Table 5 Treatment-related adverse events and reasons for discontinuation from study of patients who received at least one dose of study drug.

	micafungin (n = 53)		voriconazole (n = 54)	P
Serious	1 (1.9%)	Serious	4 (7.4%)	
Disseminated intravascular coagulation	1 (1.9%)	Ventricular extrasystoles	1 (1.9%)	
		Hepatic events	1 (1.9%)	
		Dizziness	1 (1.9%)	
		Nausea	1 (1.9%)	
Treatment discontinuation	3 (5.7%)	Treatment discontinuation	7 (13.0%)	
Rash	2 (3.8%)	Hepatic events	4 (7.4%)	
Vomiting	1 (1.9%)	Hyperkalemia	1 (1.9%)	
		Ventricular extrasystoles	1 (1.9%)	
		Dizziness	1 (1.9%)	
Overall	14 (26.4%)	Overall	33 (61.1%)	0.0004
Hepatic events	8 (15.1%)	Hepatic events	19 (35.2%)	0.025
Visual events	0 (0%)	Visual events	15 (29.6%)	<0.0001
Overall excluded visual events	14 (26.4%)	Overall excluded visual events	27 (50%)	0.012
Rash	3 (5.7%)	Hallucination	4 (7.4%)	
Vomiting	2 (3.8%)	Dizziness	2 (3.7%)	
Renal events	1 (1.9%)	Renal events	1 (1.9%)	
Blood pressure increased	1 (1.9%)	Hyperkalemia	1 (1.9%)	
Eosinophil count increased	1 (1.9%)	Ventricular extrasystoles	1 (1.9%)	
White blood cell count decreased	1 (1.9%)	Pseudomembranous colitis	1 (1.9%)	
Disseminated intravascular coagulation	1 (1.9%)	Nausea	1 (1.9%)	
		Headache	1 (1.9%)	
		Restlessness	1 (1.9%)	
		Hemoptysis	1 (1.9%)	
		Skin discoloration	1 (1.9%)	

Our study also revealed the important clinical aspects of patients with CPA in Japan. The mean age of the patients with CPA was 70.9 years old and most patients were men with malnutrition and pulmonary complications, especially tuberculosis sequelae. Smith et al. showed that tuberculosis sequelae are also a major risk factor in patients with CPA in United Kingdom.[23] Only the *Aspergillus* antibody test by the *Aspergillus* immunodiffusion system showed a reasonably high positive rate with 88.6% of all patients, which resembled the previous results by Denning et al.[2,5] The common serum antigen detecting tools for invasive *Aspergillus* infection are not suitable for CPA diagnosis.

In conclusion, this first large-scale prospective clinical trial comparing intravenous MCFG and VRCZ for patients with CPA indicated that MCFG and VRCZ showed good effectiveness without statistical difference, although significantly fewer adverse effects were observed in the MCFG group.

Appendix

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Short Communication

Micafungin Alters the Expression of Genes Related to Cell Wall Integrity in *Candida albicans* Biofilms

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SUMMARY: We investigated whether treating *Candida* biofilms with micafungin, an echinocandin that inhibits the synthesis of glucan in the fungal cell wall, alters the expression of genes related to chitin synthesis and degradation in response to cell wall stress. As expected, all four genes encoding chitin synthases—*CHS1*, *CHS2*, *CHS3*, and *CHS8*—were upregulated by micafungin treatment. Interestingly, of the four genes encoding chitinases, the expression of only *CHT2* and *CHT3* was markedly downregulated, that of *CHT1* was upregulated, and that of *CHT4* remained unaltered after micafungin treatment. Thus, the suppression of only two genes associated with chitin degradation, *CHT2* and *CHT3*, may be involved in the tolerance to the cell wall stress caused by micafungin as well as the induction of chitin synthesis. Further, micafungin markedly increased *UTR2*, which is calcineurin dependent, and *CRZ2*, which is calcineurin independent. Therefore, gene regulation possibly includes calcineurin-dependent and independent stress responses, though the regulation of genes associated with cell wall chitin has not yet been completely clarified. Our results imply that cell wall stress can be exploited to enhance the efficacy of micafungin.

Echinocandins are a relatively new class of antifungal drugs that inhibit the synthesis of glucan in the fungal cell wall. The recent clinical practice guidelines for the management of candidiasis released by the Infectious Diseases Society of America recommended echinocandins as the primary or alternative treatment for several types of candidiasis (1). Micafungin (MFG), the sole echinocandin available in Japan, is as effective as and causes fewer adverse events than liposomal amphotericin B as the first-line treatment for candidemia and invasive candidiasis (2). Though MFG is also active against *Candida albicans* biofilms, which are notorious for being resistant to antifungals, these biofilms are somewhat tolerant to MFG; therefore, a higher dose of MFG is required for killing biofilms than for inhibiting the growth of planktonic cells (3–5). A known mechanism of resistance is hot spot mutations of *FKS1*, a target of echinocandins (6); however, it was also suggested that the tolerance of *Candida* biofilms is not related to the genotype but to the phenotype, and is at least partly a response to cell wall stress (3). To further understand the mechanism underlying this resistance, we investigated whether treating *C. albicans* biofilms with MFG altered the expression of genes related to chitin synthesis and degradation in response to cell wall stress.

SC5314, a standard *C. albicans* strain from our collection, was used in this study (3). MFG was provided by Astellas Pharma Inc. (Tokyo, Japan); it was dissolved in distilled water to make the stock solution (10 mg/ml), and stored at −20°C until use. The minimum inhibitory concentration of MFG against planktonic cells has previously been determined to be 0.031 μg/ml (7). Biofilms were prepared as described in our previous study (3); briefly, small silicone elastomer (SE) disks were immersed in a *Candida* cell suspension for 90 min to allow attachment, and the biofilms were allowed to form by further incubating the disks in yeast nitrogen base (YNB) with 2% glucose for 24 h. The SE disks with the attached biofilms were soaked in a medium containing 0.5 μg/ml of MFG and incubated at 37°C for 4 h, following which the total RNA was extracted using the hot phenol method described previously (8). Approximately 800 ng of total RNA was used as a template to synthesize the cDNA (final volume, 20 μl). The gene expression levels were measured using the TaqMan Gene Expression Assay and the ABI 7000 Real-Time PCR system (Applied Biosystems, Foster City, Calif., USA) with 96-well optical reaction plates (each reaction mixture containing 1 × SYBR Green PCR Master mix (Invitrogen, Carlsbad, Calif., USA), 0.15 pmol/ml each of the forward primer and reverse primer, and 1 μl of template cDNA (equivalent to approximately 40 ng of total RNA); final volume, 10 μl). The cycling profile was as follows: 40 cycles each at 95°C for 5 s, 55°C for 30 s, and 72°C for 30 s. The real-time PCR data were acquired and analyzed using the 7000 System SDS software, version 1.0 (Applied Biosystems). The actin gene, *ACT1*, was used as the internal control, and all expression values were normalized against *ACT1*. The sequences of the RT-PCR primers are listed in Table 1. The data were analyzed using Student's *t* tests. The data are presented as fold changes in comparison to the control (untreated) and the mean ± standard error of repli-

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Table 1. RT-PCR primers used in this study

Primer	Sequence (5′→3′)	Reference
HSP90 forward	GGTTGCTGATCACGTCCAAGTT	This study
HSP90 reverse	AACTTACCACCAGCGTTAGATTCC	This study
UTR2 forward	GATTCTGGTAGTAGTGGAAGCAGTTCT	3
UTR2 reverse	ATGGAAGCAAATATACCACTG ATAACAC	3
CRZ1 forward	GGATGATGGGTCACAGCTATATGA	This study
CRZ1 reverse	TGATCAGTATCGGCTTTCCTGAT	This study
CRZ2 forward	GTCTAATTTGCCTATTGTTGCAGGTAT	This study
CRZ2 reverse	TGGTATTGACAGATCCGGAAGTG	This study
CHS1 forward	GTTGGTGGCAAAGCAGGTAAC	This study
CHS1 reverse	CTCGGTTCTGGTCAACACTTTTC	This study
CHS2 forward	GGGAAAGATTCATGGAAGAAAATTG	This study
CHS2 reverse	TGCTTGTGCTCTTTCATTAATCTTTG	This study
CHS3 forward	GCTCTTTCCAAGACTTTTAAATATACCACTA	This study
CHS3 reverse	TACCACGTTTACCATATCCGTTACG	This study
CHS8 forward	GGTAATAAATCAGGTCAAACGAATGC	This study
CHS8 reverse	CCAGCGACAATCCATAAAATGA	This study
CHT1 forward	CAAGCTTTGAGTGGATCAGGAA	This study
CHT1 reverse	TCTTGTGAGTGGTGTGTTTGGTT	This study
CHT2 forward	TGATTTATTATCCAAAGTCCCACTTG	This study
CHT2 reverse	TTGAATTGGCCATTGATTGAA	This study
CHT3 forward	TGCTACTATTCCAGATGACAAAGAAATT	This study
CHT3 reverse	TTCAGTGATGATAGCAGGTGGTTT	This study
CHT4 forward	CAACCCCATTATTTAAGACTTGTGTTT	This study
CHT4 reverse	TCAATTGGTATGTCTTGTGGGAAA	This study
ACT1 forward	TTGGTGATGAAGCCCAATCC	3
ACT1 reverse	CATATCGTCCCAGTTGGAAAC	3

cates (n = 4). The data are representative of three or more individual experiments.

The maintenance of cell wall integrity is important for growth, development, and survival, so that fungal cells can counteract environmental stress. Although chitin is only a minor component of the fungal cell wall, it provides mechanical strength, and an increase in the chitin content may compensate for damage to the cell wall structure. In *C. albicans*, four chitin synthases are currently known. As expected, MFG increased the expression of genes encoding all four chitin synthases—*CHS1*, *CHS2*, *CHS3*, and *CHS8*—approximately twofold compared to the control (Fig. 1A). This implies that induction of chitin synthesis might contribute to cell survival when the cell wall is damaged.

On the other hand, the cell wall should maintain not only strength but also flexibility. Thus chitin degradation is also required for remodeling chitin in the cell wall of fungi (9). Chitinases contribute to chitin degradation and four genes of those are known. MFG reduced the expression of the two chitinase genes, *CHT2* and *CHT3*, increased that of *CHT1*, and did not alter that of *CHT4* (Fig. 1B). The four chitinases in *C. albicans* appear to have different functions: *CHT3* is responsible for a major part of the chitinase activity in cells; *CHT2*, for a minor part; and *CHT1* and *CHT4* contribute little (9). Our results could be reflective of each function. In addition, Drakulovski et al. recently reported a FKS1-independent mechanism of resistance to caspofungin, an echinocandin in *C. albicans* (10). They showed that in a caspofungin-resistant isolate, point mutations were present in *CHT2* and *CHT3*, but that no mutations were present in *CHT1* and *CHT4*. Our results are consistent with their report, and they strongly imply that the reduced expression of *CHT2* and *CHT3* genes might be related to the tolerance against cell wall stress.

Next, we investigated whether the expression of genes related to calcineurin-dependent and -independent stress responses was altered by MFG. Cell wall stress is thought to be at least partly dependent on calcineurin, which is an important stress-response element and also an effecter of heat shock protein 90 (hsp90) (11). *UTR2* contains a calcineurin-dependent response element (CDRE) in its promoter, and was highly induced by MFG, as expected (Fig. 1C) (12). Interestingly, MFG also induced *CRZ2*, which is thought to be independent of calcineurin (12). Thus, our results indicate that not only calcineurin-dependent but also calcineurin-independent pathways are involved in the induction of cell wall stress by MFG. Further, the slight induction of *HSP90* and *CRZ1* (Fig. 1C), which can activate calcineurin-dependent gene expression through a CDRE found in the promoters of several genes such as *UTR2* (12), might partially contribute to promoting calcineurin-dependent stress responses. The relationship between such stress responses and gene regulation of chitin homeostasis is still hypothetical, but further investigation may identify the elaborate mechanism underlying the regulation.

In conclusion, we speculate that stress response and chitin metabolism could be related to the mechanism underlying the tolerance of *Candida* biofilms to antifungal agents. We previously showed that a chitin synthase inhibitor and an hsp90 inhibitor enhanced the effect of

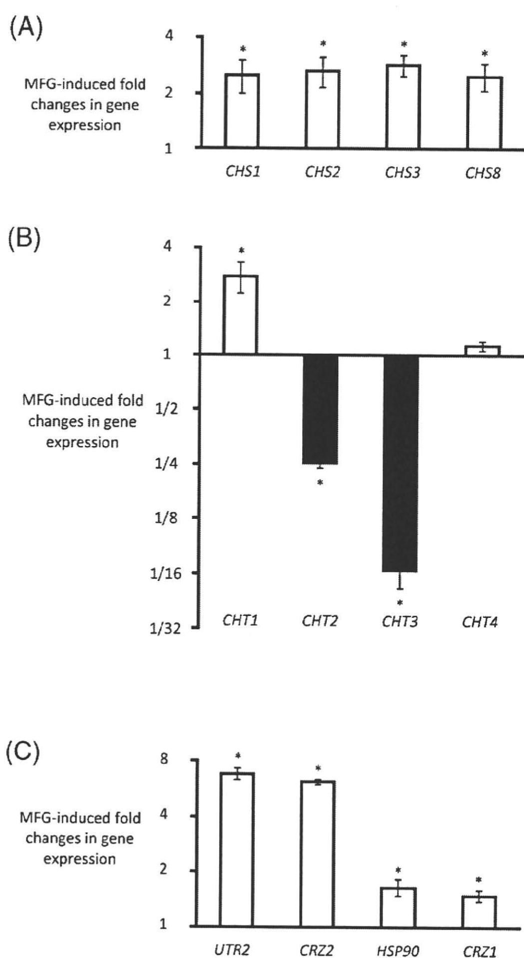

Fig. 1. Fold changes in the gene expression of four chitin synthases, four chitinases, and stress response-related factors under micafungin (MFG) treatment of *Candida* biofilms. (A) Genes encoding all four chitin synthases were induced by MFG. (B) The expression of two chitinase-encoding genes, *CHT2* and *CHT3*, was reduced more than fourfold. *CHT1* was induced and *CHT4* was not altered by MFG. (C) *HSP90* and *CRZ1* were slightly induced, and *UTR2* and *CRZ2* were markedly induced by MFG. White bar indicates an increase and black bar indicates a decrease in gene expression. *$P < 0.05$ compared to control.

MFG against *Candida* biofilms (3). Thus, our results may be beneficial for the effective management of candidiasis, because they suggest that exploiting cell wall stress could be a reasonable approach for enhancing the efficacy of MFG.

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Conflict of interest None to declare.

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EDITORIAL COMMENTARY

Posaconazole for Chronic Pulmonary Aspergillosis: The Next Strategy against the Threat of Azole-Resistant *Aspergillus* Infection

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(See the article by Felton et al, on pages 1383–1391.)

Chronic pulmonary aspergillosis (CPA) is a complex and slowly progressive inflammatory disease caused by *Aspergillus* species. There are 3 major unsolved issues regarding this disease: the complexity of the disease, the scarcity of clinical evidence for its management, and the drug resistance of *Aspergillus.*

The pathophysiology of CPA ranges widely, from aspergilloma to semi-invasive types of infection, such as chronic necrotizing pulmonary aspergillosis and chronic cavitary pulmonary aspergillosis or chronic fibrosing pulmonary aspergillosis. These subtypes of CPA have recently been proposed and the recommendations for treatment of each type are outlined in the latest guidelines from the Infectious Diseases Society of America [1,2]. However, Hope et al [3] reported that apparent distinct entities do not exist for this syndrome and these subtypes usually overlap.

This causes difficulties in comparing more recent efficacy results for antifungal drugs with previous data regarding CPA management. It is difficult to establish a simple definition for this disease entity because of the complex backgrounds of CPA patients, such as the presence of chronic underlying pulmonary diseases (eg, tuberculosis sequelae, bronchiectasis, chronic obstructive pulmonary disease, and pulmonary fibrosis) and/or mild immunosuppression (eg, due to low-dose steroid therapy, diabetes, collagen diseases, and/or alcohol abuse) as well as coinfection with other microorganisms. Thus, it is also difficult to conduct large-scale randomized clinical trails for patients with CPA.

In this issue of the journal, Felton et al [4] report on the efficacy and safety of posaconazole for treatment of CPA and describe response rates of 61% at 6 months and 46% at 12 months, with a relatively low incidence of side effects. The study evaluated a total of 79 CPA patients who were administered posaconazole. The definition of CPA was that the following findings were present: (1) progressive pulmonary cavitation with associated cavity wall or pleural thickening on chest radiography or cross-sectional imaging, (2) positive *Aspergillus* antibody titer or isolation or visualization of *Aspergillus* species in a biopsy specimen from the lung or pleura, (3) elevated values for inflammatory markers (C-reactive protein level or plasma viscosity), (4) constitutional or pulmonary symptoms lasting for at least 3 months, (5) exclusion of other causes that can mimic this syndrome (eg, pulmonary malignancy), and (6) no significant systemic immunosuppression. Denning et al [1] previously proposed enrollment criteria for prospective clinical studies of CPA, and the criteria for CPA in the study by Felton et al [4] basically follow those criteria. The definition proposed by Denning et al [1] is simple and practical for conducting studies.

These criteria, however, possess some difficulties for clear interpretation in details. For example, coinfection with other bacteria, such as *Pseudomonas aeruginosa* or mycobacteria, is not uncommon in many patients with CPA, and it would be very difficult to confirm that only *Aspergillus* is involved in each case. It is also difficult to distinguish significant and mild systemic immunosuppression, as there are no good tools for assessment. It is, however, reasonable to evaluate the overall clinical efficacy of treatment by evaluating both clinical and radiological data, since other serological and microbiological data (such as *Aspergillus* antigen or antibody level, (1,3)-β-D-glucan level, and the results of culture) are not typically correlated with the strength of response to antifungal drugs.

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We assumed that many of the cases investigated in the study of Felton et al [4] were cases of chronic cavitary pulmonary aspergillosis; however, there may be cases more accurately characterized as chronic necrotizing pulmonary aspergillosis or aspergilloma. Questions remain about the differences in the appearance of CPA on chest X-ray films and its correlation with the effectiveness of posaconazole therapy. It is difficult to compare the results given by Felton et al [4] directly with previous data on treatment with other azoles, such as itraconazole and voriconazole. It is apparent that even the newer azole, posaconazole, possesses low efficacy rates for the treatment of CPA. The reported efficacy of oral itraconazole therapy varied widely, with an approximate range of 30%–82%, and that of oral voriconazole ranges from 53% to 65% after administration for several months [5]. The efficacy of itraconazole and voriconazole in these studies was mostly assessed on the basis of clinical, radiological, and mycological improvement at the end of treatment or at a regular interval, regardless of whether there was a partial or complete response. These wide ranges in efficacy are due to the differences in the definition of CPA, the evaluation methods, and the endpoints of each study and the duration of treatment. However, it is apparent that oral azole formulations, including posaconazole, do not possess sufficient efficacy for CPA.

The prevalence of azole-resistant *Aspergillus fumigatus* isolates is reported to be increasing, mainly in the United Kingdom and The Netherlands, and is becoming major clinical concern [6]. Unlike drug resistance in bacteria and *Candida* species, resistance in *A. fumigatus* has not been paid attention in the last decade. One of the major reasons is that there was no standardized drug susceptibility test. However, in the last few years, universal methods for drug susceptibility testing have become available, such as those recommended by the Clinical and Laboratory Standards Institute (M38-A2) and the European Committee on Antimicrobial Susceptibility Testing, as well as tentative clinical breakpoints [7]. It is very important that we realize that some azole-resistant strains have been isolated from patients with CPA, and many of these case patients had been exposed to azoles for an extended duration (1–30 months) [6, 8]. Although oral administration of azoles is the mainstay of treatment for CPA, long-term administration potentially induces azole resistance. This means that the more we use azoles to treat CPA patients, the fewer treatment options we will have. Felton et al [4] note that they recovered 4 isolates that showed a minimum inhibitory concentration of >8 mg/L for posaconazole, and posaconazole treatment failure was observed in all 4 affected patients. Although Felton et al [4] did not describe in detail how they treated these patients, possible options in such cases would be intravenous amphotericin B or intravenous echinocandins, both of which are unavailable in oral formulations. Using other azoles, such as itraconazole or voriconazole, is another option, but cross-resistance among azoles is carefully considered before administration.

We recently published the results of the first large-scale prospective study comparing intravenous micafungin and intravenous voriconazole for treatment of CPA [9]. There was a favorable response rate with both micafungin therapy (60.0% of patients) and with voriconazole therapy (53.2% of patients); there were fewer side effects in the micafungin group than in the voriconazole group (26.4% vs. 61.1% of patients) [9]. Originally, the study was conducted because intravenous antifungal agents may have an important role as induction therapy for patients with CPA or may be required for patients whose infection is refractory to oral antifungal drugs or who develop severe disease. The utility of intravenous antifungal drugs has not been evaluated for CPA, as they are very expensive and require hospital admission. Infection with azole-resistant *Aspergillus*, however, needs to be considered as infection refractory to oral antifungal drug therapy; a trial would be useful. We are also currently conducting a comparative study of liposomal amphotericin B and intravenous voriconazole for treatment of CPA. We believe that data on such intravenous antifungal drug therapies will be important for future clinical management of CPA; however, these agents would be only a temporary option for treatment (for at most a month), and the problems related to maintenance therapy using oral antifungal drugs still remain. Thus, it is very important to minimize the emergence of azole-resistant strains in clinical settings.

As there may be a relationship between drug exposure and the emergence of drug resistance, the appropriate use of drugs on the basis of their pharmacokinetic and pharmacodynamic properties and therapeutic drug monitoring is important, particularly in patients with CPA. Felton et al [4] report that they maintained serum posaconazole concentrations above 0.5 mg/L, but no data on the interactions between adverse effects and serum posaconazole concentrations were discussed in the study. We believe that therapeutic drug monitoring for azoles may have an important role in achieving maximum efficacy with minimum side effects. Furthermore, it will be necessary to prevent the development of drug resistance. Importantly, studies on drug exposure and azole resistance are urgently required.

In conclusion, Felton et al [4] provide new evidence on the use of posaconazole in the management of CPA; however, efficacy remains unsatisfactory. There is a clear need for the development of better antifungal drugs and for more studies regarding drug resistance.

Acknowledgments

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Short Communication

The Effects of an Hsp90 Inhibitor on the Paradoxical Effect

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SUMMARY: It is important to conserve the effectiveness of antifungal agents because the options for currently available agents are limited. Although echinocandins, which have been developed in recent decades, are highly active against a broad spectrum of fungi, one concern is their reduced activity against *Candida albicans* at high drug concentrations, which is known as the paradoxical effect. To date, resistance related to the paradoxical effect has not been reported in clinical situations, but some in vivo data suggest that the paradoxical effect potentiates the emergence of resistance. It is valuable to investigate the underlying mechanisms of as well as strategies against this paradoxical resistance. Previous reports imply that the paradoxical effect might be related to stress responses. In this study, we report that radicicol, a heat shock protein 90 (Hsp90) inhibitor, reduces the paradoxical effect of micafungin. We also confirm that radicicol reduces the tolerance to voriconazole, one of the new azoles, which is consistent with a previous report. Our results may therefore imply that common stress responses might exist in the paradoxical resistance to micafungin and also the tolerance to voriconazole, and may suggest that inhibiting Hsp90-related stress responses could help to avoid potential resistances.

The emergence of resistance is one of the problems in intractable infections such as fungal infections. Because the options for currently available antifungal agents are still limited, it is important to maintain the effectiveness of these agents against fungi as long as possible (1,2). Though echinocandins have been developed in recent decades and are highly active against a broad spectrum of fungi, including *Candida* and *Aspergillus* spp., there are concerns regarding their reduced activity at high drug concentrations, which is known as the paradoxical effect (PE) (3). To date, the PE has been linked to upregulation of homeostatic cell wall stress responses such as calcineurin (4,5). Since heat shock protein 90 (Hsp90) is one of the key stress response components, we investigated whether an inhibitor of Hsp90 could reduce the PE of *Candida albicans*.

SC5314, a standard *C. albicans* strains from our collection, was used in this study (6). Micafungin (MFG), one of the echinocandins, and voriconazole (VRC), one of the new azoles, were kindly provided from Astellas Pharma Inc. (Tokyo, Japan) and Pfizer Japan Inc. (Tokyo, Japan), respectively. Radicicol (Rad, Hsp90 inhibitor; MIC = 8 μM) was purchased from Sigma-Aldrich (St. Louis, Mo., USA). MFG was dissolved in distilled water, and VRC and Rad were dissolved in dimethylsulfoxide (DMSO) for stock solutions, and they were stored at −20°C before use. We used yeast nitrogen base medium (YNB) (Difco Laboratories, Detroit, Mich., USA) instead of RPMI medium, which is recommended as a standard medium by the Clinical and Laboratory Standards Institute (CLSI) because growth is slow in RPMI, making it difficult to detect paradoxical growth.

After overnight preculture in YNB supplemented with 2% glucose, approximately 2×10^3 cells were inoculated in each well of 96-well plates. Each well contained 200 μl of YNB with the indicated concentrations of MFG or VRC; without Rad or with 1 μM Rad. The maximum final DMSO concentration (0.1%) was included in assays performed in the absence of Rad to control for any solvent effects. After a 24-h incubation period, the cell growth was compared using an XTT assay. Though the XTT assay is not usually used for examining planktonic cell growth, this assay is useful for detecting small amounts of cell growth such as that occurring with paradoxical growth because it is more sensitive than simple optical density measurements. The method was slightly modified from that previously described (7). In brief, the plates were centrifuged to spin down the organisms, the medium was removed, and 200 μl of PBS containing 50 μg/ml of XTT (Sigma-Aldrich) and 4 μM menadione (Sigma-Aldrich) was added to each well. After a 1-h incubation period, the absorbance at 490 nm/630 nm was measured by plate reader and values were normalized to the control (neither an antifungal agent nor Rad) as the relative cell growth. Each condition was quadruplicated. *P* values were calculated using the Student's unpaired *t* test.

As previously described for the PE, MFG severely inhibited cell growth to less than 1% at 0.031 and 0.063 μg/ml, and higher concentrations of MFG were less effective (Fig. 1A). The addition of 1 μM Rad, which alone did not alter growth, reduced the paradoxical cell growth at high concentrations of MFG. We confirmed that cyclosporin A, which is a calcineurin inhibitor, also attenuated the PE, as previously described (4) (data not shown). These results are consistent with the results from previous studies showing the relationship between the PE and stress-related cell integrity pathways (3-5,8,9). Rad also reduced the tolerance to VRC, as previously noted (Fig. 1B) (10). These results may suggest that the paradoxical resistance to MFG depends on the same stress responses as the tolerance to VRC.

Cowen et al. first reported that an Hsp90 inhibitor did not alter the sensitivity to caspofungin, an echinocandin, but they also recently reported that an Hsp90 inhibitor enhanced the

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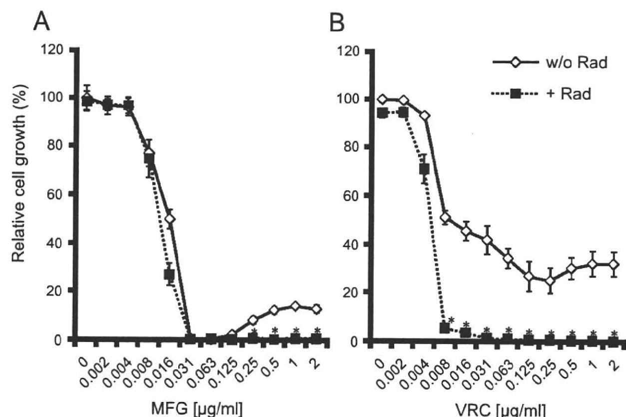

Fig. 1. Relative cell growth at various concentrations of MFG (A) or VRC (B); when used without Rad (w/o Rad) or with 1 μM Rad (+ Rad). All values are as a percentage of cell growth (XTT activity) in a control with neither antifungal agent nor Rad.
$^*P < 0.01$ compared to corresponding value without Rad.
MFG, micafungin; VRC, voriconazole; Rad, radicicol.

fungicidal effects of MFG (10,11). Their latter finding might be related to our results.

It remains controversial whether the PE phenotype is clinically important. Only a mutation of *fks1*, which is a target of echinocandins, has been proven to cause clinical failure with echinocandins to date, and it has been reported that the PE is not due to the *fks1* mutation (9,12,13). However, some studies have found the PE in vivo as well as in vitro, and accordingly it is not deniable that the PE potentiates the threat of clinically relevant resistance (14-17). Therefore, inhibiting the PE may be important for avoiding the emergence of resistance, and regulating stress responses could be useful for inhibiting the PE.

In conclusion, the present findings regarding the PE and the development of resistance in antifungal agents could be valuable. It has never before been reported that an Hsp90 inhibitor reduces the PE, and this finding may suggest that inhibiting Hsp90-related stress responses could help to avoid potential resistances.

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Efficacy of Combination Antifungal Therapy with Intraperitoneally Administered Micafungin and Aerosolized Liposomal Amphotericin B against Murine Invasive Pulmonary Aspergillosis▽

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Targeted intrapulmonary delivery of drugs may reduce systemic toxicity and improve treatment efficacy. In the current study, we evaluated the effects of a combination treatment consisting of inhalation of aerosolized liposomal amphotericin B (L-AMB) with intraperitoneal administration of micafungin (MCFG) against murine invasive pulmonary aspergillosis. The combination of aerosolized L-AMB with intraperitoneal MCFG significantly improved the survival rate, and the fungal burdens and histopathology findings after this treatment were superior to those of the control and both monotherapy groups.

Invasive pulmonary aspergillosis (IPA) results in significant morbidity and mortality in severely immunocompromised patients (6). Targeted intrapulmonary delivery of antifungals has the potential to reduce systemic toxicity and improve treatment efficacy as well as prophylaxis (1, 8) and may be used as an optional route in combination with other systemic antifungals. In the current study, we evaluated the efficacy of aerosolized liposomal amphotericin B (L-AMB) both singly and in combination with intraperitoneally administered micafungin (MCFG) in a murine model of IPA.

Aspergillus fumigatus MF13 was clinically obtained from a patient admitted to the Nagasaki University Hospital. The minimum effective concentration of MCFG (Astellas Pharmaceuticals Inc., Tokyo, Japan) and the MIC of AMB (Sigma, St. Louis, MO) were determined using the microdilution method in accordance with Clinical Laboratory Standards Institute document M38-A2 (2). Drug interactions were assessed using the checkerboard titration broth microdilution-based method (3), and the fractional inhibitory concentration index was determined as previously described (5).

Six-week-old female ICR mice (Charles River Breeding Laboratories, Shiga, Japan) were immunosuppressed and then challenged on day 0 with 5×10^6 conidia of *A. fumigatus* MF13 intratracheally for monitoring of survival, as previously described (7, 11). Eight-week-old female ICR mice were used to determine fungal burdens and for histopathological examination. Mice were immunosuppressed by subcutaneous injection of cortisone acetate (Sigma, Tokyo, Japan) at 250 mg/kg of body weight and intraperitoneally administered cyclophosphamide (Sigma) at 200 mg/kg on days −2 and 0 for the survival study. Only cortisone acetate (200 mg/kg) was used on days −1, 0, and 1 for fungal-burden analysis and histopathological examination. Mice were assigned into the following groups: (i) control mice, (ii) mice receiving MCFG intraperitoneally, (iii) mice receiving aerosolized L-AMB, and (iv) mice receiving a combination treatment of intraperitoneally administered MCFG and aerosolized L-AMB. Each group consisted of 11 and 10 mice for survival and fungal-burden analyses, respectively. MCFG was administered intraperitoneally once daily at 1 mg/kg/day. L-AMB was administered once daily in an 8-ml

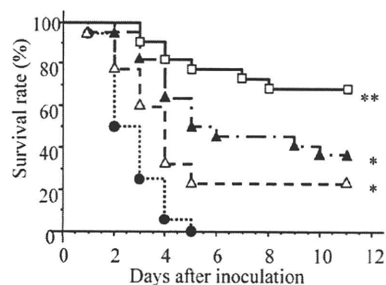

FIG. 1. Survival curves for mice with IPA (Kaplan-Meier plot). Groups of 11 mice were treated with a combination of intraperitoneal administration of MCFG (1 mg/kg/day) and inhalation of aerosolized L-AMB (8 ml at 1.2 mg/ml [open squares]), inhalation of aerosolized L-AMB (8 ml at 1.2 mg/ml [filled triangles]), intraperitoneal administration of MCFG (1 mg/kg/day [open triangles]), and no therapy (control [filled circles]). *, $P < 0.05$ versus the control; **, $P < 0.05$ versus the control group, intraperitoneal-MCFG group, or aerosolized-L-AMB group (log rank test). The survival times for all treatment groups were longer than that for controls ($P < 0.05$). The survival time for the combination treatment group was significantly longer than those of the intraperitoneal-MCFG group and the aerosolized-L-AMB group ($P < 0.05$).

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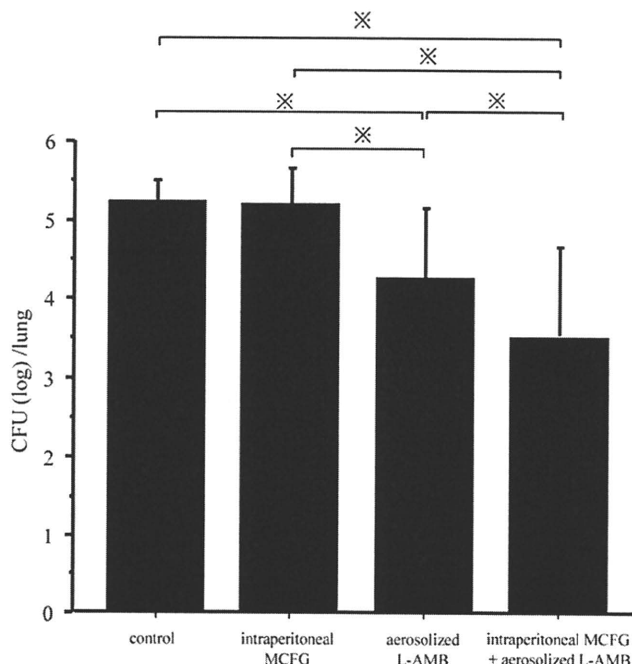

FIG. 2. Numbers of CFU from homogenized lung tissues of mice with IPA. Groups of 10 mice were treated once per day with a combination of intraperitoneally administered MCFG (1 mg/kg/day) and inhalation of aerosolized L-AMB (8 ml at 1.2 mg/ml), aerosolized L-AMB (8 ml at 1.2 mg/ml), intraperitoneal MCFG (1 mg/kg/day), and saline (control). CFU counts, as a parameter of *A. fumigatus* burden in the lungs of IPA mice at 4 h after day 3 of treatment, are shown. *, $P < 0.05$ (Student's t test).

suspension (at 1.2 mg/ml) per inhalation. Antifungals were initiated 16 h after inoculation and continued for 5 and 3 days for survival and fungal-burden analyses, respectively. The L-AMB solution was aerosolized using a nebulizer (Muromachi Kikai Co., Ltd., Tokyo, Japan), and mice were exposed to aerosol treatment for 60 min as previously described (9). Control mice were treated with sterile saline. Survival was observed until 11 days following the challenge. For fungal-burden and histopathological examinations, mice were sacrificed 4 h after the treatment on day 3. Numbers of CFU per lung tissue were calculated, and removed lungs were fixed and stained with Grocott's methenamine silver nitrate and hematoxylin-eosin as previously described (11). Survival and fungal burden data are presented from a combination of two sets of experiments. The concentration in blood and the pharmacokinetics of aerosolized L-AMB were evaluated. Uninfected mice were also exposed to several concentrations of aerosolized L-AMB for 5 days, and blood samples and lungs were collected. AMB concentration was quantified as previously described (10). Survival curves were generated using the Kaplan and Meier method, and statistical differences were evaluated by the log rank test. To assess fungal burden in lung tissue, geometric means of numbers of CFU per organ were compared by Student's t test. Statistical significance was defined as a P of <0.05.

The MIC of AMB against *A. fumigatus* MF-13 was 1.0 μg/ml, and the minimum effective concentration of MCFG was 0.0315 μg/ml. The fractional inhibitory concentration index of AMB and MCFG was 1.5, and drug interaction was classified as indifferent (5).

Survival periods of monotherapy groups, in which mice either were treated with intraperitoneally administered MCFG or inhaled aerosolized L-AMB were significantly longer than

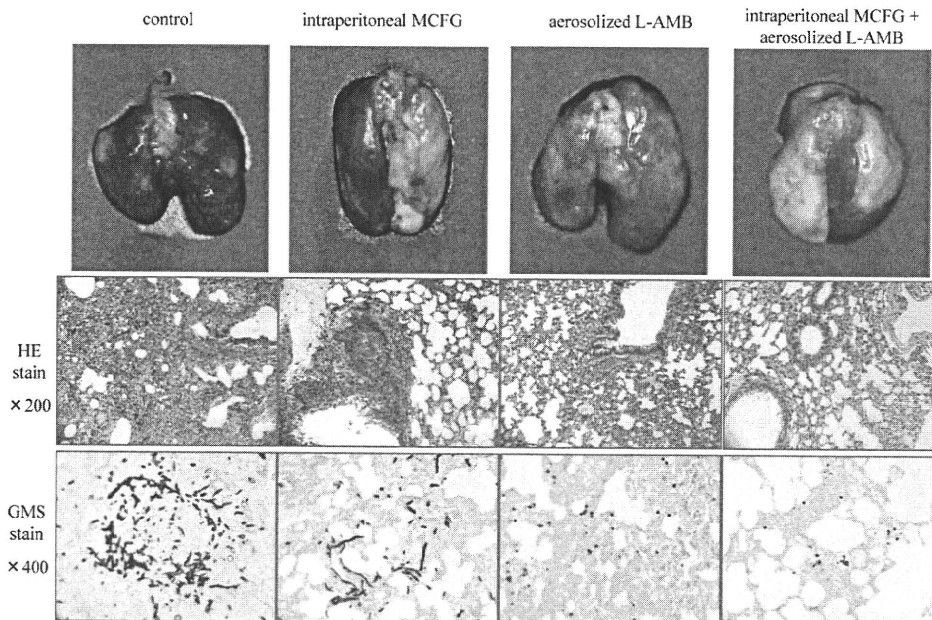

FIG. 3. Histopathology of lung tissues. Both lungs were obtained from IPA mice 4 h after 3 days of treatment with a combination of intraperitoneally administered MCFG and inhalation of aerosolized L-AMB, aerosolized L-AMB, intraperitoneal MCFG, and saline alone as a control. The lungs obtained from aerosolized-L-AMB-treated and combination treatment mice showed obviously smaller numbers of hyphae and fewer foci of inflammation than intraperitoneal-MCFG and control mice. HE, hematoxylin-eosin; GMS, Grocott's methenamine silver nitrate stain.

that of the control group (MCFG alone versus the control, $P = 0.006$; L-AMB versus the control, $P < 0.001$) (Fig. 1). The combination treatment group showed significantly longer survival than the intraperitoneal-MCFG ($P < 0.001$), aerosolized-L-AMB ($P = 0.037$), and control ($P < 0.001$) groups. Numbers of CFU in the lungs of mice in the combination treatment group were significantly reduced compared to those in each of the intraperitoneal-MCFG ($P < 0.001$), aerosolized-L-AMB ($P = 0.027$), and control ($P < 0.001$) groups (Fig. 2). The lungs of aerosolized-L-AMB-administered and combination treatment mice showed obviously smaller numbers of hyphae and fewer foci of inflammation than the intraperitoneal-MCFG and control groups (Fig. 3). The mean AMB concentrations in the lung tissue following L-AMB inhalation at 1.2, 2.6, and 4.0 mg/ml were 35.5, 73.2, and 94.2 μg/g, respectively. Recorded levels in sera were 0.02, 0.06, and 0.06 μg/ml when inhaled-L-AMB suspensions were administered at 1.2, 2.6, and 4.0 mg/ml, respectively.

The current study demonstrated the efficacy of monotherapy of aerosolized L-AMB in a murine IPA model. The AMB concentrations in lung tissue in our study were relatively higher but extremely lower in serum than those from another report of a murine model of intravenously administered L-AMB, although experimental conditions were not the same (10). These results suggested that systemic toxicity generally caused by AMB treatment may be reduced by L-AMB inhalation therapy.

The effect of combined intraperitoneal-MCFG and aerosolized-L-AMB treatment was an enhanced survival rate, even though this drug interaction was classified as indifferent in vitro. Since 78% of all control mice died in first 3 days in a survival analysis, we changed the experimental conditions for analysis of fungal burden and histopathological examination. In this model, no mice died before euthanasia, a prerequisite for the organ CFU assay. Both fungal-burden data and histopathological findings supported the survival data in our study.

Unlike in our study, Graybill et al. previously reported that combination therapy demonstrated a lack of synergistic effects following intravenous-L-AMB and intraperitoneal-MCFG treatment in a model of murine IPA (4). These discrepancies are likely due to differences between our model and Graybill et al.'s model, including (i) the route of infection, (ii) the status of immunosuppression, and (iii) the administration route of antifungal drugs. These differences also suggest that targeted intrapulmonary delivery of drugs by inhalation raises the drug concentration at the active site of infection in the lungs, thus contributing to the efficacy of combination therapy. Further comparative efficacy studies in a clinical setting are warranted.

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Biochemical properties and primary structure of elastase inhibitor AFUEI from *Aspergillus fumigatus*

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An elastase inhibitor from *Aspergillus fumigatus* (AFUEI) was isolated, and its biochemical properties and primary structure examined. The inhibitor was purified by column chromatography using DE52 cellulose and Sephadex G-75, and was found to be homogeneous as indicated by a single band following discontinuous PAGE and SDS-PAGE. A molecular mass of 7525.1 Da was observed by matrix-assisted desorption/ionization time-of-flight mass spectroscopy. The elastolytic activity of elastases from *A. fumigatus*, *Aspergillus flavus* and human leukocytes was inhibited by AFUEI. However, the elastolytic activity of porcine pancreas elastase, *Pseudomonas aeruginosa* elastase and elastase from snake venom was not affected by AFUEI. No inhibitory effect of DTT or 2-mercaptoethanol on the elastase inhibitory activity of AFUEI was observed. The amino acid sequence of AFUEI peptides derived from digests utilizing clostripain was determined by Edman sequencing. AFUEI was composed of 68 aa and had a calculated molecular mass of 7526.2 Da. The search for amino acid homology with other proteins demonstrated that aa 1–68 of AFUEI are 100 % identical to aa 20–87 of the hypothetical protein AFUA 3G14940 of *A. fumigatus*.

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INTRODUCTION

Currently, the genus *Aspergillus* has been shown to comprise more than 150 species. Approximately ten species have been isolated from clinical material, in particular *Aspergillus fumigatus.* Aspergillosis is a common mycosis occurring in immunocompromised hosts undergoing chemotherapy. It is caused by inhalation of high concentrations of spores of the species *Aspergillus.* It has been reported that various proteases are produced by *A. fumigatus* (Frosco *et al.*, 1992b; Hasegawa *et al.*, 1995; Larcher *et al.*, 1992; Monod *et al.*, 1991, 1993), *Aspergillus flavus* (Hasegawa *et al.*, 1997; Rhodes *et al.*, 1990) and *Aspergillus niger* (Inoue *et al.*, 1991; Takahashi *et al.*, 1991). As 28 % of lung tissue is composed of elastin, a strong correlation between elastase and the pathogenesis of aspergillosis has been suggested.

From a practical standpoint, elastase-producing strains promote a higher death rate in immunodeficient mice than non-elastase-producing strains. Elastase-producing strains destroy lung tissue (Kothary *et al.*, 1984; Reichard *et al.*, 1990), and an elastase produced by *Aspergillus* has been confirmed as a virulence factor of aspergillosis (Blanco *et al.*, 2002). We have previously studied and reported the pathogenicity of elastase produced by *A. flavus* in mice immunocompromised with cyclophosphamide, cyclosporine, prednisolone and carrageenan. These studies suggested that, when infected with *A. flavus*, a severe lesion in bronchioli or alveoli is induced by elastase-producing strains (Okumura *et al.*, 2007a).

Ulinastatin, an elastase inhibitor, has been administered by intravenous drip infusion concomitantly with antifungal agents to patients with pulmonary aspergillosis (Hasegawa *et al.*, 1994; Maesaki *et al.*, 1993; Ogawa, 1997; Ogawa *et al.*, 1992, Sugimura *et al.*, 1994) and it has been suggested that this elastase inhibitor could be an effective therapeutic agent for aspergillosis. It has been shown that *A. fumigatus* and *A. flavus* produce elastase inhibitors (Okumura *et al.*, 2004). We previously reported the isolation and the primary structure of an elastase inhibitor from *A. flavus* (Okumura *et al.*, 2007b). In this paper, we report the isolation, biochemical properties and primary structure of

Abbreviations: GAAPLNA, glutaryl-L-alanyl-L-alanyl-L-proryl-L-leucine *p*-nitroanilide; MALDI/TOF-MS, matrix-assisted desorption/ionization time-of-flight mass spectroscopy; Pe-AFUEI, *S*-pyridylethylated AFUEI; *p*NA, *p*-nitroanilide.

an elastase inhibitor from *A. fumigatus* (strain AFU-12) from a patient.

METHODS

Strain. *A. fumigatus* strain AFU-12 was identified and isolated from the sputum of a patient with allergic bronchopulmonary aspergillosis. Elastases from *A. fumigatus* and *A. flavus* were purified using the method of Hasegawa *et al.* (1995, 1997).

Production of elastase inhibitor. Conidia (5×10^6) were cultured in 100 ml synthetic medium comprising 0.1 % yeast carbon base (Difco) with 1 % casamino acid (Difco) as a nitrogen source. The cultures were incubated for 7 days at 37 °C. The elastase inhibitory activity of the culture supernatant was measured (Fig. 1) and determined to be approximately 65–90 % in culture supernatants of 4–7 days. The 7 day culture broth was filtered aseptically through a 0.22 μm membrane filter and the cell-free culture supernatant was used as the crude source of inhibitor.

Discontinuous PAGE. Discontinuous PAGE was carried out on 8.5 % polyacrylamide gels (0.5 × 12.5 cm, 3 mA, 4 °C), using 40 mM Tris/glycine (pH 8.3) as the running buffer. Gels were stained with 0.05 % Coomassie brilliant blue R-250 in 1 % acetic acid containing 5 % methanol for 1 h at 48 °C, followed by diffusion destaining.

Purification of AFUEI. The 7 day culture broth was applied to a column of diethylaminoethyl (DE52) cellulose (column size 2 × 45 cm; Whatman) equilibrated with 10 mM Tris/HCl (pH 7.2) containing 10 mM NaCl. The column was eluted with 10 mM Tris/HCl (pH 7.2) containing a 0–0.5 M NaCl gradient (Fig. 2a). Inhibitory activity (100–50 %) was found in fractions 232–300, which were pooled, concentrated by lyophilization and applied to a Sephadex G-75 column (2.5 × 100 cm; Pharmacia) equilibrated with 10 mM Tris/HCl (pH 7.2) containing 10 mM NaCl. Inhibitory activity of 100 % was found in fractions 115–130 (Fig. 2b). These fractions were analysed for purity by PAGE.

Assay for elastolytic inhibitory activity. The elastolytic inhibitory activity of AFUEI was assayed by the method of diazo coupling. Using 50 mM glutaryl-L-alanyl-L-alanyl-L-prolyl-L-leucine *p*-nitroanilide (GAAPLNA; Peptide Institute) in DMSO as the substrate, the amount of *p*-nitroanilide (*p*NA) released was measured. Fifty microlitres of AFUEI (3 ng) was mixed with 50 μl elastase (30 ng) and the mixture was incubated for 15 min at 37 °C. Next, 900 μl 50 mM Tris/HCl (pH 7.5) and 20 μl 50 mM GAAPLNA solution were added and incubated for 60 min at 37 °C. The reaction was stopped by adding 1 ml 10 % (w/v) trichloroacetic acid. Sodium nitrite (0.2 ml, 0.1 %), 0.2 ml 0.5 % ammonium sulfamate and 0.2 ml 0.1 % N-1-naphthylethylenediamine dihydrochloride were then added to the solution and the absorbance was read at 550 nm. One unit of elastolytic inhibitory activity was defined as the amount of AFUEI (mg) that did not release *p*NA from GAAPLNA in 1 min.

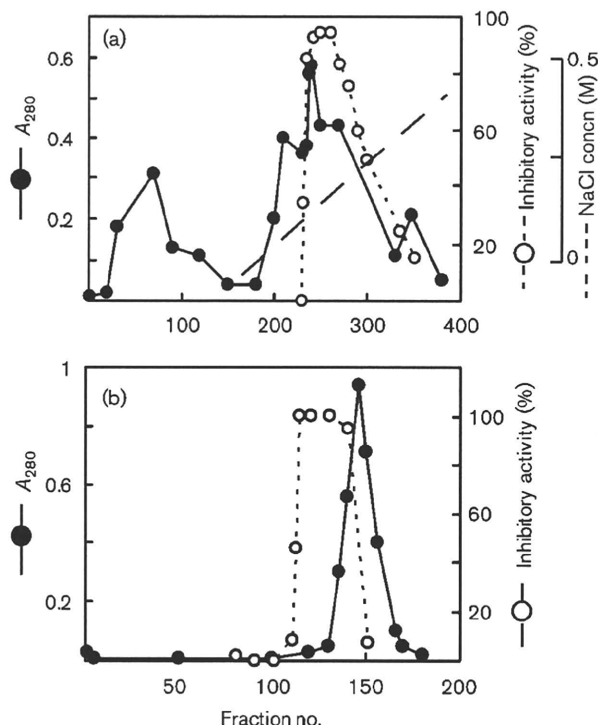

Fig. 2. Column chromatography of *A. fumigatus* culture filtrate. (a) DE52 cellulose column chromatography. *A. fumigatus* culture filtrate (100 ml) was applied to a DE52 cellulose column (2×45 cm) equilibrated with 10 mM Tris/HCl (pH 7.2) containing 10 mM NaCl. The column was eluted with 10 mM Tris/HCl (pH 7.2) containing a 0–0.5 M NaCl gradient. Fractions (6 ml) were collected at a flow rate of 18 ml h^{-1}. (b) Sephadex G-75 column chromatography. DE52 cellulose column fractions 232–300 were pooled, concentrated by lyophilization and applied to a Sephadex G-75 column (2.5×100 cm) equilibrated with 10 mM Tris/HCl (pH 7.2) containing 10 mM NaCl. The column was eluted with 10 mM Tris/HCl (pH 7.2) containing 10 mM NaCl. Fractions (3 ml) were collected at a flow rate of 9 ml h^{-1}. ●, A_{280}; ○, inhibitory activity; dashed line, NaCl concentration.

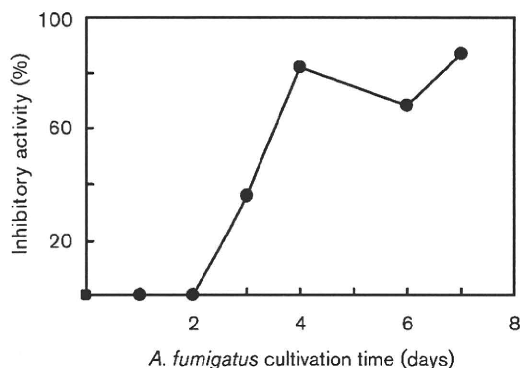

Fig. 1. Elastase inhibitory activity of *A. fumigatus* culture filtrate.

Effects of AFUEI on fibrinogenase and collagenase activities of elastase. Elastase from *A. fumigatus* in 10 mM Tris/HCl (pH 8.5) was incubated with the AFUEI supernatant for 15 min and then with 1 mg fibrinogen (Katayama Chemical) or collagen (types I, II, IV and V; Wako Pure Chemical) at 37 °C for various time intervals. Simultaneously, 100 μl of the reaction mixture was pipetted into a small test tube and 100 μl buffer containing 10 M urea, 4 % SDS, 10 mM phosphate buffer (pH 7.2) and 3 % 2-mercaptoethanol were added. This solution was incubated at 37 °C for 6 h. An aliquot of 20 μl was analysed by 12 % SDS-PAGE.

Enzymic cleavage. Native AFUEI (40 μg) was digested with 5 μg clostripain (Roche) for 24 h at 37 °C in 50 mM Tris/HCl (pH 7.2) containing 1 % (v/v) 2-mercaptoethanol and 10 mM $CaCl_2$. Native AFUEI was dissolved in 1.6 ml 5 mM Tris/HCl (pH 7.5) containing 8 M urea, and 200 μl 2-mercaptoethanol was added to this solution. The reaction mixture was bubbled through with nitrogen for 10 min and incubated at 37 °C for 1 h. 4-Vinylpyridine (200 μl) was added and the reaction mixture was stirred at room temperature for another 2 h. The excess reagents were removed by dialysis against distilled water. *S*-Pyridylethylated AFUEI (Pe-AFUEI) was also digested with clostripain as above. All digests were then separated by reversed-phase HPLC using a Gilson model 302 chromatograph with a Develosil 300 ODS-HG5 column (0.46 × 25 cm).

Sequence analysis. The amino acid sequence of AFUEI was analysed using an Applied Biosystems 491 protein sequencer. The phenylthiohydantoin derivatives of amino acids were identified using an Applied Biosystems model 120A PTH analyser in accordance with the manufacturer's instructions.

Determination of molecular mass. AFUEI was desalted using a ZipTip column (Millipore) and analysed by matrix-assisted desorption/ionization time-of-flight mass spectroscopy (MALDI/TOF-MS) using a Voyager PK2 (Applied Biosystems).

RESULTS AND DISCUSSION

AFUEI isolation

AFUEI was isolated from *A. fumigatus* by DE52 cellulose and Sephadex G-75 column chromatography as described in Methods. Fractions 115–130 (Fig. 2b) were analysed by discontinuous PAGE (pH 8.3) and SDS-PAGE (Fig. 3), and were found to be electrophoretically homogeneous. These fractions were pooled, and this preparation was designated the purified AFUEI (elastase inhibitor from *A. fumigatus*), which was used for the biochemical characterization. The total yield of purified AFUEI from 100 ml culture supernatant was 97 μg.

Properties of AFUEI

The molecular mass of the inhibitor isolated from *A. fumigatus* was 7525.1 Da as determined by MALDI/TOF-MS (Fig. 4). The carbohydrate content of AFUEI was calculated to be 0 % with glucose as a standard using the method of Morris (1948). The heat stability of AFUEI (0.1 μg) in 10 mM Tris/HCl (pH 7.5) containing 10 mM NaCl was investigated. The inhibitor was heated for 10 min at 37, 50, 60, 80 and 100 °C, cooled quickly to 4 °C and the inhibitory activity was determined. AFUEI was stable to heat treatment, retaining 33.3 % activity after treatment at 100 °C. The effect of reducing agents on AFUEI was also determined. AFUEI was mixed with the reducing agent and incubated for 15 min at 37 °C prior to the addition of GAAPLNA as substrate to determine whether reduction had an effect on its inhibitory activity. AFUEI activity was not inhibited by 2.5 mM DTT or 1.9 mM 2-mercaptoethanol.

The effect of AFUEI on various elastases was determined and is shown in Table 1. Elastases from *A. fumigatus*, *A. flavus*, human leukocytes and porcine pancreas were inhibited 100, 98.9, 83.8 and 11.7 % by AFUEI, respectively. However, *Pseudomonas aeruginosa* (Komori *et al.*, 2001) and snake venom elastases were not inhibited. The effects of AFUEI on the fibrinogenase and collagenase activities of elastase from *A. fumigatus* were measured. Both activities of the elastase were completely inhibited by AFUEI. The Michaelis constant (K_m) for GAAPLNA of elastase from *A. fumigatus* at pH 7.5 was 3.8×10^2 μM. The inhibition constant (K_i) of AFUEI for elastase was determined by

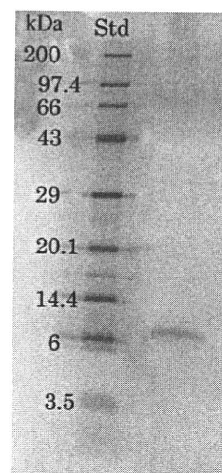

Fig. 3. SDS-PAGE of purified AFUEI. SDS-PAGE was performed using the method of Weber & Osborn (1969). Protein standards used were insulin B chain (3.5 kDa), aprotinin (6 kDa), lysozyme (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), BSA (66 kDa), phosphorylase *b* (97.4 kDa) and myosin (200 kDa). AFUEI was treated with 4.3 % SDS, reduced with 10 % 2-mercaptoethanol for 3 min at 100 °C and then run on a polyacrylamide gel (MULTIGEL II Mini 15/25; Daiichi Pure Chemicals).

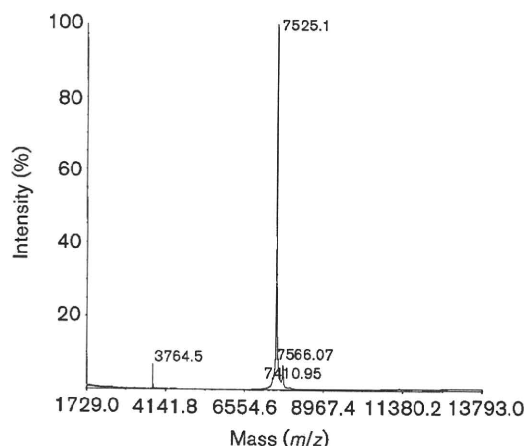

Fig. 4. MALDI-TOF MS results for AFUEI.

Table 1. Inhibitory activity of AFUEI on elastases

Elastase/proteinase	Substrate	Inhibitory activity (%)
Purified elastase from *A. fumigatus*	GAAPLNA	100.0
Purified elastase from *A. flavus*	GAAPLNA	98.9
Purified elastase from human leukocytes	SAPANA	83.8
Purified elastase from porcine pancreas	STANA	11.7
Elastase from *P. aeruginosa*	GAAPLNA	0
Elastase from *Crotalus atrox* venom	STANA	1.7
Fibrinogenase activity of elastase from *A. fumigatus*	Fibrinogen	IN
Collagenase activity of elastase from *A. fumigatus*	Collagen (types I, II, IV and V)	IN

IN, Complete inhibition; SAPANA, succinyl-Ala-Pro-Ala-*p*NA; STANA, succinyl-Ala-Ala-Ala-*p*NA.

measuring the initial rate of hydrolysis of GAAPLNA at pH 7.5. The inhibition of AFUEI was non-competitive and an inhibition constant of 1.6×10^{-3} μM was obtained.

Primary structure of AFUEI

The first 44 residues of AFUEI were determined by N-terminal sequence analysis (Fig. 5). AFUEI was incubated with clostripain. The digested fragments were purified by reversed-phase HPLC (Fig. 6a) and their sequences analysed (Table 2). Pe-AFUEI was also incubated with clostripain and analysed by reversed-phase HPLC (Fig. 6b), and the amino acid sequence of each fragment (Pe-R-1 and Pe-R-2) was examined (Fig. 5). The AFUEI sequence showed two cysteinyl residues. From these results, a total of 68 aa residues of AFUEI were identified (Fig. 5). Thus, AFUEI is composed of 68 aa and the molecular mass of the protein portion of AFUEI was estimated to be 7526.2 Da. The molecular mass of AFUEI based on the amino acid sequence was identical to the molecular mass obtained by MALDI/TOF-MS. The hypothetical protein AFUA 3G14940 of *A. fumigatus* has been reported (Nierman *et al.*, 2005) and its amino acid sequence from aa 20 to 87 was found to be identical to aa 1–68 of AFUEI. It is likely that this hypothetical protein also has an inhibitory activity. No tryptophan residue was found and a total of three aromatic amino acids (two tyrosines and one phenylalanine) were present. As a result, the absorption at 280 nm was minimal (Fig. 2b).

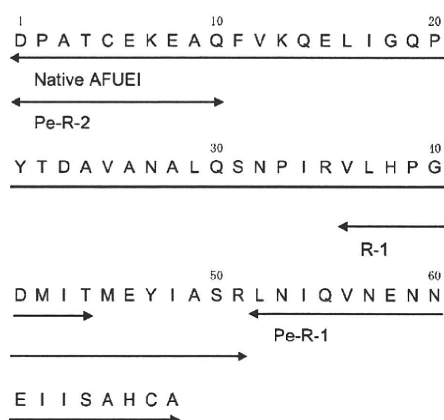

Fig. 5. Amino acid sequence of AFUEI. Arrows indicate residues determined by sequence analysis.

Comparison with other protease inhibitors

Comparative studies of the inhibitory activity of AFUEI with four other protease inhibitors were performed (Table 2). Ulinastatin, nafamostat mesilate, sivelestat sodium hydrate and gabexate mesilate are proteinase inhibitors normally used for the treatment of acute pancreatitis, acute lung disturbance and intravascular coagulation disease. Our results showed that AFUEI inhibited elastase from *A. fumigatus*. Inhibition by nafamostat mesilate was also observed; however, all four protease inhibitors demonstrated very weak inhibitory activity in comparison with AFUEI. These results clearly demonstrated that AFUEI has a strong inhibitory effect on elastase from *A. fumigatus* and that only low concentrations of AFUEI are needed for inhibition.

Aspergillosis is a common mycosis occurring in immunocompromised hosts undergoing chemotherapy. The pathogenesis of aspergillosis is not clearly understood. However, the published results on pathogenesis strongly indicate that proteases are significant factors in the pathogenic process. Several of these proteases have been isolated: Frosco *et al.* (1992a) purified an elastase of 32 000 Da from *A. fumigatus* that was inhibited by EDTA, Monod *et al.* (1991) purified an alkaline protease of 33 000 Da, Rhodes *et al.* (1990) purified an elastolytic protease of 23 000 Da from *A. flavus*, and Kolattukudy *et al.* (1993) purified an elastolytic serine protease of 33 000 Da from *A. fumigatus* and determined the primary structure by using an elastin medium. The latter study compared the lethal activity of the elastase-producing and non-elastase producing strains and reported that the former exhibited lethal activity. They also found that the non-elastase-producing strain possessed lethal activity, but this was at a negligible minimum level. Kothary *et al.* (1984) compared the lethal activity of six elastase-producing strains and four non-elastase-producing

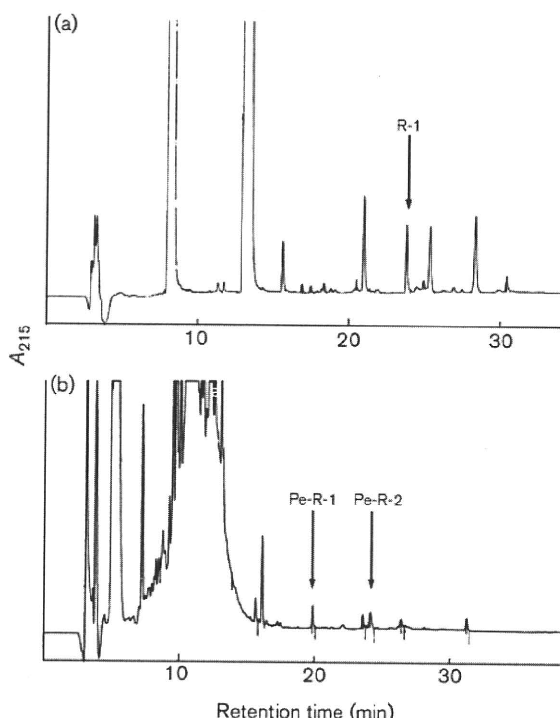

Fig. 6. Reversed-phase HPLC fractionation of the peptides obtained by clostripain cleavage of AFUEI. Native AFUEI (a) or Pe-AFUEI (b) was incubated with clostripain and aliquots were taken for analysis by reversed-phase HPLC using a Develosil 300 ODS-HG-5 column (0.46×25 cm). Solvent A was 0.1 % trifluoro-acetic acid (TFA) in H_2O, solvent B was 0.1 % TFA in acetonitrile and the flow rate was 1.0 ml min^{-1}. Elution was achieved over 60 min with a linear gradient from 0 to 100 % using solvent B.

strains of *A. fumigatus*. Their results were comparable to those of Kolattukudy *et al.* (1993). Furthermore, when the dead murine pulmonary tissue was examined, it was found that elastase-producing strains had broadly invaded the tissue more than non-elastase-producing strains. Monod *et al.* (1991) reported a similar result with an alkaline protease-deficient mutant, and Blanco *et al.* (2002) presented results on the participation of elastase in pathogenicity. As lung tissue contains large amounts of elastin, we focused on elastase as a factor of infection. Our laboratory purified an elastolytic protease from *A. fumigatus* with a molecular mass of 32 000 Da and pI of 9.1 (Hasegawa *et al.*, 1995) that has been shown to cause pathogenicity. Additionally, we purified an elastolytic glycoprotein protease from *A. flavus* that was inhibited by diisopropyl fluorophosphate. This protease had a molecular mass of 40 000 Da and pI of 8.6 (Hasegawa *et al.*, 1997) and has also been shown to cause pathogenicity (Okumura *et al.*, 2007a). It is clear from these results that elastases are related to pathogenicity and therefore that an elastase inhibitor could be an important treatment for aspergillosis.

Table 2. Effect of various inhibitors on elastase from *A. fumigatus*

Inhibitor	IC_{50} (μg)
AFUEI	0.0026
Ulinastatin	>830
Nafamostat mesilate	72
Sivelestat sodium hydrate	>500
Gabexate mesilate	2000

In a previous report, screening of the elastase inhibitor from *Aspergillus* was conducted and it was found that both *A. fumigatus* and *A. flavus* produced an elastase inhibitor (Okumura *et al.*, 2004). In addition, both the elastase activity and inhibitory activity of the AFU-12 strain were found to be stronger than those in the previous 13 strains used. In this report, the elastase inhibitor from *A. fumigatus* (AFUEI) was purified, and its biochemical properties and primary structure examined.

Development of a substance that inhibits leukocyte elastase for the purpose of suppressing internal inflammation is a current focus. For example, ONO-5046 (Kawabata *et al.*, 1991) is used for the treatment of acute lung disturbance with a general inflammatory reaction symptom complex. The results of our studies reveal an inhibitor capable of inhibiting leukocyte elastase. AFUEI is therefore a promising agent for suppressing inflammatory diseases effectively, as it is a potent inhibitor of human leukocyte elastase.

Elastase from *A. fumigatus* displays strong degrading effects on fibrinogen and collagen (types I, II, IV and V). AFUEI strongly inhibited the activity of the elastase and therefore could prevent the degrading effect of *A. fumigatus* on fibrinogen and collagen. Type IV collagen is the main component of the basal membrane of blood vessels and alveoli, and the bleeding seen on histopathological examination is thought to be caused by injury to the basal membrane of these structures. Moreover, elastase has an injurious effect locally at the infection site, and is thought to be related in some way to the pathogenicity manifested by the tissue infiltration and inflammation exhibited by *A. fumigatus*. Taken together, these findings suggest that AFUEI inhibits tissue infiltration and inflammation seen with aspergillosis and thereby weakens pathogenicity. Ogawa *et al.* (1992) reported a clinical effect with the protease inhibitor ulinastatin. When ulinastatin was administered to a patient with lung aspergillosis who had recurrent haemoptysis and haemosputum, the symptoms disappeared within a short period. Maesaki *et al.* (1993) and Sugimura *et al.* (1994) reported that cases of pulmonary aspergilloma and invasive pulmonary aspergillosis were successfully treated with the combined use of ulinastatin and amphotericin B.

The elastase inhibitor from *A. flavus* (AFLEI) has been purified and its biochemical properties and primary structure examined previously (Okumura *et al.*, 2006,

2007b). The biochemical properties and primary structure of AFUEI were identical to AFLEI. It is interesting that *A. fumigatus* and *A. flavus* produce elastase inhibitors with the same primary structure. Most cases of aspergillosis are caused by *A. fumigatus* and *A. flavus*. If AFUEI inhibits elastases from *A. fumigatus* and *A. flavus in vivo*, it is predicted that AFUEI will be an effective agent against most cases of aspergillosis.

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Original Article

Biological properties of elastase inhibitor, AFLEI from *Aspergillus flavus*

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Abstract

The biological properties of elastase inhibitor from *Aspergillus flavus* (AFLEI) were investigated. AFLEI was produced at the highest rate when casamino acid was used as the nitrogen source. When a mixture of AFLEI (approx. molecular weight, 7,500) and elastase from *A. flavus* (approx. molecular weight, 40,000) was detected using anti-AFLEI antibody, molecular weight of the detected mixture was approximately 48,000, indicating that AFLEI and elastase bound at a proportion of 1 : 1. When immunocompromised mice administrered of immunosuppressive (cyclophosphamide) were infected by inhalation of *A. flavus* and administered amphotericin B (AMB) alone or in combination with AFLEI, survival rate tended to be higher with combination treatment than with AMB alone.

Moreover, although extensive bleeding was seen in pathology sections taken from rat lung resected 24 hr after purified elastase was administered to the lung via the bronchus, this bleeding was inhibited by AFLEI. These findings indicate that for the treatment of aspergillosis, combination of an existing antifungal agent with AFLEI can be expected to provide greater therapeutic benefits than administration of an antifungal agent alone.

Key words : *Aspergillus flavus*, elastase inhibitor, cyclophosphamide, virulence

Introduction

A major host-related factor involved in the onset of aspergillosis is compromised immune function. Bacterium-related factors in this type of infection include proteolytic enzymes, mycotoxins and adhesion factors. Numerous reports have examined the properties of proteolytic enzymes [1-5] and the relationship between proteolytic enzymes and pathogenesis [6,7]. For reasons such as the fact that elastin constitutes approximately 28% of lung tissue, we thought that proteolytic enzymes, particularly elastase, are likely to be a pathogenic factor in aspergillosis, and examined elastases produced by *Aspergillus flavus* [8,9] and *A. fumigatus* [10-12]. We also identified AFLEI, a substance that inhibits elastase produced by *A. flavus*, and described its properties [13-15]. Ogawa *et al.* [16,17], Maezaki *et al.* [18] and Sugimura *et al.* [19] reported that ulinastatin, an agent that inhibits proteolytic enzymes, was effective in treating

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