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Research Paper

Biochemical and cellular activity of chemically synthesized elastase inhibitor (S-AFUEI) from *Aspergillus fumigatus*

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ABSTRACT

Purpose. – Elastase, produced by *Aspergillus fumigatus* and *A. flavus*, is an important pathogenic factor in pulmonary aspergillosis. We investigated the possibility of using *A. fumigatus*-derived *A. fumigatus* elastase inhibitor (AFUEI) as a therapeutic agent. As native-AFUEI (N-AFUEI) has an extremely low yield, we generated a synthetic-AFUEI (S-AFUEI) and investigated whether S-AFUEI has a biological activity against *A. fumigatus* elastase (AFUE) and inhibits cytotoxicity.

Methodology. – *A. fumigatus* was cultured in Yeast Carbon Base (YCB) -elastin culture medium for 3–7 days, and AFUE was purified by chromatography using DE52 cellulose and Sephadex G-75 column. Elastolytic activity was examined using Glt-Ala-Ala-Pro-Leu-pNA (GAAPLNA) as the substrate. The hydrolytic activity of AFUE was determined using the characteristic substrates, fibrinogen and collagen (Type IV), and human cell cytotoxicity was measured colorimetrically. Furthermore, the inhibitory effect of S-AFUEI on these activities was examined.

Results. – We confirmed that S-AFUEI demonstrated elastase inhibitory activity and heat stability equivalent to that demonstrated by N-AFUEI, and inhibited human collagen hydrolytic activity and human fibrinogen hydrolytic activity. Further, S-AFUEI inhibited cytotoxicity in AFUE human pulmonary artery endothelial cells (HPAEC), human small airway epithelial cells (HSAEC), and human pulmonary alveolar epithelial cells (HPAEpiC).

Conclusion. – As S-AFUEI strongly inhibited cytotoxicity induced by elastase in human-derived cells, it could prove beneficial for the treatment of pulmonary aspergillosis.

1. Introduction

Invasive pulmonary aspergillosis (IPA), a severe opportunistic infection, has an increased incidence in immunosuppressed or hospitalized patients with severe underlying disease [1]. IPA is thought to occur mainly in patients with risk factors such as neutropenia, hematologic malignancy, allogeneic bone marrow transplantation, solid organ transplantation, solid cancer, or HIV infection [2]. However, in recent years, the incidence of IPA has also increased in non-neutropenic patients requiring high-dose steroid therapy and in those with autoimmune diseases requiring immunosuppressive therapy, including new monoclonal agents [3]. Despite the introduction of new antifungal agents, fungal infections still pose a threat because of factors such as the occurrence of drug-resistant strains, toxicity to the host, and drug bioavailability [4]. Voriconazole and amphotericin B are first-line drugs for IPA; however, their response rate, 52.8% and 31.6%, respectively, is inadequate [5], and thus, new treatments are needed. One of the major issues regarding *Aspergillus* infections is azole-resistance in environmental and clinical isolates of *Aspergillus fumigatus* [6]. Recently, combinatorial therapy with antifungal drugs has been reported to be useful; however, its scope is limited [7]. In addition to antifungal agents with fungicidal activity, drugs targeting virulence factors are garnering attention as candidates for developing new antifungal agents [8].

We have focused on the relationship between elastase and aspergillosis. When elastase-producing and non-producing strains were inoculated in immunosuppressed mice, elastase-producing strains displayed an increased mortality rate [9]. When an elastase-deficient mutant was generated and inoculated in

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immunocompromised mice, mouse mortality rate decreased, compared with that in the elastase-producing strain [10]. Clinical isolates of *A. fumigatus* isolated from patients with IPA have demonstrated potent elastolytic activity and previous reports have indicated a relationship between elastase activity and pathogenicity [11]; therefore, elastase is considered a pathogenic factor in aspergillosis. Elastase induces bleeding in rat lungs (observed in the alveoli and bronchioles) and infiltration of neutrophils and exudation of a fibrin-like substance; thus, it was hypothesized that this enzyme is involved in lung tissue damage [12]. Inhibition of elastase reduces pulmonary tissue damage, which is beneficial for the treatment of pulmonary aspergillosis. Okumura et al. identified an elastase inhibitor produced by *Aspergillus* in the culture supernatant of *A. fumigatus* [13] and named it *A. fumigatus* elastase inhibitor (AFUEI). In liquid medium, *A. fumigatus* produces both *A. fumigatus* elastase (AFUE) and Native-AFUEI (N-AFUEI), both of which react with one another. While N-AFUEI has an extremely low yield, Synthetic-AFUEI (S-AFUEI) was successfully synthesized at the Peptide Institute Inc. (Osaka, Japan) in 2015 for medical application. However, the functional analysis of S-AFUEI has not been performed so far.

In this study, we investigated the physiological and biological activity of S-AFUEI. In addition, we investigated whether S-AFUEI inhibits the cytotoxicity of AFUE in normal human small airway epithelial cells (HSAEC), normal human pulmonary artery endothelial cells (HPAEC), and normal human pulmonary alveolar epithelial cells (HPAEpiC), to explore the clinical application of S-AFUEI to human pulmonary aspergillosis.

2. Methods

2.1. Materials

Three *A. fumigatus* strains derived from the sputum of three patients with pulmonary aspergillosis (Higashinagoya National Hospital, Nagoya, Japan) were used. Potato dextrose agar (PDA) medium was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Collagen (type IV) was obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Human fibrinogen and sodium dodecyl sulfate (SDS) were obtained from Sigma-Aldrich Japan LLC (Tokyo, Japan). PVDF membranes were purchased from Millipore Co., (Danvers, MA, USA). Anti-AFUEI peptide antibody was purchased from Medical & Biological Laboratories Co., Ltd (Nagoya, Japan). Anti-Rabbit Antibody HRP-linked IgG was purchased from Cell Signaling Technology, Inc., (Danvers, MA, USA). Chemiluminescence (ECL) reagent was purchase from GE Healthcare (Chicago, IL, USA). The Glt-Ala-Ala-Pro-Leu-*p*NA (GAAPLNA) product was obtained from Peptide Institute Inc. (Osaka, Japan). HPAEC, HSAEC, their respective cell culture media (HuMedia EG-2 and BronchiaLife Comp Kit), and other cell culture supplements and reagents were obtained from Kurabo (Osaka, Japan). HPAEpiC and their culture media were purchased from ScienCell Research Laboratories, Inc. (Carlsbad, CA, USA). Cell Counting Kit-8 was obtained from Dojindo Lab Co., Ltd (Kumamoto, Japan) and the CellTracker Green fluorescent probe was purchased from Lonza Japan (Tokyo, Japan). A spectrophotometer was purchased from Bio-Rad Lab, Inc., (Hercules, CA, USA). Membrane filters were purchased from Advantec Toyo Co., Ltd. (Tokyo, Japan). Other chemicals used were of analytical grade and purchased from commercial sources.

2.2. Production of elastolytic protease

A. fumigatus was isolated and cultured from the sputum of a patient receiving antifungal therapy for pulmonary aspergillosis and subcultured in PDA medium. A sufficient number of *A. fumigatus* spores were cultured in 100 ml YCB-elastin medium (a synthetic medium containing 1% yeast carbon base and 1% elastin) as a nitrogen source. The cultures were incubated for 3–7 d at 37 °C with agitation at 100 rpm. The culture broth was filtered through an aseptic 0.22-μm pore size membrane filter and the cell-free culture supernatant was used as the crude enzyme source of elastase. AFUE was purified by chromatography using DE52 cellulose and Sephadex G-75 column [14].

2.3. Elastolytic activity assay

The elastolytic activity of the elastase was assayed using the diazocoupling method that measures p-nitroanilide (p-NA) released from 50 mM (GAAPLNA) in DMSO used as the substrate [13]. To determine the extent of GAAPLNA digestion, 0.1 ml of the enzyme solution, 0.9 ml of 50 mM Tris-HCl buffer, [pH 7.5], and 20 μl of the 50 mM GAAPLNA solution were incubated for 30 min at 37 °C. The reaction was stopped by adding 1 ml of 10% trichloroacetic acid (TCA). Thereafter, 0.2 ml of 0.1% sodium nitrite, 0.5% ammonium sulfamate, and 0.1% N-1-naphtyletylenediamine dihydrochloride were added to the reaction solution and the absorbance was measured at 550 nm. One unit of GAAPLNA hydrolase activity was defined as the amount of enzyme that hydrolyzed 1 μg of substrate per minute.

2.4. Western blot analysis

Western blot analysis was performed essentially as previously described [15]. Samples (0.5 mg S-AFUEI and culture filtrate) were separated via SDS-PAGE using 4–20% resolving gels and then electrophoretically transferred to a polyvinylidene difluoride membrane. After blocking with Tris-buffered saline containing 3% skimmed milk and 0.05% tween 20, the membranes were incubated sequentially with a primary antibody (anti-AFUEI peptide antibody; 1:1000) and a secondary antibody (Anti-Rabbit Antibody HRP-linked IgG). Bands were detected by chemiluminescence (ECL). Anti-AFUEI antibody was generated by immunizing rabbits with C-E-K-E-A-Q-F-V-K-Q-E-I-G-Q-P-Y-T (5–22 residues) of whole primary structure of N-AFUEI.

2.5. Elastase inhibitory activity assay

Fifty microliters of elastase inhibitor solution was mixed with 50 μl of the purified elastase from *A. fumigatus* and incubated for 15 min at 37 °C prior to the addition of the GAAPLNA substrate. Thereafter, 0.9 ml of 50 mM Tris-HCl buffer [pH 7.5] and 20 μl of the 50 mM GAAPLNA solution were added and incubated for 30 min at 37 °C. The reaction was stopped by adding 1 ml of 10% TCA. Subsequently, 0.2 ml of 0.1% sodium nitrate, 0.5% ammonium sulfamate, and 0.1% N-1-naphthylethylenediamine dihydrochloride were added to the solution and the absorbance was measured at 550 nm.

2.6. Heat stability of S-AFUEI

Thermostability of S-AFUEI (0.2 μg) was determined using 10 mM Tris-HCl buffer [pH 7.5] containing 10 mM NaCl. The inhibitor was heated for 10 min at 37 °C, 50 °C, 60 °C, and 80 °C and then quickly cooled to 4 °C, and the elastase inhibitory activity on GAAPLNA was determined.

2.7. Effect of S-AFUEI on the fibrinogenase and collagenase activities of elastase

The effect of S-AFUEI was examined according to the method of Okumura et al. [16]. Elastase from *A. fumigatus* in 10 mM Tris-HCl

buffer [pH 7.5] was incubated with S-AFUEI for 15 min and with 1 mg of fibrinogen or collagen (type IV) at 37 °C for various time intervals. Following incubation, 0.1 ml of the above reaction mixture was used for a clottable fibrinogen assay. Simultaneously, 0.1 ml of the reaction mixture was pipetted into a small test tube and 0.1 ml of buffer containing 10 M urea, 4% SDS, 10 mM phosphate buffer [pH 7.2], and 3% β-mercaptoethanol was added. This solution was incubated at 37 °C for 3 h. An aliquot (20 μl) was then electrophoresed on a 12% SDS-polyacrylamide gel.

2.8. Cytotoxicity assay

Cytotoxicity of AFUE was determined in accordance with the method of Komori et al. [17]. Cultured human cells (HSAEC, HPAEC, HPAEpiC) were trypsinized, re-suspended in medium, and seeded into 96-multiwell plates at a density of 1.5×10^4 cells/well. AFUE was serially diluted with sterile physiological saline and then added to the cells (5 wells each), which were then incubated at 37 °C for 24 h. Viable cell numbers were determined colorimetrically using a cell counting kit based on the tetrazolium salt/formazan system [18,19]. For fluorescence microscopy, cell staining was performed with 0.5 mM of CellTracker Green CMFDA (5-chloromethyl-fluorescein diacetate) in serum-free medium in accordance with the instructions of the supplier. A BZ-X700 fluorescence microscope system (Keyence, Osaka, Japan) was used for observation. The inhibitory activity of S-AFUEI on AFUE cytotoxicity was measured in the same manner as described above, wherein the S-AFUEI molar ratio was 0, 0.25, 0.5, and 0.75 with respect to 1 mol of AFUE. The control group comprised only S-AFUEI (equivalent to 0.75 mol).

2.9. Statistical analysis

The experiments were performed at least in duplicate. Results are presented as means ± standard deviations. Quantitative data were compared using Student's *t*-test. Results with a *P*-value of < 0.05 were considered to be significant.

3. Results

3.1. Elastase activity of purified AFUE

Elastase activity of AFUE purified from three *A. fumigatus* strains derived from patients with pulmonary aspergillosis was set as 1 unit (U) based on the rate of hydrolysis of 1 μg substrate by 1 mg of enzyme in 1 min. The activity of the AFUE obtained from the three strains was 8.09–8.77 μg/min/mg (AFU-1, 8.46; AFU-2, 8.09; and AFU-3, 8.77).

3.2. Western blot analysis

S-AFUEI was detected via western blot analysis, using anti-AFUEI antibodies raised with N-AFUEI as the antigen. A band corresponding to the molecular weight of S-AFUEI (7525.1 Da) was observed. SDS-PAGE revealed a homogeneous substance recognized in the same band as that of N-AFUEI [13].

3.3. Primary structure and elastase inhibitory activity

In the previous study, we synthesized a substance with the same primary structure as N-AFUEI but with insufficient inhibitory activity. Analysis of the primary structure of N-AFUEI via three-dimensional analysis revealed that the cysteines at the 5th and 67th positions form a three-dimensional structure via disulfide bonds [20]. We considered the possibility that the three-dimensional structure site is important for inhibitory activity. Here, we performed partial synthesis of S-AFUEI with a disulfide bond; AFUEI with the same primary structure, but lacking a disulfide bond; AFUEI 39-47 (P-G-D-M-I-T-M-E-Y); and 39-51 (P-G-D-M-I-T-M-E-Y-I-A-S-R). Next, we determined the inhibitory activity of each synthesized compound. AFUE and S-AFUEI were reacted at a molar ratio of 1:1. S-AFUEI inhibited 94.9 ± 1.2% of the GAAPLNA hydrolytic activity of AFUE. AFUEI without disulfide bonds inhibited 51.7 ± 5.8% of the activity, partially synthesized AFUEI 39-47 inhibited 1.9 ± 1.5% of the activity, and partially synthesized AFUEI 39-51 inhibited 21.5 ± 5.5% of the activity. S-AFUEI demonstrated equivalent elastase inhibitory activity as N-AFUEI [13], while it had stronger inhibitory activity than AFUEI without disulfide bonds (94.9% vs. 51.7%, $P < 0.05$) and the partially synthesized AFUEIs. Taken together, these results indicate that formation of the three-dimensional structure with disulfide bonds at the N-terminal 5th residue and the C-terminal 67th residue [20] is important for elastase inhibitory activity (Fig. 1).

3.4. S-AFUEI heat stability

The residual activity of S-AFUEI was 100 ± 0.5% at 37 °C, 98.6 ± 1.0% at 50 °C, 95.1 ± 1.6% at 60 °C, and 53.9 ± 4.3% at 80 °C, confirming the heat-resistant properties of S-AFUEI (Fig. 2). These results were equivalent to those obtained for N-AFUEI [13].

3.5. S-AFUEI human collagen (type IV) hydrolytic inhibitory activity

We next examined the human collagen (type IV) hydrolytic inhibitory activity of S-AFUEI (3.75 μg). AFUE began to hydrolyze collagen after 30 min and hydrolysis was nearly complete after 180 min. In contrast, S-AFUEI strongly inhibited the collagen

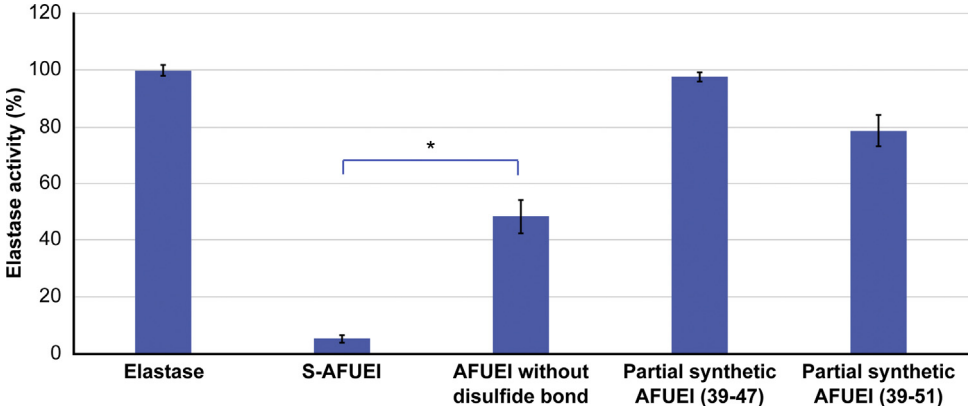

Fig. 1. Comparison of the elastase inhibitory activity of the synthetic inhibitors. S-AFUEI has the same amino acid sequence and three-dimensional structure as N-AFUEI (=AFLEI).

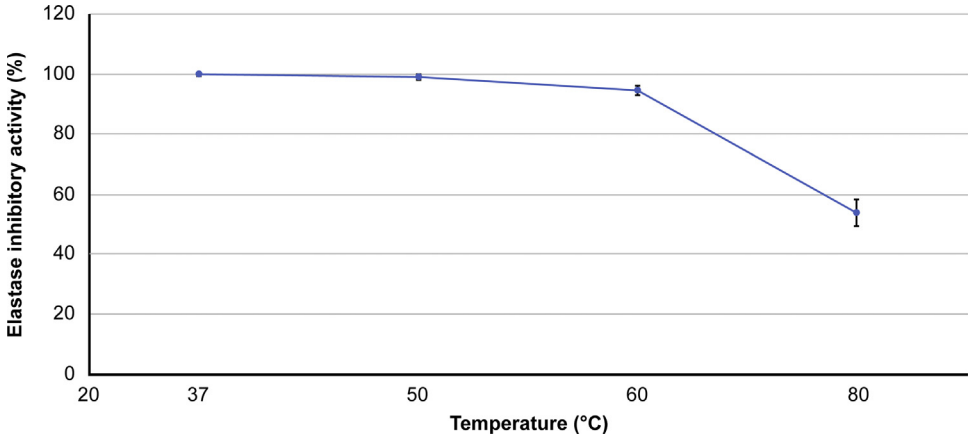

Fig. 2. Heat stability of S-AFUEI.

hydrolytic activity of AFUE; following the addition of S-AFUEI, there was no collagen hydrolysis even after 180 min (Fig. 3a).

3.6. S-AFUEI human fibrinogen hydrolytic inhibitory activity

AFUE (3.75 μg) began to hydrolyze fibrinogen after 10 min and hydrolysis was nearly complete after 30 min. In contrast, S-AFUEI strongly inhibited the fibrinogen hydrolytic activity of AFUE; following the addition of S-AFUEI, no fibrinogen hydrolysis was apparent even after 30 min (Fig. 3b).

3.7. Cytotoxic activity

Human cell cytotoxicity of AFUE and inhibitory activity of S-AFUEI on AFUE cytotoxicity was examined in HPAEC, HSAEC, and normal HPAEpiC. The cellular survival rate of HPAEC, HSAEC, and HPAEpiC with 2.5 μg/ml AFUE was approximately 21.8 ± 3.4%, 47.1 ± 15.2%, and 66.1 ± 8.3%, respectively, indicating differences in cellular survival rates (Fig. 4a–c). HPAEC had the highest degree of sensitivity to AFUE with the cellular survival rate rapidly decreasing with increasing AFUE concentrations; a similar trend was observed

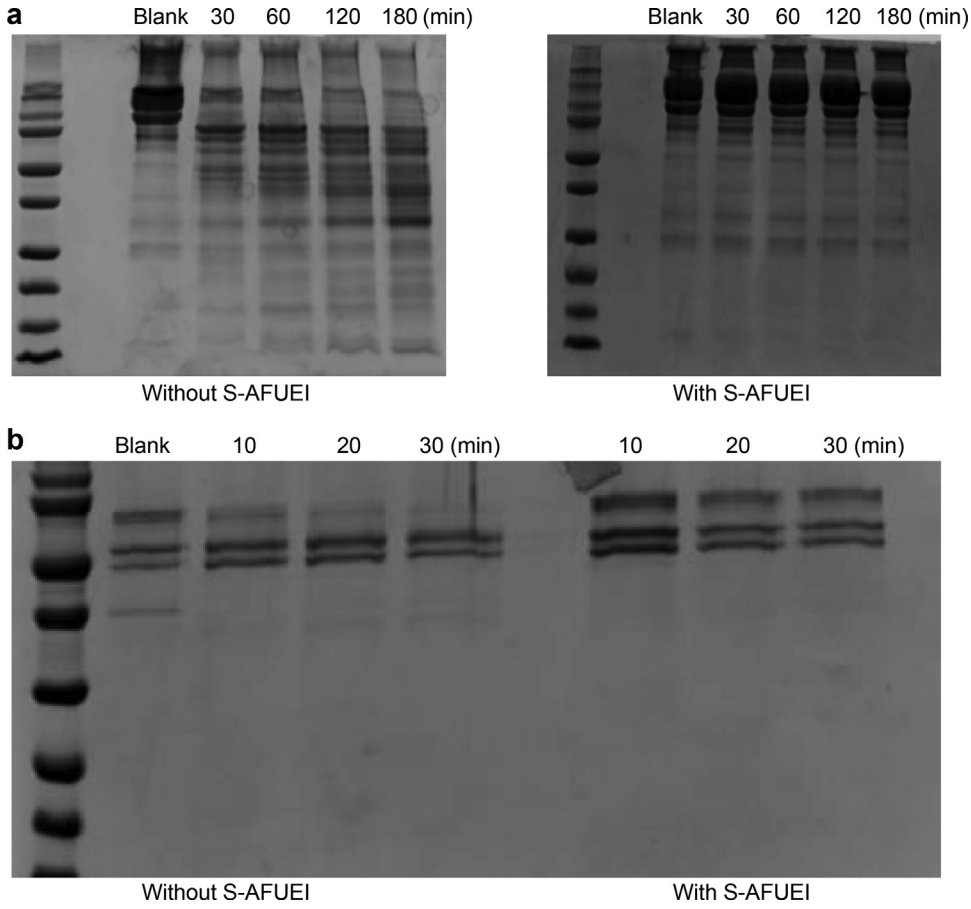

Fig. 3. AFUE collagen (type IV) hydrolytic activity with or without S-AFUEI (a). AFUE fibrinogen hydrolytic activity with or without S-AFUEI (b).

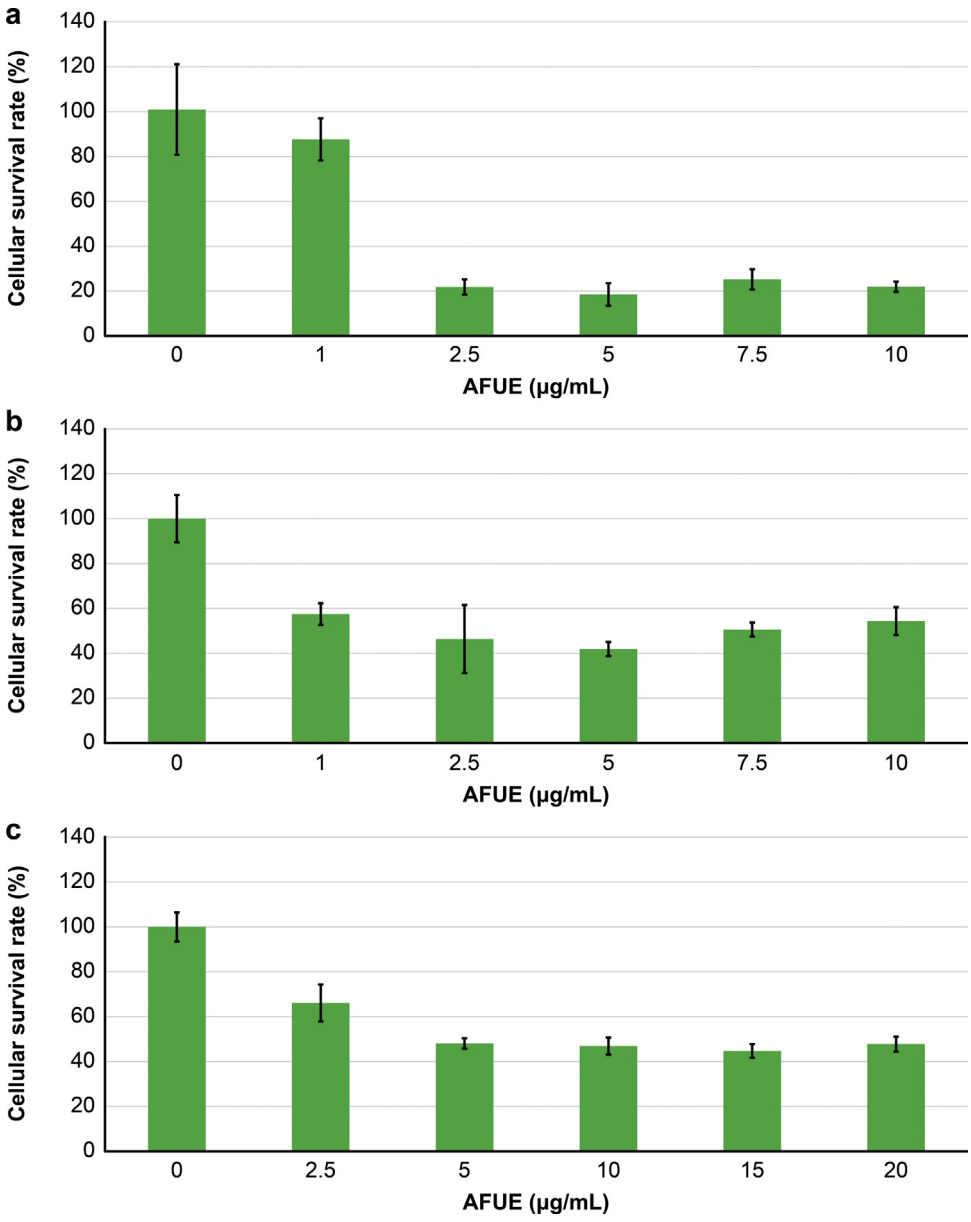

Fig. 4. AFUE cytotoxicity in human pulmonary artery endothelial cells (a). AFUE cytotoxicity in human small airway epithelial cells (b). AFUE cytotoxicity in human pulmonary alveolar epithelial cells (c).

with *Aspergillus nidulans*-derived elastase [17]. The cellular survival rate of HSAEC and HPAEpiC exceeded 40% even with 10.0 μg/ml AFUE, indicating that AFUE had a milder cytotoxic effect on these cells than on HPAEC Fig. 4a–c.

Thereafter, we reacted various molar ratios of S-AFUEI with 1 mol AFUE to assess the inhibition of AFUE cytotoxicity. The cellular survival rate of HPAEC with AFUE alone was 40.7 ± 0.8%, compared with 53.8 ± 14.8%, 83.8 ± 5.6%, and 100 ± 18.3% with 0.25 mol, 0.50 mol, and 0.75 mol S-AFUEI, respectively. The cellular survival rate of HSAEC with AFUE alone was 21.7 ± 14.4%, compared with 60.8 ± 18.8%, 80.1 ± 25.0%, and 95.3 ± 22.1% with 0.25 mol, 0.50 mol, 0.75 mol S-AFUEI, respectively; and the cellular survival rate of HPAEpiC with AFUE alone was 54.6 ± 7.0%, compared with 66.8 ± 1.0%, 81.8 ± 1.1%, and 94.6 ± 4.0% with 0.25 mol, 0.50 mol, and 0.75 mol S-AFUEI, respectively. These results demonstrate an increase in the survival rate of these cells in presence of S-AFUEI, with 0.75 mol S-AFUEI significantly inhibiting the cytotoxicity of 1 mol AFUE in human cells (Fig. 5a–c).

4. Discussion

In this study, we first examined the similarities in the biochemical properties of S-AFUEI and N-AFUEI. Furthermore, because the cellular physiological properties of S-AFUEI are unknown, we investigated whether S-AFUEI inhibits AFUE cytotoxicity.

It is known that pulmonary aspergillosis causes tissue destruction with vascular invasion. Elastin comprises approximately 20% of the lungs [21] and is considered an important element of lung tissue destruction. Moreover, elastin is abundant in the elastic fibers of the tunica media of arteries, while type IV collagen, which comprises the basement membrane, is abundant in the tunica intima of arteries. AFUE may cause vascular invasion through hydrolysis of both elastin and collagen; thus, S-AFUEI may inhibit vascular invasion by inhibiting AFUE activity. In this study, S-AFUEI strongly inhibited human fibrinogen hydrolytic activity of AFUE, similar to N-AFUEI. Fibrinogen is an important component

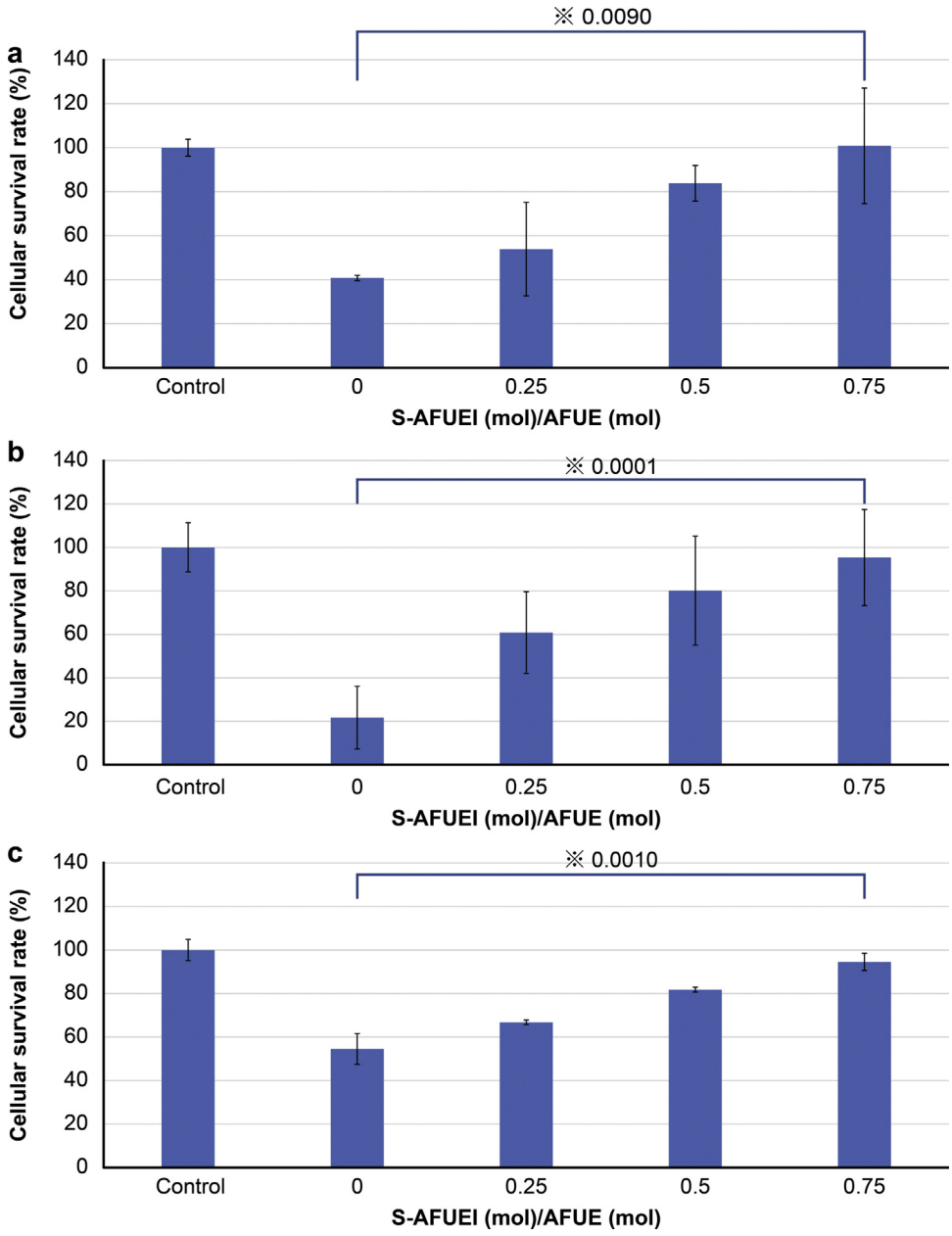

Fig. 5. Inhibition of cytotoxicity by S-AFUEI in human pulmonary artery endothelial cells (a). Inhibition of cytotoxicity by S-AFUEI in human small airway epithelial cells (b). Inhibition of cytotoxicity by S-AFUEI in human pulmonary alveolar epithelial cells (c).

for blood coagulation, and S-AFUEI inhibition of the human fibrinogen hydrolytic activity of AFUE suggests that it may inhibit the bleeding tendency observed under this condition [13].

The *Aspergillus flavus*-derived *A. flavus* elastase inhibitor (AFLEI) is similar to N-AFUEI [13]; a combinational therapy with AFLEI and amphotericin B resulted in a higher survival rate in a pulmonary aspergillosis pathology mouse model than with amphotericin B alone [22]. AFUE induces bleeding in rat lungs (observed in the alveoli and bronchioles), as well as infiltration of neutrophils and exudation of a fibrin-like substance; thus, it was hypothesized that this enzyme is involved in lung tissue damage. However, AFLEI inhibits lung tissue damage by inhibiting AFUE [22]. In this study, S-AFUEI demonstrated elastase inhibitory activity, heat stability, and inhibition of human collagen hydrolytic activity and human fibrinogen hydrolytic activity equivalent to that of N-AFUEI (=AFLEI), suggesting that the use of S-AFUEI in combination with amphotericin B could have the same effect as AFLEI in a pulmonary aspergillosis pathology model.

Furthermore, we investigated whether S-AFUEI inhibits the cytotoxicity of elastase derived from the most commonly encountered fungi, *A. fumigatus* (AFUE), in normal HSAEC, HPAEC, and HPAEpiC, to explore the clinical application of S-AFUEI in human pulmonary aspergillosis. The cytotoxic effect of AFUE is thought to cause hemoptysis in pulmonary aspergillosis in HPAEC and tissue invasion in pulmonary aspergillosis in HSAEC and HPAEpiC. S-AFUEI inhibited the cytotoxic effect of AFUE on human cells, suggesting that S-AFUEI may reduce vascular invasion and bronchial invasion in human pulmonary aspergillosis.

In conclusion, we synthesized S-AFUEI with the same amino acid sequence and three-dimensional structure as N-AFUEI (=AFLEI) and confirmed that S-AFUEI inhibition of AFUE elastolytic activity, heat stability, and inhibition of human collagen hydrolytic

activity and human fibrinogen hydrolytic activity is equivalent to that of N-AFUEI. Moreover, AFUEI inhibited AFUE cytotoxicity in human-derived cells (pulmonary artery endothelial cells, small airway epithelial cells, and pulmonary alveolar epithelial cells). This study is the first, to our knowledge, to report that S-AFUEI inhibits AFUE-induced injury with regards to biochemical and cellular aspects. S-AFUEI is considered capable of reducing vascular invasion and tissue injury in pulmonary aspergillosis, and it may be useful as a novel therapeutic agent; especially, its combination with antifungal drugs is expected to be effective. However, the in vitro nature of the present study limits the applicability of our results in vivo; hence, in vivo studies are warranted in the future.

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Disclosure of interest

The authors declare that they have no competing interest.

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