Inhibitory Activity to Protein Prenylation and Antifungal Activity of Zaragozic Acid D3, a Potent Inhibitor of Squalene Synthase Produced by the Fungus, *Mollisia* sp. SANK 10294

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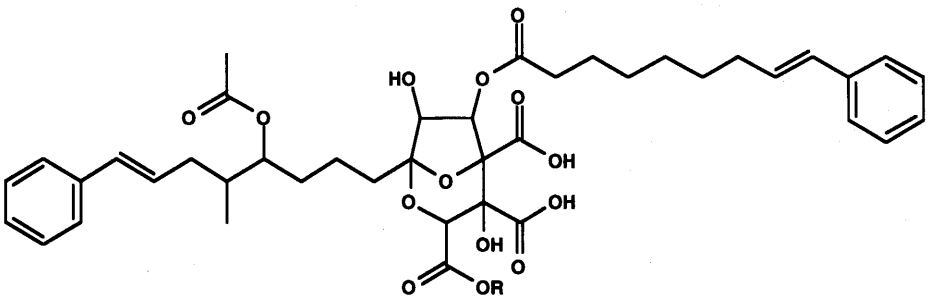
Recently we found novel zaragozic acids (ZAs), F-10863A (zaragozic acid D3, ZAD3), B, C and D in the culture broth of the fungus *Mollisia* sp. SANK 10294 as potent inhibitors of squalene synthase. There are several other enzymes that use farnesylpyrophosphate as their substrate. Among them we chose farnesyl-protein transferase and examined whether ZAD3 and F-10863B inhibit this enzyme's activity. ZAD3 and F-10863B inhibited farnesyl-protein transferase with IC_{50} values of 0.60 and 3.7 μM, respectively. They also inhibited geranylgeranyl-protein transferase at similar concentrations. In addition, they exhibited potent antifungal activity.

Zaragozic acids are a family of fungal metabolites having potent inhibitory activities on squalene synthase (SQS)[1]. Recently we found novel zaragozic acids, F-10863A (zaragozic acid D3, ZAD3) and related compounds (Fig. 1), in the culture broth of the fungus *Mollisia* sp. SANK 10294 as potent inhibitors of SQS[2,3]. Since both SQS and farnesyl-protein transferase (FPTase) use farnesylpyrophosphate (FPP) as their substrate, we examined the inhibitory activities of F-10863A and B on FPTase and a related enzyme, geranylgeranyl-protein transferase I (GGPTase I). Isoprenylation is a post-translational modification that involves the formation of thioether bonds between cysteine and isoprenyl groups derived from pyrophosphate intermediates of the cholesterol biosynthetic pathway[4,5]. FPTase catalyses the transfer of the farnesyl group of FPP to proteins ending with the carboxyl-terminal sequence CAAX where A is an aliphatic amino acid, and X is serine or methionine. GGPTase I catalyses the transfer of the geranylgeranyl group to proteins ending with the carboxyl-terminal sequence CAAX where X is leucine or phenylalanine. Many types of inhibitors of these protein prenylation enzymes have been reported[6,7]. Among them, ZAD3 and F-10863B are moderately potent inhibitors of FPTase and GGPTase. In addition, since many inhibitors of cholesterol synthesis have antifungal activities, we also

Fig. 1. Structures of ZAD3 and F-10863B.



R=H; F-10863A (Zaragozic acid D3)
R=Me; F-10863B

examined the antifungal activities of ZAD3 and F-10863B. In this paper we describe the inhibitory activities of ZAD3 and F-10863B to FPTase and GGPTase, and their antifungal activities.

Materials and Methods

Materials

[1-^{3}H]-FPP (22.5 μCi/μmol) and [1-^{3}H]-geranylgeranyl pyrophosphate (GGPP, 21.5 μCi/μmol) were purchased from New England Nuclear Corp. (Boston, MA, USA). Recombinant wild type human H-ras protein and recombinant CVLL type human H-ras protein were purchased from Calbiochem-Novabiochem (La Jolla, CA, USA).

Cells and Preparation of Enzymes

Human THP-1 cells were purchased from ATCC. The cells were cultured in RPMI 1640 medium supplemented with 10% FBS at 37°C. The fraction containig both FPTase and GGPTase activities was partially purified from the lysate of THP-1 cells by the method of OMURA *et al.*[8].

Assay Method for FPTase Activity

FPTase activity was determined by measuring the amount of [^{3}H]-farnesyl moiety transferred from [^{3}H]-FPP to H-ras protein. Assays of FPTase were done by the method of OMURA *et al.*[8]. Briefly, the standard reaction mixture contained 50 mM Tris-HCl (pH 7.5), 50 μM $ZnCl_2$, 4 mM $MgCl_2$, 4 mM dithiothreitol, 1.3 μM recombinant wild type human H-ras protein, 0.03 μM [1-^{3}H]-FPP, 3 μl of inhibitor solution in MeOH and 10 μg of enzyme fraction in a final volume of 60 μl. The reaction mixture in a 13 × 100 mm borosilicate tube was incubated at 37°C for 30 minutes. The reaction was stopped by addition of 0.5 ml of 1% SDS and 0.5 ml of 30% TCA. The tube was vortexed and left on ice for 60 minutes. Then the mixture was filtered on a Whatman GF/C filter and washed with 5 ml of 6% TCA. The filter was dried and counted in a liquid scintillation counter.

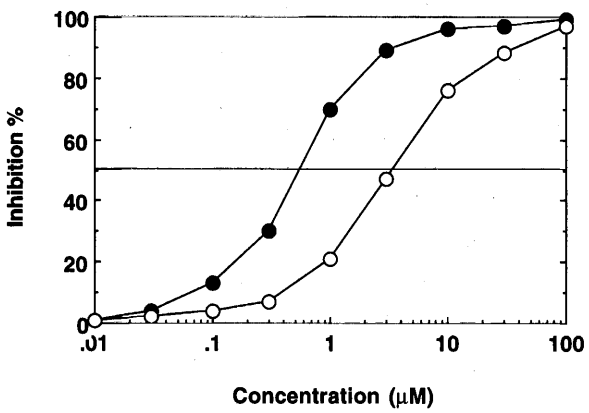
Assay Method for GGPTase I Activity

Assays of GGPTase I were done using essentially the same method as FPTase described above. Instead of recombinant wild type human H-ras protein and [1-^{3}H]-FPP, the same concentrations of recombinant CVLL type human H-ras protein and [1-^{3}H]-GGPP were used as the substrates for the GGPTase assay.

Assay Method for Antifungal Activities

Antifungal activities of ZAD3 and F-10863B were determined by the agar dilution method using Sabouraud media. Compounds were dissolved in DMSO and diluted serially with 50% acetone. One hundred μl of sample solution was mixed with 900 μl of Sabouraud agar medium in a 24-well microplate to make agar plates. Test strains were grown on Sabouraud slant agar and suspended in 5 ml of saline. The agar plates were inoculated with 5 μl of the cell suspensions and incubated at 27°C for 2 days for yeasts or 7 days for filamentous fungi. The minimal inhibitory concentration (MIC) was expressed as the lowest concentration of the compounds that inhibited visible cell growth after incubation.

Fig. 2. Inhibition of FPTase by ZAD3 and F-10863B.



Human FPTase activity was determined as described in Materials and Methods. Symbols represent ZAD3 (●), F-10863 B (○).

Results

Inhibitory Activities to FPTase

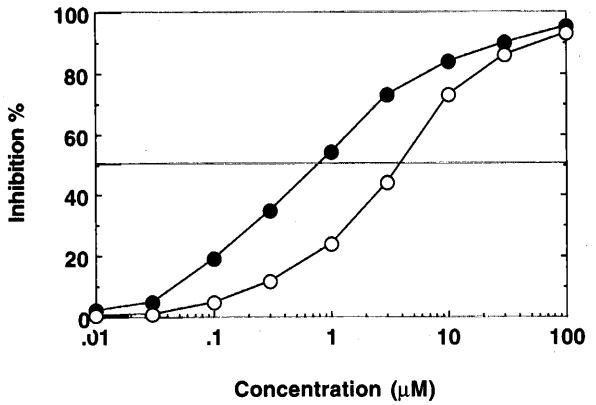
FPTase activity was measured in the presence of various concentrations of ZAD3 and F-10863B. The two compounds showed potent FPTase inhibitory activities

Table 1. Inhibitory activities of F-10863 compounds to enzymes of prenylation

Compound	IC_{50} (μM)		
	SQS	FPTase	GGPTase
Zaragozic acid D3	0.0007[a]	0.60	0.78
F-10863B	0.0013[a]	3.7	3.8

[a] Data from reference 2.

Fig. 3. Inhibition of GGPTase I by ZAD3 and F-10863 B.



Human GGPTase I activity was determined as described in Materials and Methods. Symbols represent ZAD3 (●), F-10863 B (○).

Table 2. Antifungal activities of ZAD3 and F-10863B.

Organism	MIC (μg/ml)	
	ZAD3	F-10863B
Candida albicans Sc.	3.1	>50
Candida albicans 427	1.5	12.5
Cryptococcus neoformans 58063	6.2	25
Mucor mucedo 14358	>50	>50
Aspergillus fumigatus 10569	0.8	3.1
Microsporum gypseum 11268	1.5	6.2
Trichophyton mentagrophytes Sc.	0.1	0.4
Trichophyton rubrum Sc.	0.1	0.4

in a dose-dependent manner. The IC_{50} values of ZAD3 and F-10863B were 0.60 and 3.7 μM, respectively (Fig. 2, Table 1).

Inhibitory Activities to GGPTase

GGPTase activity was measured in the presence of various concentrations of ZAD3 and F-10863B. The two compounds showed potent GGPTase inhibitory activities in a dose-dependent manner. The IC_{50} values of ZAD3 and F-10863B were 0.78 and 3.8 μM, respectively (Fig. 3, Table 1).

Antifungal Activities

Antifungal activities were expressed as MICs (Table 2). ZAD3 showed potent *in vitro* antifungal activity with low MIC values against *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Microsporum gypseum*, *Trichophyton mentagrophytes* and *T. rubrum*. F-10863B has weaker activity than ZAD3 against the fungi described above.

Discussion

ZAD3 and F-10863B are novel members of ZAs having highly potent inhibitory activities to SQS. F-10863B is the monomethyl ester of the carboxyl group at C3 of ZAD3 (Fig. 1). ZAD3 and F-10863B also inhibited both FPTase and GGPTase in dose-dependent manner (Fig. 2 and 3). Inhibitory activities of ZAD3 and F-10863B to SQS, FPTase and GGPTase are summarized in Table 1. Comparing with their highly potent inhibitory activities to SQS, ZAD3 and F-10863B are moderately potent inhibitors of FPTase and GGPTase. No selectivity was observed between the two protein prenylation enzymes. As with the case of SQS, ZAD3, which has three free carboxylic acid residues, is a more potent inhibitor of FPTase and GGPTase than F-10863B, which has only two. Although the inhibitory potency of the two compounds to SQS differ less than twofold, the potency to FPTase and GGPTase differ five- to sixfold. Having three free carboxylic acid residues is more important for recognition by protein prenylation enzymes than by SQS. Zaragozic acid A (ZAA) is reported to have inhibitory activities on these two protein prenylation enzymes at a comparable potency with the F-10863 compounds[6].

The F-10863 compounds showed potent antifungal activities (Table 2). Ergosterol is essential for the maintenance of the fungal cell wall, and inhibition of its biosynthesis pathway disturbs fungal growth. For example, many azols, which inhibit lanosterol 14-demethylase, and allylamines, which inhibit squalene epoxidase, have been reported or already used as potent antifungal agents. The F-10863 compounds inhibited the pathway at the step catalyzed by SQS. ZAA was also reported to have antifungal activity[1]. As with the case of enzyme-inhibition, ZAD3 is also a more potent antifungal agent than F-10863B. The potency of SQS inhibitory activity of the two compounds parallels the antifungal activities.

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