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ORIGINAL ARTICLE

Discovery of a new antifungal agent ASP2397 using a silkworm model of *Aspergillus fumigatus* infection

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Natural products are the major source of currently available drugs. However, screening natural product presents several challenges, including the time-consuming and labor-intensive steps required for the isolation of a drug from crude extracts as well as the differences between the activities of compounds *in vitro* and *in vivo*. To address these challenges, we used silkworm larvae infected with *Aspergillus fumigatus* to screen a natural products library for potent drugs to treat invasive aspergillosis. A rationally designed library was constructed using numerous, geographically diverse fungal species and then screened to collect extracts of microorganisms that had detectable anti-*Aspergillus* activity. We evaluated this library using cultures of *A. fumigatus* and a silkworm model system of *A. fumigatus* infection. With this model, we identified the novel antifungal compound ASP2397 that not only cured infected silkworm larvae but also increased the rates of survival of mice infected with *A. fumigatus*. These findings strongly support the utility of the silkworm screening system for the simple and rapid isolation of antibiotics from natural products libraries.
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INTRODUCTION

Invasive aspergillosis, which includes invasive pulmonary aspergillosis, is a life-threatening infection with increasing rates among patients with neutropenia, a bone marrow or solid-organ transplant or congenital or acquired immunodeficiency.[1] The mortality rates of invasive aspergillosis and invasive pulmonary aspergillosis exceed 50% in patients with neutropenia and 90% in recipients of hematopoietic stem cell transplants.[2,3] The clinical success rate of voriconazole (VRCZ) for treating patients with invasive aspergillosis is a disappointing 52.8%, despite its greater efficacy over deoxycholate amphotericin B (>31.6%).[4,5] Therefore, new and more effective drugs are required to treat aspergillosis.

Natural products are an excellent source of drugs.[6–8] Drug discovery currently employs mainly high-throughput screening of libraries of compounds synthesized in the laboratory. In contrast, bioassay-guided purification is required to screen natural products to identify bioactive secondary metabolites in extracts with complex compositions. Nevertheless, natural products are an attractive source of diverse compounds with unpredictable activities or modes of action.

Despite laborious and rigorous isolation processes, we and others sometimes encounter discrepancies between *in vitro* and *in vivo* activities of potential drugs. Thus, primary *in vivo* screening is an important step in the identification of potentially useful natural products. For example, we used mice infected with *Candida albicans* during the early drug discovery stage to identify novel antifungal compounds such as FR109615 (cispentacin)[9] and FR901379 (an intermediate of micafungin (MCFG))[10] in a library of natural products. In contrast, we were unsuccessful in our attempts using mice to establish such an assay for rapid (2 days) identification of antibiotics active against *Aspergillus fumigatus* infection. To address this problem, we decided to use silkworm larvae infection model that was previously shown to be as effective as mammalian models in evaluating the activities of antimicrobials.[11,12] However, this silkworm model[11,13] required modification to develop a simple and rapid method for evaluating the effects of natural products on infections caused by *A. fumigatus*. Both the screening method and a diverse, high-quality screening library are important for identifying novel compounds. For this purpose, we constructed a library of natural products derived from numerous fungal species isolated from geographically diverse regions of the tropics. We refined the library to prepare extracts from only those fungi with detectable anti-*Aspergillus* activity.

We demonstrate here the isolation of compound ASP2397 from this library and clarify its efficacy in treating mice with systemic aspergillosis infection.

MATERIALS AND METHODS

Compounds

ASP2397, AS2488059, AS2488053 and MCFG were prepared at Astellas Pharma (Tokyo, Japan). VRCZ, caspofungin (CAS) and amphotericin B (AMPHB)

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were purchased from Pfizer Japan (Tokyo, Japan), Merck (Kenilworth, NJ, USA) and Bristol-Myers Squibb (Tokyo, Japan), respectively.

Construction of a library of natural products with activity against *Aspergillus*

Colonies of microorganisms that inhibited the spread of *A. fumigatus* on agar plates were collected to construct a library with anti-*Aspergillus* activity. Briefly, a piece of an agar slant with a lawn of a test microorganism was placed on a CM-agar plate 1 cm from a drop of a solution containing 1×10^3 spores of *A. fumigatus*. CM medium contained glucose 0.5%, soluble starch (Nacalai Tesque, Kyoto, Japan) 1.5%, yeast extract (Wako Pure Chemical Industries, Osaka, Japan) 0.5%, KCl 0.02%, $MgSO_4 \cdot 7H_2O$ 0.02%, KH_2PO_4 0.1%, $NaNO_3$ 0.2% and Bacto agar 2%. After incubation for 1–2 weeks at 20 °C, we selected microorganisms that formed colonies that inhibited the growth of *A. fumigatus*. These microorganisms were cultured on a CM-agar slant (4 ml) for 7 days at 20 °C. An equal volume of acetone was added to the slant, which was incubated overnight at room temperature, to extract secondary metabolites. After centrifugation, the supernatant was concentrated under reduced pressure, and distilled water was added to adjust the volume to 400 µl.

Primary (*in vitro*) and secondary (*in vivo*) screening of compounds that inhibited *A. fumigatus* infection

The clinical isolate *A. fumigatus* FP1305 was provided by the Teikyo University Institute of Medical Mycology (Tokyo, Japan) and cultured on a potato dextrose agar slant for 4 days at 37 °C, after which spores were harvested in sterile saline and filtered through gauze. *In vitro* anti-*A. fumigatus* activity was measured in 384-well culture plates containing 20% silkworm hemolymph (Katakura Industries, Tokyo, Japan) medium with 0.165 M MOPS, pH 7.0. *A. fumigatus* FP1305 was inoculated into each well at a final concentration of 2×10^3 CFUs per well, and the plates were incubated for 20 h at 37 °C. Antifungal activity was defined by microscopic observation of a 50% reduction of fungal growth compared with the control well.

We modified a silkworm model of infection[11] to screen the library. Silkworm eggs (Fuyo Tukubane) were purchased from Ehime Sansyu (Ehime, Japan), and the hatched larvae were fed Silkmate 2S (Nosan Corporation, Yokohama, Japan) at 27 °C until the fourth molt. Fifth instar day 2 silkworm larvae fed Silkmate 2S overnight were first infected with *A. fumigatus* FP1305 conidia (50 µl, 3×10^7 CFUs per ml) injected into the hemolymph through the dorsal surface and then injected 1 h later with a sample of the library (50 µl per larva, $n = 2$) at 30 °C. All silkworms infected with *A. fumigatus* died and then turned black because of melanization at 43–46 h after infection, and the outcome was defined as the number of survivors at the time of each observation.

Antifungal activity

The anti-*A. fumigatus* activities of ASP2397, AS2488059 and AS2488053 were measured using the microbroth dilution method with 96-well culture plates containing RPMI-1640 medium (Invitrogen Japan, Tokyo, Japan), 50% silkworm hemolymph or 50% mouse serum (Nippon Bio-Supp. Center, Tokyo, Japan) buffered with 0.165 M MOPS, pH 7.0. *A. fumigatus* FP1305 was inoculated into each well at a final concentration of 1×10^4 CFUs per well. The plates were incubated for 20 h at 37 °C. Microscopy was used to determine the end point, defined as the lowest drug concentration that prevented discernible growth (MIC).

Effect of ASP2397 on mice with disseminated *A. fumigatus* infection

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Astellas Pharma. We injected female mice (ICR, 5 weeks old; Japan SLC, Shizuoka, Japan) intraperitoneally with 200 mg kg^{-1} cyclophosphamide on days −4 and 1 post infection (p.i.) to immunosuppress the mice. The inoculum was prepared from *A. fumigatus* FP1305 grown for 4 days on a potato dextrose agar slant. The spores were harvested in sterile saline and filtered through gauze, and then 2.5×10^5 spores were intravenously injected into the mice. ASP2397 and AS2488053 were prepared in 10% HCO-60 (polyoxyethylene (60) hydrogenated castor oil) in sterile saline, and MCFG was prepared in sterile saline. Drugs were intravenously administered to mice 1 h after infection and then once daily for 3 days ($n = 5$). Outcomes were determined using the Kaplan–Meier method, and statistical analysis was performed using the log-rank test (GraphPad Prism ver. 5.04; GraphPad Software, La Jolla, CA, USA).

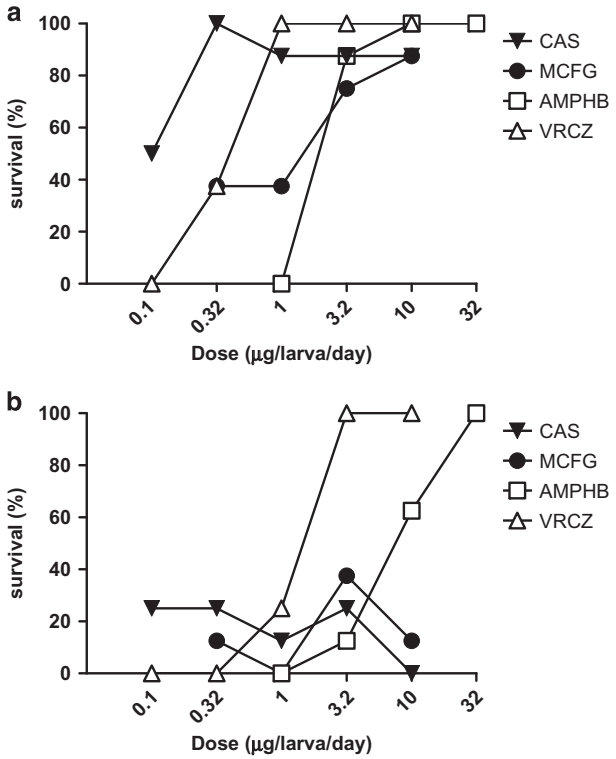

Figure 1 Effects of antifungals on the survival of silkworms infected with *Aspergillus fumigatus* ($n = 8$) at (**a**) 32 h and (**b**) 46 h post infection (p.i.).

RESULTS

Establishment of a silkworm model of *A. fumigatus* infection

Hamamoto *et al.*[11] established a model system of microbial infection using silkworms injected with *Staphylococcus aureus*, *Stenotrophomonas maltophilia*, *Candida albicans* and *Candida tropicalis*. Using this method, we established a silkworm model of *A. fumigatus* that required increasing the temperature from 27 to 30 °C to sustain infection, leading to 100% mortality within 48 h.

At 32 h p.i., 75% of untreated infected larvae were dead, and their bodies were white. In contrast, infected larvae treated with antifungal agents were still alive at this point (Figure 1a). All untreated infected larvae succumbed to the infection by 46 h p.i., and their bodies were black because of melanization. The fungistatic agents (CAS and MCFG) had no effect on survival, whereas the fungicidal drugs (AMPHB and VRCZ) were efficacious (Figure 1b).

Construction of a rationally designed library of potential anti-*Aspergillus* agents

We tested 4997 fungal strains for their ability to inhibit the growth of *A. fumigatus*, and 310 strains were selected as potential producers of anti-*Aspergillus* agents.

Screen for antifungal activity *in vitro* and *in vivo*

As described in 'Materials and methods', we first conducted an *in vitro* assay and then applied the samples with antifungal activity to the silkworm model. We identified one fungal extract that strongly inhibited the infection of silkworm larvae with *A. fumigatus*. We then

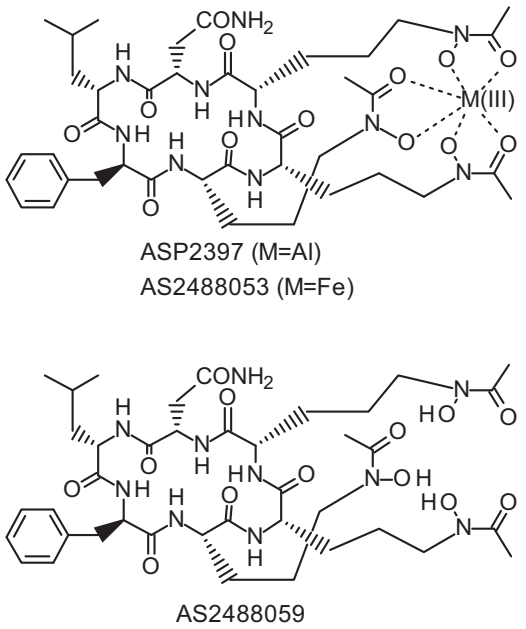

Figure 2 Structure of ASP2397, AS2488059 and AS2488053.

Table 1 Efficacies of ASP2397, AS2488059, AS2488053 and VRCZ against *Aspergillus fumigatus* infection of silkworm larvae 43 h p.i.

		AS compound (μg per larva per day)					*VRCZ (μg per larva per day)*		
Treatment	*Non treatment*	1.2	3.7	11.1	33.3	100	0.1	1	10
ASP2397	5/5	5/5	0/5	0/5	0/5	0/5	3/3	2/3	0/3
AS2488059	4/5			3/3	2/3	3/3	3/3	3/3	0/3
AS2488053	5/5	5/5	5/5	5/5	5/5	5/5	2/2	2/2	0/2

Abbreviations: p.i., post infection; VRCZ, voriconazole.
Number mortality/number inoculated.

used the silkworm system to guide the purification of the active compound, culminating with the isolation of the novel compound designated ASP2397 (Figure 2). We isolated AS2488059 that did not form a complex with Al(III) and prepared AS2488053 that chelated with Fe(III) as ASP2397 derivatives. We will report the identification in a subsequent study.[14]

ASP2397 protects silkworm larvae from *A. fumigatus* infection

We evaluated the effect of ASP2397 on silkworm larvae infected with *A. fumigatus* (Table 1). All untreated infected larvae were dead 43 h p.i., and their bodies were black. In contrast, all infected larvae treated with ASP2397 and VRCZ survived at the highest respective dose of each compound. In contrast, the infected larva with AS2488059 and AS2488053 did not survive.

Antifungal activity of ASP2397, AS2488059 and AS2488053 against *A. fumigatus*

We found that the MIC evaluated in serum-containing medium correlated accurately with preclinical *in vivo* effects of antifungals.[15] Using the broth-dilution method, ASP2397 was effective against *A. fumigatus* in 50% silkworm hemolymph medium, 50% mouse serum and RPMI medium at MIC values (μg ml^{-1}) of 0.16, 0.16 and 0.20, respectively (Table 2). On the other hand, AS2488059 and AS2488053 were not effective against *A. fumigatus* in serum-containing medium.

Table 2 *In vitro* antifungal activities of ASP2397, AS2488059, AS2488053 and AMPHB

	Antifungal activity (Aspergillus fumigatus FP1305) MIC (μg ml^{-1})		
Test substrate	*50% Silkworm hemolymph*	*50% Mouse serum*	*RPMI*
ASP2397	0.16	0.16	0.2
AS2488059	>50	12.5	0.2
AS2488053	>50	>50	0.1
AMPHB	2.5	1.25	1.25

Abbreviation: AMPHB, amphotericin B.
MIC is the lowest drug concentration that prevented any discernible growth.

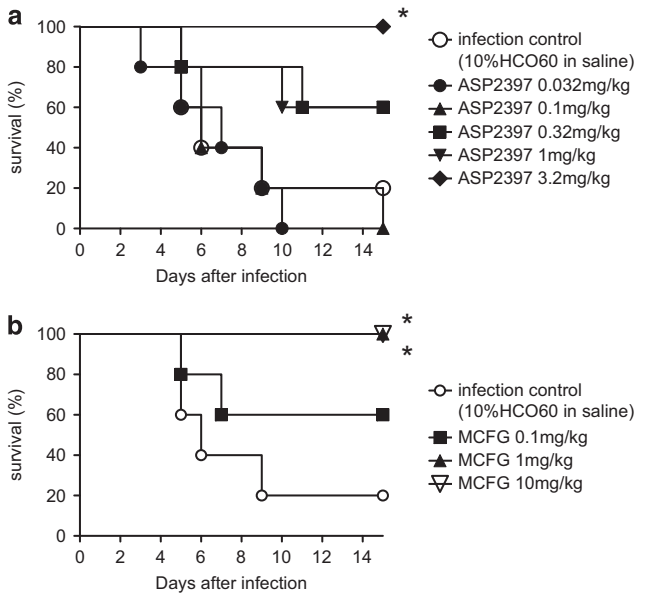

Figure 3 Efficacies of ASP2397 (**a**) and micafungin (MCFG) (**b**) against mice infected with *Aspergillus fumigatus* (*$P<0.05$, log-rank test, $n=5$).

Evaluation of the effects of ASP2397 and AS2488053 on the survival of mice infected with *A. fumigatus*

We next assessed whether ASP2397 and AS2488053 administered intravenously protected mice from infection with *A. fumigatus*. The data in Figure 3 show that 3.2 mg kg^{-1} of ASP2397 significantly prolonged survival. MCFG was effective as well. On the other hand, AS2488053 did not show the survival effect at 32 mg kg^{-1}.

DISCUSSION

In the present study, we describe a rationally designed library of natural products that we used to rapidly and readily identify a novel compound (ASP2397) that protected mice from infection with *A. fumigatus*. An important component of the success of this system was a simple modification (increasing the incubation temperature) of a published method for infecting silkworm larvae with bacterial and yeast pathogens.

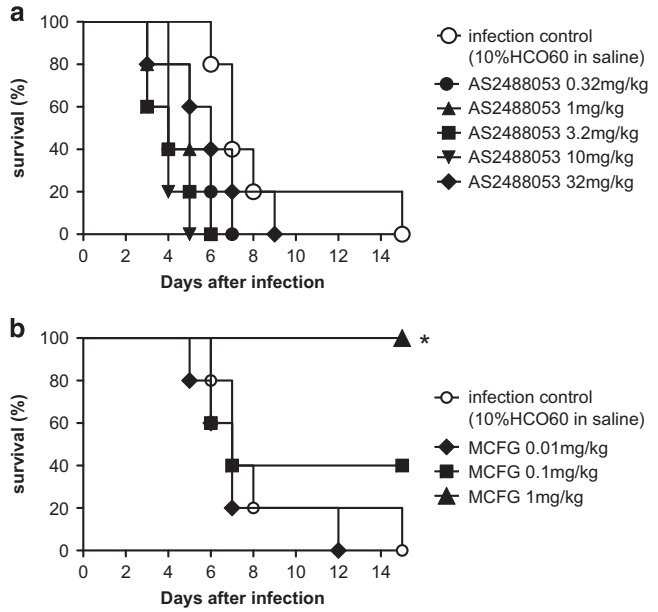

Figure 4 Efficacies of AS2488053 (**a**) and micafungin (MCFG) (**b**) against mice infected with *Aspergillus fumigatus* (*$P<0.05$, log-rank test, $n=5$).

Libraries of natural products derived from microorganisms comprise a mixture of secondary metabolites. Although such mixtures are a rich source of novel compounds, many components interfere with the detection of pharmacologically active molecules. For example, certain compounds may be highly cytotoxic to the host (cells or organisms), and the optical properties of these or other compounds may interfere with detection techniques. In an attempt to circumvent these frequently encountered problems, we evaluated the following variables: (1) diversity of producing microorganisms, (2) quality of assay samples comprising a mixture of secondary metabolites and (3) an *in vivo* method that circumvents the rate-limiting step in screening for natural products called dereplication.

First, we presumed that the diversity of a library depends on its source of microorganisms. Here we isolated geographically diverse fungi, and isolated ASP2397 from an *Acremonium* sp. collected from a tropical forest. Second, because countless strains of microorganisms are present in soil or leaf litter, we constructed a library of fungal extracts that inhibited the growth of *A. fumigatus*. We screened ~38 000 samples and identified only 1 compound, ASP2397, with acceptable antifungal activity. The success rate of the rationally designed library was ~100-fold higher compared with that of a random library (0.3% versus 0.0026%). Third, we implemented a new screening method that can be evaluated after 2 days for activity against *A. fumigatus* infection. The key element of this method was raising the temperature of the silkworm larvae rearing environment from 27 to 30 °C. We attempted to use different screening methods that employ cultured cells, genetic manipulations using gene disruption or overexpression as well as rodent models. Phenotypic screening using an animal model may more faithfully represent findings from clinical trials. Although laboratory rodents serve this purpose, the silkworm system[11,13] is advantageous for the following reasons: only a single injection of test compound is required because of the silkworm larva's small size, the assay is fast, the assays' end point is easy to measure (color of dead or living larvae) and silkworm larvae are much easier to maintain and manipulate than rodents.

During our first attempts to purify the active compound, we used the *in vitro* antifungal assay; however, we were unable to isolate an active fraction that was effective in the silkworm model. AS2488059 did not affect the survival of infected silkworm larvae, despite demonstrating *in vitro* activity (Tables 1 and 2). Next, we relied solely on the silkworm assay to guide the purification and succeeded in isolating ASP2397. ASP2397 was active against infected silkworm larvae and was fungicidal, similar to VRCZ. Furthermore, ASP2397 significantly improved the rate of survival in mice with disseminated *A. fumigatus* infection. In contrast, AS2488053 was not active in serum-containing medium and did not affect the survival of infected silkworm larva and mice (Tables 1 and 2 and Figure 4).

In conclusion, we established the silkworm infection model and a rationally designed library to discover unique natural products with potent anti-*A. fumigatus* activity. Using this system, we isolated ASP2397 that exhibited a potent effect on the survival of infected mice. The detailed characterization of the biological and physiochemical properties of this newly discovered antifungal agent and those of its derivatives as well as the identification of its source and fermentation techniques will be reported in a subsequent study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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