Anti-fungal Activity of Sulfamethoxazole toward *Aspergillus* Species

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Invasive mycosis has significantly increased in frequency among immunocompromised hosts leading to excessive morbidity and mortality. The combination of sulfamethoxazole (SMX) and trimethoprim (TMP) has been used extensively for the treatment and prophylaxis of infections by various microbes. The purpose of this study is to estimate the anti-fungal activity of SMX-TMP and examine the mechanism of activity. To investigate the antimicrobial activity of SMX-TMP *in vitro*, a mixture of SMX and TMP at 5 : 1 was serially diluted and added to potato dextrose agar medium or C-limiting agar medium. *Aspergillus* species were inoculated on the medium plate with SMX-TMP. The growth of *A. fumigatus* and *A. oryzae* was inhibited by addition of SMX-TMP. The anti-*Aspergillus* effect depended on not TMP but SMX and that was inhibited by *p*-aminobenzoic acid (PABA). *A. niger* was not sensitive against SMX-TMP in PDA medium, but sensitive in C-limiting medium. Those results showed that the activity depends on culture medium. Furthermore, addition of human serum did not influence the activity of SMX. The finding in this study suggested that SMX might be effective against *Aspergillus* species in clinical practice and prophylaxis treatment.

Key words sulfamethoxazole; trimethoprim; *Aspergillus* species; *Aspergillus fumigatus*; chemotherapy; prophylaxis

Opportunistic infections are becoming increasingly common due to the growing number of individuals immunocompromised by chemotherapy or immunosuppressants, such as cyclosporine and steroids.[1] For example, immunosuppressants are necessary for the treatment of autoimmune diseases such as rapidly progressive glomerulonephritis (RPGN), nephrotic syndrome.[2] Recently, fugal infections have especially become a problem.[3—5] Invasive Aspergillosis induced by *Aspergillus* species has significantly increased in frequency among immunocompromised hosts leading to excessive morbidity and mortality.[6,7] The high mortality of Aspergillosis is due in part to difficulties of diagnosis in that signs and symptoms are often nonspecific and usually appear at the late stages of disease.[8] Therefore, prophylaxis of *Aspergillus* infection is important in therapy for autoimmune disease.[9]

Sulfamethoxazole (SMX) is a sulfa drug that inhibits the metabolism of folate in microorganisms. It acts as a competitive antagonist of *p*-aminobenzoic acid (PABA), which is a component of the biosynthesis of folic acid. Then it inhibits the enzyme dihydropteroate synthase (DHPS), which catalyzes the condensation of PABA with pteridine, forming dihydropteroic acid. Clinically, SMX has been used in combination with trimethoprim (TMP) which inhibits another enzyme, dihydrofolate reductase (DHFR), involved in the metabolism of folate. Because SMX and TMP inhibit different enzymes in the same pathway, their combination (SMX-TMP) shows antimicrobial activity synergistically and against a broad spectrum of gram-positive and gram-negative bacteria.

SMX-TMP has been used extensively for treatment and prophylaxis to prevent *pneumocystis carinii* pneumonia in AIDS patients and in other immunocompromised individuals.[10—12] Moreover, it was reported that SMX is active *in vitro* against *Aspergillus* species and therefore might help to prevent invasive Aspergillosis in AIDS patients receiving SMX-TMP.[13,14] Such reports suggest that sulfa drugs have anti-fungal activity. Although there have been many papers about the anti *P. carinii* activity of sulfonamides, there have been few about anti-*Aspergillus* species and *Candida* species. Therefore, we investigated the anti-*Aspergillus* activity of SMX-TMP in detail.

The purpose of this study is to evaluate the anti-fungal activity of SMX-TMP and examine the mechanism of this activity. Furthermore, we estimated *in vitro* whether SMX-TMP actually acts in the human body by adding human serum to medium with drugs and fungi.

MATERIALS AND METHODS

Materials SMX, TMP, folic acid (FA) and PABA were purchased from Sigma Chemical Company (U.S.A). Filter paper was purchased from ADVANTEC Toyo Roshi kaisha, Ltd, Japan. *Aspergillus fumigatus* IFO 30870, *Aspergillus niger* IFO 6342, *Aspergillus oryzae* IFO 30103, *Candida albicans* IFO 1385, and *Candida parapsilosis* IFO 1068 were purchased from the Institute for Fermentation, Osaka, Japan (IFO). The fungi were maintained on plate of Potato dextrose agar (PDA) purchased from Difco, U.S.A. at 27 °C and transferred to a new medium once every three months. Human sera were collected from healthy donors.

Media PDA and C-limiting medium, originally described by Shepherd and Sullivan,[15] were used in experiments on antimicrobial activity. The C-limiting agar medium that we used contained (per liter) sucrose 10 g, $(NH_4)_2SO_4$ 2 g, KH_2PO_4 2 g, $CaCl_2 \cdot 2H_2O$ 0.05 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $ZnSO_4 \cdot 7H_2O$ 1 mg, $CuSO_4 \cdot 5H_2O$ 1mg, $FeSO_4 \cdot 7H_2O$ 0.01 g, biotin 25 μg, and agar 10 g, with a final pH of 5.2.

Measurement of Anti-fungal Activity by Plate Culture Added with Antimicrobial Agent SMX and TMP were dissolved in methanol at an initial concentration of 50 mg/ml. SMX is now used mainly as a mixture with TMP[32] and a

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mixture SMX and TMP at a ratio of 5 : 1 has marketed in Japan. Therefore, SMX-TMP as an antimicrobial agent was prepared by mixing SMX and TMP at a ratio of 5 : 1, and dissolved similarly. Then, the agents were diluted 2 times serially in methanol at a concentration of 25, 12.5, 6.25 or 3.125 mg/ml. The diluted forms were added to PDA or C-limiting agar medium in order to obtain final concentrations of 500, 250, 125, 62.5 and 31.25 μg/ml while the medium melted.

A. fumigatus, *A. niger*, *A. oryzae*, *C. albicans*, and *C. parapsilosis* were passaged at an interval of 7 d at 27 °C by subculturing on PDA plates to obtain adequate sporulation. Conidia of *Aspergillus* species were collected and suspended in saline. In the case of *Candida* species, a single colony was removed and suspended in saline. This homogeneous suspension (10 μl) was inoculated onto the center of a plate of PDA or C-limiting agar medium. The inoculation was performed in four plates. Each strain was incubated stationary for 7 d at 27 °C.

After incubation, the diameter of the giant colony was measured.

Anti-fungal Activity of SMX Alone, TMP Alone or the Combination SMX and TMP were dissolved in methanol at an initial concentration of 10 mg/ml. SMX-TMP was prepared at 10 mg/ml. The agents were added to C-limiting agar medium in order to obtain final concentrations of 100 μg/ml while the medium melted. As described in protocol, suspension of *A. fumigatus*, *A. niger* or *A. oryzae* were prepared and placed onto the center of a plate. After incubation for 7 d, the colony size was measured.

Reversal of the Inhibition of the Antimicrobial Agent by Exogenous Folic Acid and *p*-Aminobenzoic Acid FA and PABA were dissolved in saline at 1 mg/ml. Then they were diluted 2 times serially in saline at a concentration of 500, 250, 125, or 0 μg/ml. The diluted FA and PABA were added to PDA plates with SMX-TMP at 100 μg/ml in order to obtain final concentrations of 10, 5, 2.5, 1.25 and 0 μg/ml while the medium melted. A PDA plate with methanol instead of SMT-TMP was prepared as a control.

As described above, *A. fumigatus* was inoculated, the diameter of the giant colony was measured.

Reversal of Anti-*A. niger* Activity by Exogenous PABA in C-Limiting Agar Medium with SMX-TMP PABA was dissolved in saline at 1mg/ml and was diluted 2 times serially in saline at a concentration of 500, 250, 125 or 0 μg/ml. The diluted PABA was added to PDA plate with SMX-TMP at 100 μg/ml in order to obtain final concentrations of 10, 5, 2.5, 1.25 and 0 μg/ml while the medium melted. A C-limiting agar plate with methanol instead of SMT-TMP was prepared as a control.

As described above, conidia of *A. niger* were collected and inoculated into the center of a plate of C-limiting agar. The diameter of the giant colony was measured.

Detection of Inhibitors for the Anti-*A. niger* Activity of SMX-TMP in PDA Water soluble fraction of PDA (sPDA) was prepared from Potato dextrose agar (Difco, U.S.A.) by centrifugation. After centrifugation, the supernatant was filtrated for sterilization. A part of sPDA was dialyzed to remove low molecular weight materials including PABA. After the dialysis, supernatant fluid (dPDA) was centrifuged and filtrated for sterilization. These fractions were prepared before use.

Pieces of filter paper cut in a circle of diameter 6 mm were sterilized by autoclave. After adequately drying, the pieces were soaked with saline as a control, PABA in saline at 100 μg/ml, the fraction of sPDA and the fraction of dPDA. Conidia of *A. niger* were collected and suspended in saline. The suspension (1 ml) of *A. niger* was inoculated homogeneously onto a plate of C-limiting agar with SMX-TMP at 100 μg/ml or 0 μg/ml. Then, pieces of filter paper soaked with saline (upper left), PABA (upper right), sPDA (lower left) or dPDA (lower right), were placed on PDA plates with or without SMX-TMP. *A. niger* was incubated stationary for 2 d at 27 °C.

After incubation, it was observed whether *A. niger* grew around the filter paper with saline, PABA, sPDA or dPDA.

Detection of Inhibitors for the Activity of SMX-TMP in Human Serum Pieces of filter paper cut in a circle 6 mm in diameter were sterilized by autoclave. After adequately drying, those pieces were soaked with saline as a control, PABA in saline at 100 μg/ml, Folic acid in saline at 100 μg/ml or human serum.

Conidia of *A. fumigatus* were collected and suspended in saline as described above. The suspension (1 ml) was inoculated homogeneously onto a plate of PDA medium with SMX-TMP at 100 μg/ml or 0 μg/ml. The inoculation was performed in duplicate.

Then, the circular pieces of filter paper soaked with saline (upper left), FA (upper right), PABA (lower left) or human serum (lower right), were placed on PDA plates with or without SMX-TMP. *A. fumigatus* on the plate was incubated stationary for 2 d at 27 °C.

After incubation, it was observed whether *A. fumigatus* grew around the filter paper with saline, FA, PABA or human sera.

RESULTS

SMX Shows Antimicrobial Activity *in Vitro* against *Aspergillus* Species and *C. albicans*, but the Activity Depended on Culture Medium *A. fumigatus*, *A. niger* and *A. oryzae* as *Aspergillus* species were incubated on PDA medium. Colony sizes of *A. fumigatus* and *A. oryzae* decreased with the increase of SMX-TMP in medium. But the colony of *A. niger* was unchanged despite of a larger dose of drug (Fig. 1A). Notably, the colony of *A. fumigatus* decreased in size by 50% on addition of SMX-TMP at about 100 μg/ml.

With a similar protocol, *A. fumigatus*, *A. niger* and *A. oryzae* were incubated on C-limiting agar medium that contained serially diluted SMX-TMP. In the absence of drugs, the colony of *Aspergillus* species in C-limiting agar medium was smaller than that in PDA medium (Fig. 1B). Moreover, the 50% inhibition dose of SMX-TMP against *Aspergillus* species was lower than that in PDA medium. It is of note that *Aspergillus niger* which was resistant to SMX-TMP in PDA medium, was sensitive to SMX-TMP in C-limiting agar medium.

Figure 1 showed that the anti fungal activity of SMX-TMP differed among *Aspergillus* species. Therefore, the activity against *Candida albicans* and *Candida parapsilosis* were examined using a similar protocol. Neither *C. albicans* nor *C.*

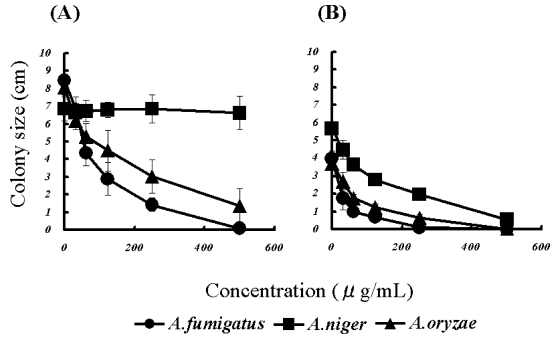

Fig. 1. Anti-*Aspergillus* Activity of the Combination of SMX and TMP

Conidia from *Aspergillus* species were suspended in saline, and the suspension was placed onto the center of PDA (A) or C-limiting agar (B), a synthetic medium with SMX-TMP at serial concentrations. After incubation for 7 d at 27 °C, giant colonies were observed. Symbols in the figure represent ●; *A. fumigatus*, ▲; *A. oryzae*, ■; *A. niger*. The error bars here represent standard deviations.

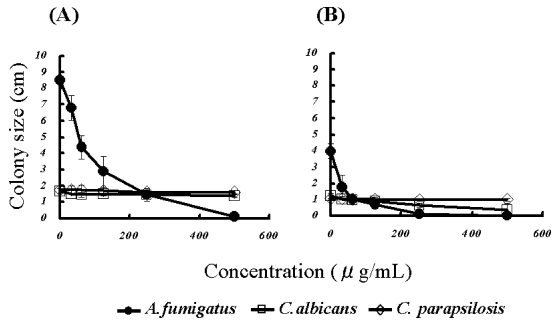

Fig. 2. Anti-*Candida* Activity of the Combination of the SMX and TMP

A single colony from *Candida albicans* and *Candida parapsilosis* was suspended in saline, and the suspension was placed at the center of PDA (A) or C-limiting agar (B) medium with SMX-TMP at serial concentrations. *A. fumigatus* was incubated as a control. After incubation for 7 d at 27 °C, the diameter of the giant colony was. Symbols in the figure represent ●; *A. fumigatus*, □; *Candida albicans*, ◇; *Candida parapsilosis*. The error bars here represent standard deviations.

parapsilosis was sensitive to SMX-TMP in PDA medium (Fig. 2A). In C-limiting medium, *C. albicans* was sensitive to SMX-TMP (Fig. 2B), but *C. parapsilosis* was not sensitive despite of high medicine dose at 500 μg/ml. This result shows that effect of SMX-TMP varies in fungal strain.

Clinically, SMX and TMP are used as a mixture of 1 : 5. To examine whether either SMX or TMP has an anti-*Aspergillus* effect, SMX or TMP was added to PDA medium and *A. fumigatus* was cultured on these medium. Figure 3A shows that SMX exhibited anti *A. fumigatus* activity but TMP did not. Furthermore, The activity of SMX or TMP alone was investigated against other *Aspergillus* species. All other *Aspergillus* species were sensitive against SMX alone and SMX-TMP as well as *A. fumigatus* (Fig. 3B).

Next, to examine whether *A. niger* produces inhibitors of antibiotics, we performed a coculture of *A. niger* with *A. fumigatus* in PDA medium with SMX-TMP. If *A. niger* produces anti antibiotic components, they may affect the growth of *A. fumigatus*. But the colony size of *A. fumigatus* was the same as in the case of the single culture (Fig. 4). The result ruled out that *A. niger* produces components against anti chemotherapeutic agent.

***p*-Aminobenoate Reversed the Effects of the Active Sulfa Drugs, But Folate Did Not** Sulfonamides such as SMX act as a competitor antagonist of PABA and demonstrate anti microbial activity by inhibiting folate synthesis. To evaluate whether the anti fungal activity depends on inhibition of folate synthesis, folic acid or PABA were added to PDA medium with SMX-TMP at 100 μg/ml and *A. fumigatus* was cultured in this medium. However, despite a high concentration (10 μg/ml), addition of folate could not reverse the inhibition by SMX-TMP (Fig. 5A). It was suggested that *A. fumigatus* could not use folic acid for the metabolism of folate. Therefore, PABA that is competed by SMX was added to PDA medium with drugs. The addition of PABA reversed the effects of SMX-TMP (Fig. 5A). The quantity that had an effect on reversion was smaller than 1 μg/ml. The result suggested that *Aspergillus* species needs PABA for folate usage.

From the results in Fig. 1, it was clear that the sensitivity of *A. niger* to SMX-TMP differed with the culture media. Therefore, to examine whether the sensitivity of *A. niger* depends on PABA in the medium, *A. niger* was cultured in the C-limiting medium plate with PABA and SMX-TMP at 100 μg/ml. Colony size of *A. niger* increased dependent on the dose of PABA added to the plate (Fig. 5B). Furthermore, because PDA may contain inhibitors of SMX-TMP like

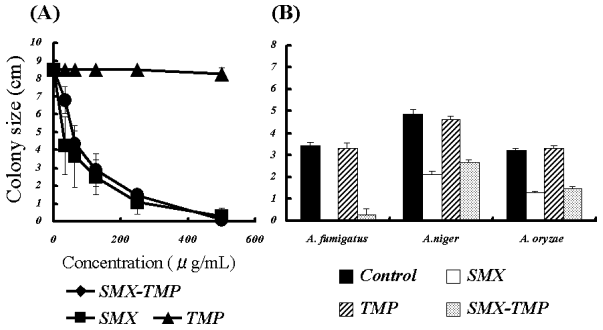

Fig. 3. Anti-fungal Activity of SMX Alone, TMP Alone, or the Combination

Conidia from *A. fumigatus*, *A. niger*, *A. oryzae* and the yeast of *C. albicans* was suspended in saline, (A) the suspension of *A. fumigatus* was placed at the center of PDA medium with SMX-TMP (◆), SMX alone (■) or TMP alone (▲) at serial concentrations. After incubation, the colony size was measured. (B) The other fungal suspension was placed at the center of C-limiting medium with SMX-TMP, SMX alone, or TMP alone at 100 μg/ml. According to similar protocol, the colony size was measured. The error bars here represent standard deviations.

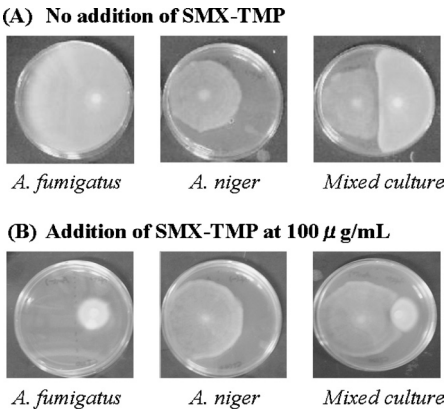

Fig. 4. Mixed Culture of *A. fumigatus* and *A. niger* in PDA Medium with SMX-TMP

Conidia from *A. fumigtus* and *A. niger* were suspended in saline, and the suspension was placed onto PDA medium with SMX-TMP at 0 (A) or 100 μg/ml (B). The culture was performed with *A. fumigatus* alone (Left), *A. niger* alone (middle) or both *A. fumigatus* and *A. nigar* (Right). No interaction of *A. fumigatus* and *A. niger* in plates with or without SMX-TMP was observed.

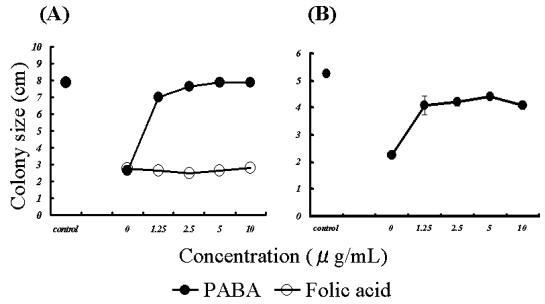

Fig. 5. Reversal of Anti-chemotherapeutic Agent Inhibition by Exogenous Folic Acid and *p*-Aminobenzoic Acid

(A) Serially diluted FA (○) and PABA (●) were added to PDA with SMX-TMP at 100 μg/ml. Conidia from *A. fumigatus* were suspended in saline, and the suspension was placed onto the center of prepared plates. A PDA plate without SMX-TMP was used as a control. After incubation for 7 d at 27 °C, the diameter of the giant colony was measured the colony size. (B) Serially diluted PABA (●) was added to C-limiting agar medium with SMX-TMP at 100 μg/ml. Conidia from *A. niger* were suspended in saline, and the suspension was placed onto the center of the prepared plates. C-limiting agar medium without SMX-TMP was used as a control. After incubation for 7 d at 27 °C, the diameter of the giant colony was measured. The error bars here represent standard deviations.

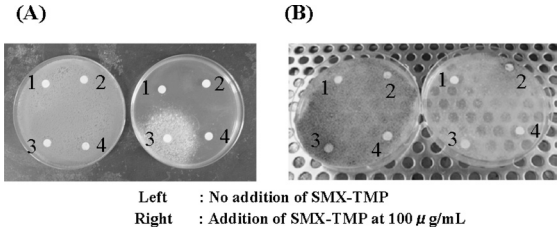

Fig. 6. Detection of Inhibitors for the Activity of SMX-TMP in Human Serum and PDA Medium

(A) Conidia from *A. fumigatus* were suspended in saline, and the suspension was placed in PDA medium with SMX-TMP at 0 (left plate in Fig. 6A) or 100 μg/ml (right). Filter paper treated with saline (No. 1), Folic acid (No. 2), PABA (No. 3) or human serum (No.4), was placed on the PDA plate with agents and *A. fumigatus*.

(B) The suspension of *A. niger* was placed in C-limiting agar medium with SMX-TMP at 0 (left plate in Fig. 6B) or 100 μg/ml (right). Filter paper treated with saline (No. 1), PABA (No. 2), water-soluble fraction of PDA (No. 3) or dialyze water-soluble fraction of PDA (No. 4), was placed on the plate with the agents and *A. niger*. *A. niger* grew around the circular pieces treated with PABA and sPDA.

PABA as a cause of why SMX-TMP did not affect *A. niger* in PDA medium, we examined whether the addition of the water-soluble fraction of PDA inhibits the effect of SMX-TMP on *A. niger*. A part of the water-soluble fraction of PDA was dialyzed to remove low weight molecules such as PABA (Mw 137). Round pieces of filter paper were soaked with saline, PABA, the sPDA and dPDA, and were placed on *A. niger* culture in the presence of SMX-TMP at 100 μg/ml in C-limiting agar medium. The culture was performed for 48 h at 27 °C. Colonies of *A. niger* germinated around the paper treated with PABA and sPDA, but not dPDA (Fig. 6B). The results suggested that sPDA contains inhibitors of SMX-TMP whose molecular weights are low such as PABA and *A. niger* is insensitive to SMX-TMP because of these inhibitors.

No Factors that Inhibit the Activity of SMX-TMP Exist in Human Serum As shown in Fig. 6, SMX-TMP does not have anti-fugal activity in the presence of PABA. Similar inhibitors may be present in patient's blood. Therefore, it was examined whether the addition of human serum inhibits the effects of SMX-TMP. Pieces of filter paper with a diameter of 6 mm were soaked with saline, PABA, Folic acid or human serum, and placed on *A. fumigatus* culture in the presence of SMX-TMP at 100 μg/ml. Cultures were performed for 48 h at 27 °C. After 48 h, colonies of *A. fumigatus* germinated around the paper treated with PABA but not human serum (Fig. 6A). In the case of folic acid, growth was not as good as the result in Fig. 5A. This result suggested that factors which inhibit the activity of SMX-TMP do not exist in human serum and SMX-TMP shows anti-fungal activity *in vivo*.

DISCUSSION

In the present study, we confirmed the anti-fungal effect of SMX and TMP used against a broad spectrum of gram-positive and gram-negative bacteria. The addition of approximately 100 μg/ml of SMX-TMP into media decreased of the colony size by 50%. Cmax of SMX and TMP is respectively 50—60 μg/ml and 1.5—2.5 μg/ml. Therefore, the concentration of 100 μg/ml may be a little high level, but actually it can be this concentration.[33,34] Furthermore, we have shown that the anti-fungal effect depends on not TMP but SMX and that it is inhibited by PABA which antagonizes SMX. Those results suggested that SMX-TMP used as a bactericidal agent has not only an anti-bacterial effect but also an anti-fungal one. In other words, treatment with SMX-TMP may protect immunocompromised patients from fungal infections. There may, therefore be other medicines that have unknown beneficial effects.

SMX inhibited the growth of *A. fumigatus* and *A. oryzae* but not *A. niger*, *Candida albicans* or *Candida parapsilosis* in PDA (Figs. 1A, 2A). However, in C-limiting agar medium a synthetic medium, the growth of *A. niger* and *C. albicans* were suppressed by adding SMX (Fig. 1B). Moreover, addition of PABA, not folic acid, restored the growth of *Aspergillus* species (Fig. 5, 6). These results suggested that the effect of SMX depends on the components of the culture medium. Verweij *et al.* reported anti-*Aspergillus* activity of SMX.[13] They used two media, RPMI 1640 and yeast nitrogen base (YNB). RPMI medium contains four times more PABA than YNB. The result showed that the MICs obtained with RPMI 1640 medium were significantly higher than those obtained with YNB medium. In the present study, we also used two media, C-limiting medium and PDA medium. C-limiting medium is a synthetic medium and does not contain PABA, whereas PDA medium is a natural medium that may contain many nutrients, such as PABA and folic acid, for the growth of microorganism. Figure 6B shows that PDA medium contains inhibitors of SMX-TMP activity. Therefore, the presence of PABA explains the difference in the anti-*A. niger* activity of SMX differs between the two culture media. But, *A. fumigatus* was inhibited in both C-limiting and PDA medium. Furthermore, in C-limiting medium, the colony of *A. fumigatus* was smaller than that in PDA medium, whereas there was little change with the two media in the colony size of *A. niger* (Fig. 1). The difference in colony growth of *A. fumigatus* and *A. niger* may depend on the folate synthetic pathway. *A. fumigatus* and *A. oryzae* which are highly sensitive to sulfa drugs such as SMX may need many folate components, whereas *A. niger* may require little folate. Therefore, Fig. 5 suggested that *Aspergillus* species have folate synthesis pathway and need for growth. In study for utilization of exogenous folaes in yeast, one mutant lacked DHPS and DHFS grow up by addition of exogenous folinic acid but not folic acid.[35] *Aspergillus* species

may not intake folic acid despite of eukaryotic cells. It may help with the development of new antimicrobial agents and application of conventional antimicrobial agents to research the metabolic biochemistry of the fungus.

The combination SMX-TMP has been used extensively for the treatment and prevention of *Pneumocystis carinii* pneumonitis.[10—12] Although *Pneumocystis carinii* lacks the major fungal sterol, ergosterol, *P. carinii* is closely related to fungi.[16] Moreover, it has been reported that the combination of SMX and TMP acts synergistically against the dimorphic fungus *Paracoccidioides brasiliensis*.[17,18] Those reports suggest that SMX-TMP acts toward fungus. In the present study (Fig. 1) and other reports,[13,14] SMX acted against *Aspergillus* species. The results suggest that SMX-TMP has a general anti-fungal effect. However, the effect of SMX-TMP toward *Candida* species was weaker than toward *Aspergillus* species. (Fig. 2). Some research suggests that sulfonamides, such as SMX, alone have anti-*P. carinii* activity without Trimethoprim.[19—21] Figure 3 shows also that the anti-*Aspergillus* effect depended on SMX, not TMP. Thus, the dihydropteroate synthase (DHPS) that is a target enzyme of sulfonamides may differ between sulfonamide-sensitive fungi, such as *P. carinii*, *Aspergillus* and non-sensitive fungi, such as *C. albicans*. The gene sequence of DHPS from *Saccharomyces cerevisiae* and *P. carinii* was already revealed.[11,22,23] Phylogenetic analysis of the DHPS sequence revealed that it's closely related to ascomycete fungi.[23] Furthermore, a gene similar to the DHPS gene from *C. albicans* was also revealed.[24] However, no gene sequence of DHPS from *Aspergillus* species has been reported yet. The sequence of DHPS from *Aspergillus* species may resemble the sequence from *P. carinii* more than that from *Candida* species, especially, *Candida parapsilosis*, given the effect of the sulfonamides. Research on the *Aspergillus* DHPS gene will be needed for the understanding and usage of sulfonamides such as SMX-TMP.

In this study, the anti-*Aspergillus* activity of SMX was restored by the addition of a small amount of PABA, less than 1 μg/ml (Fig. 5). If inhibitors of sulfonamides, such as PABA, do exist in human serum and tissue, anti-microbial susceptibility testing *in vitro* may be meaningless clinically. However, Figure 6A shows that factors inhibiting the activity of SMX-TMP do not exist in human serum. This result suggests that SMX-TMP would act against *Aspergillus* species in a clinical setting. Although it is important to use animal models of infection for the assessment of medicines,[21,25—27] such experiments are complex and not easy for non-professionals of microbiology.

Tang *et al.* demonstrated that PABA requiring mutant of *A. nidulans* was nonphathogenic in murine models of invasive pulmonary Aspergillosis.[36] In research for pathogenicity of *A. fumigatus*, Sandhu *et al.* reported that the anxotrophic mutants which have absolute growth requirement for PABA were completely avirulent to mice.[37] Those results supports that SMX could be used as therapeutic agent of Aspergillosis. Further studies of folate metabolism in *Aspergillus* species are needed for clinical application of sulfamides.

Adverse reactions to SMX-TMP have frequently been reported.[28—31] The most frequent reactions include rash, nuetropenia, and fever. However, some evidence suggests that at least some of the adverse reactions to SMX-TMP might be due to the TMP component[19,28,31] and that sulfonamides such as SMX alone have anti-*P. carinii* activity.[20,21] In the present study, SMX alone had anti-*Aspergillus* activity. Thus, sulfonamides may be useful for the prophylaxis of fungal infections by *Aspergillus* species and *P. carinii etc*.

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