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Heteronemin as a Protein Farnesyl Transferase Inhibitor

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Abstract

The known metabolite heteronemin was isolated from the marine sponge *Hyrtios reticulata* Thiele, using a bioguided fractionation with the enzyme protein farnesyl transferase as a target (IC_{50} = 3 μM). In contrast, 12-epi-heteronemin, isolated from a related sponge, *Hyrtios erecta Keller*, did not show any noticeable inhibitory activity.

Keywords: Heteronemin, *Hyrtios*, marine natural products, protein farnesyl transferase, sponges.

Introduction

Protein farnesyl transferase (PFTase) catalyses the transfer of the farnesyl group of farnesyl pyrophosphate to proteins containing the carboxy-terminal sequence CAAX (where A is usually an aliphatic amino acid and X is the carboxy-terminal amino acid). One of the substrates of PFTase is the 21-kDa Ras protein. Because activation of Ras by a point mutation is found in a large number of cancer cells, *Ras* has been identified as a predominant oncogene in human cancer cells. Farnesylation of the mutant forms of Ras is crucial for membrane integration and the subsequent transformation of normal cells into cancerous cells. Thus, inhibition of Ras function, using PFTase inhibitors, is thought to be a crucial target for cancer chemotherapy (Cohen et al., 2000; Hill et al., 2000; Prendergast, 2000; Sebti & Hamilton, 2000; Adjei, 2001).

An enzymatic bioassay was run to screen a library of natural extracts for inhibitors of PFTase. The extract of a sponge, *Hyrtios reticulata* Thiele (Thorectidae), collected in Vanuatu, was found to be a definite PFTase inhibitor. Bioguided fractionation led to the isolation of the bioactive compound identified as a known sesterterpene, heteronemin (**1**; Fig. 1) (Kazlauskas et al., 1976). This is the first example of an inhibitor of PFTase isolated from a marine sponge.

Another sponge, *H. erecta* Keller (Thorectidae), collected in the north of New Caledonia, was known to produce 12-epi-heteronemin (**2**) and to display a broad spectrum of biological activities, including that of cytotoxicity (Bourguet-Kondracki et al., 1994), but did not show any notable inhibition of PFTase in our screen. Pure compound **2** was proved inactive against the enzyme, except at higher concentration.

Materials and Methods

Collection and extraction

Hyrtios reticulata was collected in Vanuatu in 1999 by IRD and was identified as such by Pr. John Hooper. A voucher specimen is located at the Queensland Museum (Brisbane, Australia) under the accessing number G318495. A freeze-dried sample (100 g) was extracted with ethanol for 1 h (6 times). The extract was partitioned between hexane and methanol. The hexanic extract yielded heteronemin (200 mg), which readily crystallized from petroleum ether.

H. erecta was collected in the north of New Caledonia in 1988 and 1990 by IRD and was identified by Pr. Claude Levi, Muséum National d'Histoire Naturelle (Paris, France). Original samples of methylene chloride extracts were purified by successive column [cyclohexane/chloroform (50 : 50)] and preparative thin-layer chromatography using chloroform to afford pure heteronemin and a mixture of 12-epi-heteronemin and of its acetate. Preparative HPLC [Lichrospher 60 Å Silica (5 μm), 250 × 10.5 mm, Merck Eurolab (Darmstadt, Germany), cyclohexane/chloroform (65 : 35), light scattering detection] afforded pure 12-epi-heteronemin and its acetate.

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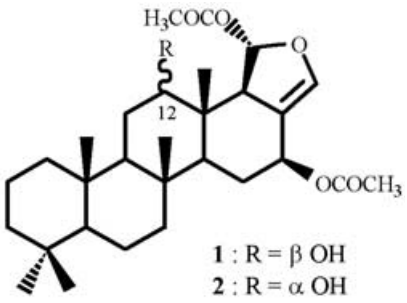

Figure 1. Heteronemin **1** and its epimer **2**.

Partial purification of PFTase

All steps were carried out at 4 °C (Sangkyou et al., 1998). Bovine brains were obtained from the slaughterhouse in Castres, France. They were homogenized (200 ml) in ice-cold buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, [ethylene glycol-*bis*(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], and 0.2 mM AEBSF protease inhibitor (Sigma A8456). Homogenate was centrifuged at 10,000 × *g* for 10 min. The supernatant was kept on ice, and the precipitated material was resuspended in 500 ml of the same buffer. Supernatants were pooled and brought to 25% saturation with solid ammonium sulfate, stirred on ice for 30 min, and centrifuged at 10,000 × *g* for 10 min. The precipitated material was dissolved in 500 ml of buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM DTT (dithiotreitol), 20 µM $ZnCl_2$, and dialyzed for 2 h against 2 l of the same buffer and 2 l of fresh buffer for 12 h. The dialyzed fraction was centrifugated at 10,000 × *g* for 10 min to remove insoluble precipitate. The supernatant was applied to a 16/20 column of Q-Sepharose Fast Flow equilibrated in 50 mM Tris-HCl (pH 7.5) containing 1 mM DTT, 20 µM $ZnCl_2$, and 0.1 M NaCl. The column was washed with 200 ml of the same buffer and with 200 ml of a 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 20 µM $ZnCl_2$, and 0.23 M NaCl buffer at a flow rate of 2 ml/min. The PFT enzyme was eluted with a 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 20 µM $ZnCl_2$, and 0.6 M NaCl buffer at a flow rate of 2 ml/min. The peak fractions (OD at 280 nm) were pooled and concentrated by ultrafiltration to 25 ml.

Purification of H-Ras-GST fusion protein

Escherichia coli expressing H-Ras-GST [glutathione *S*-transferase] were a generous gift from Pr. Allan Hall (University College of London, London, UK) (Sangkyou et al., 1998). A 1 ml volume of *E. coli* strain culture was resuspended in 500 ml T. Broth medium, 0.28 mM ampicillin, and incubated overnight at 37 °C. The culture was diluted 10-times in the same buffer and incubated for 2 h at 37 °C. Induction was started with the addition of 1 mM IPTG and incubated for 4 h at 37 °C. The culture was centrifuged at 3000 × *g* for 15 min at 4 °C. The clotting material was resuspended in 60 ml of a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, protease inhibitors (Roche 1 697 498, 1 tablet for 50 ml), sonicated 4-times for 10 s, and centrifuged at 10,000 × *g* at 4 °C. The supernatant was mixed with agarose/glutathione beads, previously washed with 50 ml lysis buffer, incubated for 30 min at 4 °C, and centrifuged at 2000 × *g* for 2 min. Beads were washed three-times with 20 ml of lysis buffer, and the GST-Ras protein was eluted 2-times with 5 ml of a 50 mM Tris-HCl (pH 8), 150 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mM DTT, protease inhibitors (Roche 1 697 498, 1 tablet for 50 ml) elution buffer. Fractions were pooled, centrifuged twice at 2000 × *g*, and concentrated by ultrafiltration.

PFTase enzyme assay

PFTase activity was determined by measuring the amount of [^{3}H]farnesyl transferred from [^{3}H]farnesyl pyrophosphate to GST-Ras protein (Sangkyou et al., 1998). The standard reaction mixture contained the following components in a final volume of 100 µl : 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 5 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.2% (w/v) *n*-octyl-β-D-glucopyranoside, 0.2 µM [^{3}H]farnesyl pyrophosphate, 3.2 µM of GST-Ras substrate, 10 µl of PFTase (adjusted to obtain 10,000 cpm when tested with DMSO only), and various concentrations of heteronemin as indicated. The reaction started with the addition of PFTase, and the reaction mixture was incubated for 45 min at 37 °C before transfer by pipette onto a Whatmann P81 filter. The filter was washed 3-times with 0.5 ml 50% ethanol/0.5% orthophosphoric acid mixture, immersed in 4 ml scintillant, and counted in a Packard 1900 TR liquid scintillation analyzer (Downers-Grove, USA).

Purified compounds

Pure heteronemin and 12-epi-heteronemin were dissolved in DMSO to afford 10^{-4} M, 5×10^{-5} M, 10^{-5} M, 5×10^{-6} M, 10^{-6} M, 5×10^{-7} M, 10^{-7} M, 5×10^{-8} M, and 10^{-8} M solutions.

Measurement of inhibitory activity

The effect of heteronemin on the activity of PFTase was determined as a percentage inhibition compared to that of the control of DMSO only. These percentages of inhibition were calculated as follows: % inhibition = (cpm with test compound/cpm control DMSO) × 100. Graph Pad Prism Software was used to calculate nonlinear regression curves of inhibition with the sigmoidal dose response mode. The concentration of heteromemin that reduced the signal of the control by 50%, termed the IC_{50} value, was also calculated and used as a measure of the inhibition. Results were reproducible in two independent experiments.

Results

Of the two pure compounds tested, only heteronemin showed significant inhibitory activity against PFTase, with an IC_{50}

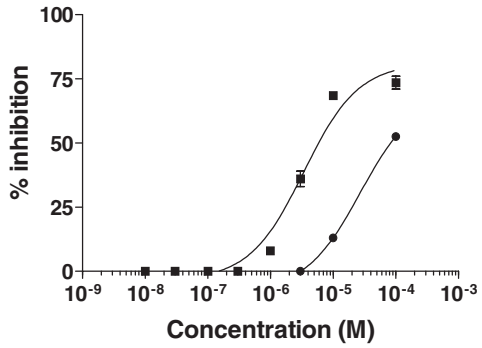

Figure 2. Inhibition of PFTase activity by heteronemin (■) or 12-epi-heteronemin (●). Data were plotted using a windows-based Prism program. The % inhibition was calculated as: % inhibition = (cpm with test compound/cpm with control of DMSO) × 100.

value of 3 µM, whereas 12-epi-heteronemin proved inactive in this low micromolar range. Only after testing higher concentrations of the 12-epi isomer was an IC_{50} value of 100 µM recorded (Fig. 2).

Discussion

The only difference between compounds **1** and **2** is the configuration of the hydroxyl at C-12, shown by ^{1}H NMR to be equatorial in heteronemin and axial in its isomer. It therefore seems that this position is of importance for the biological activity of the molecule.

By analogy with known and toxic terpene dialdehydes of marine (scalaradial) and terrestrial origin (iridiodial, warburganal, etc.), it could have been expected that this masked functionality in compounds **1** and **2** might play a role in their biological activity. The significantly lower activity in compound **2**, however, suggests that this is not the case in this *in vitro* assay.

With regard to natural products known to inhibit PFTase, heteronemin bears no resemblance to the most potent ones (manumycin, oreganic and zaragozic acids). It should be noticed, however, that rings A, B, and C of heteronemin contain all the atoms of the farnesyl skeleton, although this is also true of a wealth of natural terpenes that do not show any PFTase activity.

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