INHIBITION OF FARNESYL-PROTEIN TRANSFERASE BY GLIOTOXIN AND ACETYLGLIOTOXIN

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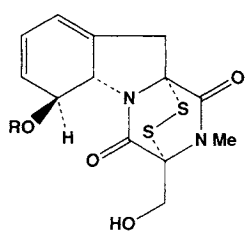
Ras p21 proteins have been shown to be post-translationally farnesylated on a specific carboxy-terminal cysteine[1~3]. Inhibition of this isoprenylation would alter membrane localization and activation of *ras* oncogene[4]. Therefore during our screening for farnesyl-protein transferase (FTase) inhibitors of microbial origin we have isolated gliotoxin (GT) and acetylgliotoxin (AGT) from the fermentation broth of a fungus. GT and AGT are known as fungal epipolythiodiketopiperazine toxin[5] and showed a broad spectrum of activity including inhibition of fungi, bacteria and viruses[6]. This paper reports the inhibition of FTase by GT and AGT likely in their reduced form. In addition we show that bisdethiobis(methylthio)-GT and bisdethiobis(methylthio)-AGT chemically prepared from GT and AGT failed to inhibit FTase.

GT and AGT (Fig. 1) were isolated from the culture of fungus strain FO2047. After fermentation at 27°C for 4 days, the whole broth (20 liters) was extracted with $CHCl_3$ (20 liters). The extract was concentrated to dryness and the residue (28.4 g) was applied to a silica gel column. The active fraction (1.3 g) was eluted with a mixture of toluene and ethyl acetate (8:2). Further purification was carried out by HPLC (column: Capcell Pak C18, 20×250 mm, solvent: 55% MeOH in H_2O, flow rate 8 ml/minute, UV at 205 nm) to give 250 mg of acetylgliotoxin and 150 mg of gliotoxin. The bioactive material was identified as the known metabolites GT and AGT according to spectroscopic data (UV, IR, NMR and MS) already published[7].

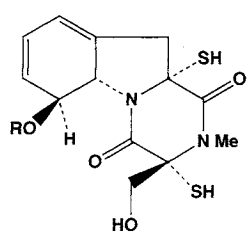
Farnesyl-protein transferase was partially purified from the cytosol of human monocyte THP-1 (ATCC TIB 202). Culture cells were washed with ice cold phosphate buffered saline (PBS) and incubated at 4°C for 30 minutes in lysis buffer (10 mM HEPES pH 7.4, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 0.5 μM pepstatin, 1 μM leupeptin, 10 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate). The lysate was centrifuged at 10,000 *g* at 4°C for 15 minutes after addition of NaF (100 mM final) and the supernatant was further centrifuged at 100,000 *g* for 45 minutes at 4°C. The supernatant (S100) was then submitted to fractionation by ammonium sulfate. The 30~60% ammonium sulfate fraction was dissolved in 30 ml of 50 mM Tris-HCl (pH 7.5) buffer containing 1 mM DTT and 0.02 mM $ZnCl_2$ and then dialysed for 15 hours against 4 liters of the same buffer and then 10 liters of fresh buffer for 20 hours at 4°C. The dialysed material (10.4 units/mg) was aliquoted and stored at −70°C.

Ha-ras p21 protein was produced in a bacterial

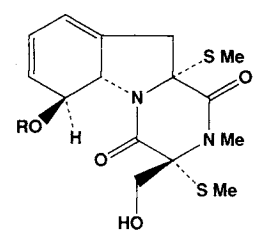
Fig. 1. Chemical structures of gliotoxin and analogues.



Disulfide form



Dithiol form



R=H Gliotoxin
R=Ac Acetylgliotoxin

R=H Bisdethiobis(methylthio)-gliotoxin
R=Ac Bisdethiobis(methylthio)-acetylgliotoxin

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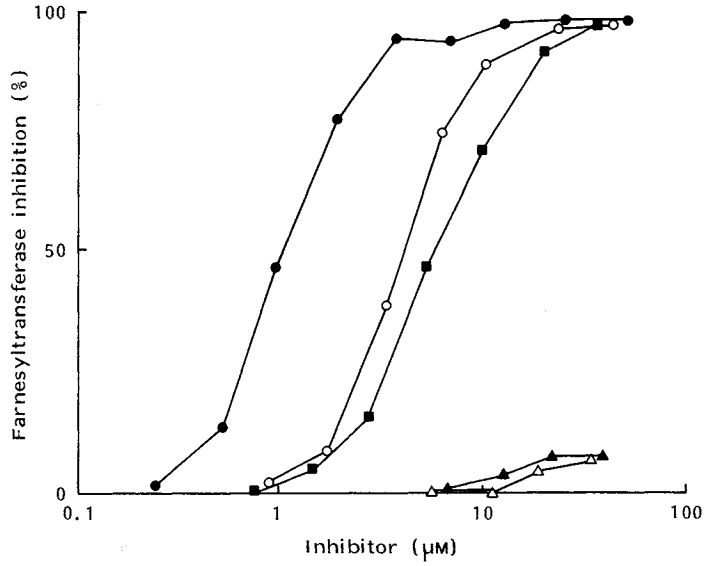
Fig. 2. Inhibition of FTase by GT (●), AGT (○), bisdethiobis(methylthio)-GT (▲), bisdethiobis(methylthio)-AGT (△) and CVLS (■).



expression system and purified as previously described[8].

FTase standard reactions were carried out in a final volume of 60 μl containing: 13 μg of partially purified FTase from THP-1 cells (ATCC), 1.3 μM of recombinant p21 protein, 0.03 μM of [^{3}H]farnesyl pyrophosphate (FPP), 50 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, and 4 mM DTT. The reaction was initiated by addition of enzyme and incubated for 30 minutes at 37°C. The reaction was stopped by addition of 0.5 ml of 1% SDS in methanol and 0.5 ml of 30% TCA. After vortexing the tubes were left for 60 minutes on ice. The mixture was then filtered on glass fiber filter, washed with 5 ml of 6% TCA using Skatron cell harvester. Dried filter was finally counted in a liquid scintillation counter. Blank value was determined in parallel incubation without p21 substrate; this blank value was subtracted before calculating % inhibition.

As shown in Fig. 2, GT and AGT inhibited FTase with IC_{50} values of 1.1 μM and 4.4 μM, respectively, since the peptide control CVLS (NH_2-Cys-Val-Leu-Ser-COOH) corresponding to the carboxy-terminal sequence of Ha-ras p21 protein showed an IC_{50} of 6.6 μM. Shifted mobility on TLC was observed when spotting a mixture of our reaction mixture and GT or AGT (Table 1), suggesting that these metabolites favor the dithio structure rather than the thiosulfinate structure in our reaction mixture containing 4 mM DTT. Based on these observation, it was assumed that the metabolites inhibited FTase under reduced forms. On the other hand, it has been reported that the bridged disulfide in GT is essential for antimicrobial activity[9]. We also confirmed that antimicrobial activity against *Candida albicans* and *Bacillus subtilis* decreased when GT or AGT was incubated with our reaction mixture (data not shown). Thus, it is likely that the inhibitory mechanism of GT and AGT on FTase is different from that of antimicrobial activity. However, we could not confirm this speculation because FTase activity can not be assayed in the absence of DTT.

Table 1. Shifted mobility of reduced GT and AGT.

Compounds	Rf
GT	0.69
GT reduced	0.02
AGT	0.78
AGT reduced	0.09

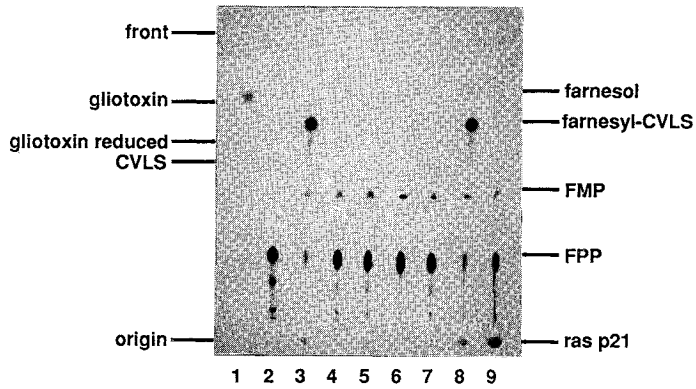
The Kieselgel TLC was developed with $CHCl_3$-MeOH (9 : 1) and examined after vanillin-sulfuric spray.

Since epipolythiodiketopiperazine toxin exhibited broad spectral activity due to the bridged disulfide moiety[6] we have chemically prepared bisdethiobis(methylthio)-derivatives (Fig. 1) as described by G. W. KIRBY[10]. Unfortunately these compounds failed to inhibit FTase (Fig. 2).

In order to examine the possibility that GT could

Fig. 3. [^{3}H]Farnesyl transfer as detected by autoradiography of TLC.

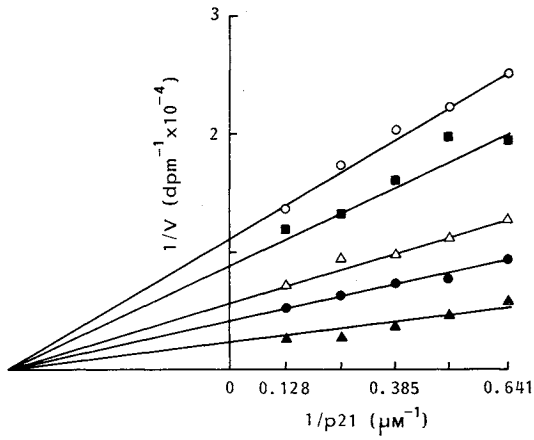
Lane 1 (FPP+HCl 1 N), lane 2 (FPP+buffer), lane 3 (enzyme+FPP+CVLS 20 μM), lane 4 (enzyme+FPP+GT 20 μM), lane 5 (enzyme+FPP), lane 6 (enzyme+FPP+CVLS 20 μM+GT 20 μM), lane 7 (enzyme+FPP+p21+GT 20 μM), lane 8 (enzyme+FPP+p21+CVLS 20 μM), lane 9 (enzyme+FPP+p21) were visualized by autoradiography.



Parallel migration of standards GT, GT reduced and CVLS were visualized with UV 254 nm.

Fig. 4. Lineweaver-Burk plot for FTase inhibition with gliotoxin.

○ GT 5 μM, ■ GT 4 μM, △ GT 2 μM, ● GT 0.8 μM, ▲ no inhibitor.



serve as substrate for the enzyme and thereby farnesylated on thiol residue like the peptide CVLS[11], we have performed an autoradiography of enzyme reaction mixture developed on TLC (Fig. 3); since CVLS was farnesylated (lane 3), GT was not an acceptor of [^{3}H]farnesyl (lane 4). In addition GT inhibited the farnesylation of CVLS (lane 6) and as expected CVLS and GT inhibited farnesylation of p21 (lanes 8 and 7).

Furthermore, the Lineweaver-Burk plot of GT versus p21 protein showed noncompetitive inhibition against p21 protein (Fig. 4). From the Dixon plot analysis, the apparent *Ki* values was found to be about 1.5 μM.

Thus, this might be the first report for FTase inhibitor from microbial origin.

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