and the underlying molecular genes and mechanisms involved in acquired resistance to this new compound are warranted. Nevertheless, it is comforting that no other isolates of *A. fumigatus* were found to be ASP2392 resistant either in our study or in a previous study of 53 isolates (16).

The *in vitro* activities of ASP2397 against *A. terreus* were higher than those found against *A. fumigatus*, which may suggest that ASP2397 is in general less efficacious against this organism, a trend that has been observed previously (16). Moreover, the MICs against the isolate harboring the M217I Cyp51A azole resistance mechanism were elevated. This finding was slightly surprising, as CYP51A is not seen as the target for ASP2397. Further studies are needed to explore the underlying mechanisms behind the MIC

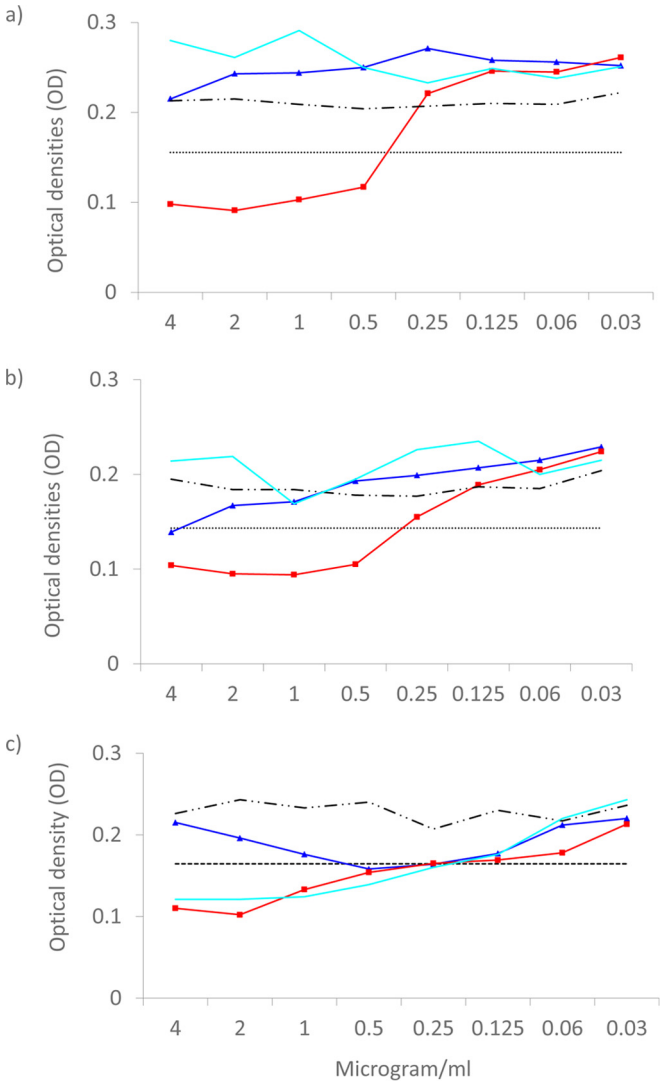

FIG 2 ASP2397 growth inhibition curves (light blue) for the atypical G54E and M220K mutant *A. fumigatus* isolates compared to those for itraconazole (dark blue) and posaconazole (red). (a and b) Growth curves for the G54E isolate determined by the EUCAST method (a) and a modified EUCAST method using a lower inoculum (b) show the fully resistant phenotype of this specific isolate. (c) In contrast, the phenotype of partial growth inhibition of the M220K isolate tested using the EUCAST method is shown. Results of growth in 8 growth control wells are shown by the upper broken black line. The horizontal dotted lines indicate 50% growth inhibition. Background absorbance in the medium corresponds to an optical density (OD at 490 nm) of approximately 0.1.

elevation against this isolate and whether it is a coincidence that the two isolates in this study that were ASP2397 resistant both harbored a CYP51A alteration, particularly if the molecular resistance mechanism is somehow related to *CYP51A* mutations or to compensatory molecular changes induced by the *CYP51A* mutations. However, a greater number of isolates and efficacy studies in animal models would be needed to understand the clinical implications of this preliminary finding.

In conclusion, the results of this study support the notion that ASP2397 exhibits good *in vitro* activity against a broad range of clinically relevant azole-resistant mutants. This is truly interesting in the context of increasing azole resistance.

TABLE 2 MICs for ASP2397 in comparison with amphotericin B, itraconazole, posaconazole, and voriconazole against *A. terreus* with or without *CYP51A* mutations

Antifungal agent and isolate category	MIC (mg/liter) obtained by the specified method				
	CLSI	EUCAST	EUCAST—partial inhib.[a]	Modified EUCAST	EUCAST spec-50%
ASP2397					
Wild-type isolates	0.5–1	0.5–1	0.5	0.5–4	0.25–0.5
Cyp51A mutant	2	>4	4	4	4
Amphotericin B					
Wild-type isolates	1–4	2–4	ND	2–4	1–2
Cyp51A mutant	2	1	ND	1	1
Itraconazole					
Wild-type isolates	≤0.03	≤0.03	ND	≤0.03–0.06	≤0.03
Cyp51A mutant	0.125	0.125	ND	0.125	0.06
Posaconazole					
Wild-type isolates	≤0.03	≤0.03	ND	≤0.03	≤0.03
Cyp51A mutant	≤0.03	≤0.03	ND	≤0.03	≤0.03
Voriconazole					
Wild-type isolates	0.25	0.5	ND	0.25–0.5	0.125–0.25
Cyp51A mutant	2	2	ND	2	1

[a] partial inhib., partial-inhibition endpoint; spec-50%, spectrophotometer reading using a 50% growth inhibition endpoint; ND, not done.

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