Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry Letters 13 (2003) 1935–1937

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Antifungal Activity of a *Candida albicans* GGTase I Inhibitor-Alanine Conjugate. Inhibition of Rho1p Prenylation in *C. albicans*

Krishna K. Murthi,[a,*] Susan E. Smith,[b] Arthur F. Kluge,[a] Gustave Bergnes,[a] Patrick Bureau[a] and Vivian Berlin[b]

[a]*GPC Biotech, Inc., Department of Chemistry, 610 Lincoln Street, Waltham, MA 02451, USA*
[b]*GPC Biotech, Inc., Department of Yeast Genetics, 610 Lincoln Street, Waltham, MA 02451, USA*

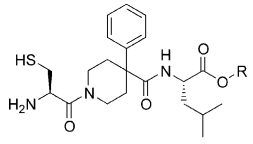
Received 15 November 2002; accepted 10 February 2003

Abstract—An alanine conjugate of a *Candida albicans* geranylgeranyl transferase I inhibitor was synthesized to facilitate its uptake into the fungal cell. The antifungal activity of CaGGTase-Ala conjugate is demonstrated. It is also shown that the CaGGTase-Ala conjugate affects prenylation of endogenous Rho1p, but has no effect on prenylation of endogenous Ras1p.

β-1,3 D-glucan is an essential component of the fungal cell wall[1] and the enzyme β-1,3 D-glucan synthase, which regulates the synthesis of β-1,3 D-glucan has been targeted for antifungal drug discovery.[2] *Candida albicans* Rho1p is required for cell viability, plays a role in cell integrity and is a key regulator of β-1,3 D-glucan synthase.[3,4] Rho1p is a small GTP binding protein that plays an essential role in cellular integrity and morphology of fungal cells.[5] Geranylgeranyl transferase I (GGTase I), a zinc metalloenzyme, catalyses the transfer of a geranylgeranyl group to Rho1p which is required for its functional localisation to the membrane.[6] The essentiality of GGTase I in *C. albicans* was validated genetically by knock-down experiments.[7] Our antifungal drug discovery strategy targeted *C. albicans* GGTase I with the premise that a GGTase I inhibitor would block the β-1,3 D-glucan synthase pathway, disrupt cell wall formation and result in lysis and cell death.

The substrate specificity of the prenyl transferases, GGTase I and farnesyl transferase (FTase) has been reported.[8] We designed *C. albicans* GGTase I inhibitors based on the terminal tetrapeptide sequence CA_1A_2L, which is the key recognition element of GGTase I. Replacement of the A_1A_2 dipeptide with a 4-phenyl piperidine spacer lead to the very potent *C. albicans* GGTase I inhibitor (IC_{50}: <5 nM) **1**.

Compound **1** showed no antifungal activity in cellular assays. We reasoned that the lack of activity of **1** in whole cells was due to its inability to enter the fungal cell.



1. R = H
1a. R = CH_3

Fungal cells utilize active transport systems such as permeases to transport nutrients such as short peptides and amino acids into the cell. This transport system has been used to transport meta-fluorophenylalanine[9] into fungal cells and achieve inhibition of growth. By analogy to this work we decided to modify the CaGGTase I inhibitor **1** to be a potential substrate for the permease transport system.

Chemistry

The alanine conjugate **3** was designed to be a substrate for active transport and was synthesized as shown in Figure 1.[10] Boc-4-phenylpiperidine-4-carboxylic acid was coupled to the dipeptide Leu-Ala-O^{t}Bu (EDC, HOBt, DIEA, CH_2Cl_2) to give intermediate **2**. *Tert*-butoxycarbamate deprotection (TFA, CH_2Cl_2), S-Tr-N-Boc-cysteine coupling (EDC, HOBt, DIEA, CH_2Cl_2), followed by overall deprotection (TFA, CH_2Cl_2, Et_3SiH) gave **3**.

*Corresponding author. Fax: +1-781-890-9005; e-mail: krishna.murthi@gpc-biotech.com

0960-894X/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0960-894X(03)00320-2

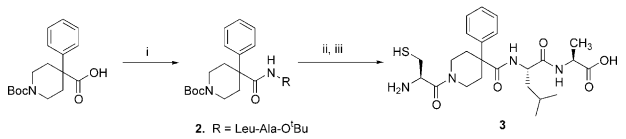


Figure 1. Synthesis of alanine conjugate.

Discussion

As shown in Table 1, compounds **1**, **1a** and **3** are all potent inhibitors of CaGGTase I but not CaFTase, as revealed by using recombinant components in appropriate in vitro prenylation assays similar to those previously described.[11] Neither **1** nor **1a** afforded any antifungal activity against SC5314 cells, a well-studied *C. albicans* clinical isolate (MIC $>128\,\mu g/mL$). In contrast, the alanine conjugate **3** showed potent antifungal activity with a minimum inhibitory concentration (MIC) of $1\,\mu g/mL$ and a minimum fungicidal concentration (MFC) of $2\,\mu g/mL$ (Table 1).

Presumably compound **3** is transported into the fungal cell by an amino acid or peptide permease. To test this hypothesis, the MIC was determined in the presence of amino acids in the media which could compete for the permease. The addition of amino acids at a concentration of 1 mM abolished the activity of compound **3** as indicated by an MIC $>64\,\mu g/mL$ suggesting that transport by a peptide transport system was responsible for its activity.

Next, given that compound **3** has antifungal activity and appeared to enter into cells, we asked whether this compound affected the activity of GGTase I in *C. albicans* cells. To do this, we studied the cellular localisation of the GGTase I substrate, Rho1p. Treatment of *C. albicans* cells with compound **3** should, by inhibition of the CaGGTase I, prevent the membrane targetting of Rho1p mislocalising it to the cytosol. Initially, a kill curve experiment[13] was carried out to determine both the appropriate concentration and timeframe over which compound **3** acted. We found that at a concentration of $3\,\mu g/mL$, cell killing occurred 5 h after exposure to compound **3**. These conditions were then used in a prenylation assay;[14] SC5314 cells were treated with compound **3**, extracts were generated and subjected to high speed centrifugation. The localisation of Rho1p was studied by Western blot analysis using a specific Rho1p peptide antibody.[6] In mock, DMSO treated *C. albicans* cells, Rho1p was exclusively localised to the membrane fraction (Fig. 2).

In contrast, cells treated with compound **3** showed a significant proportion of Rho1p in the cytosolic fraction (Fig. 2). As a control, we also analysed the localisation

Table 1. Antifungal activity[12] of compounds **1**, **1a** and **3**

Compd	CaGGTase I IC_{50}, (μM)	CaFTase IC_{50}, (μM)	MIC μg/mL	MFC μg/mL
1	<0.005	0.2	>128	>128
1a	0.005	NT	>128	>128
3	0.01	3	1	2

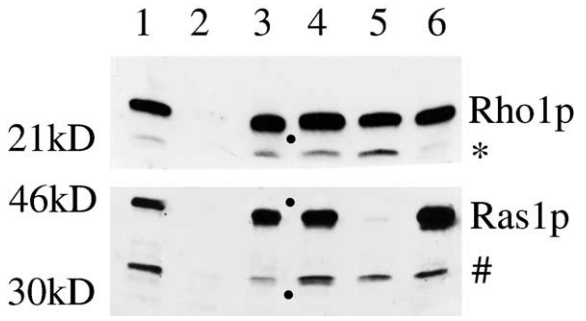


Figure 2. Compound **3** affects the localisation of *C. albicans* Rho1p but not Ras1p. Lanes 1–3 DMSO treated samples, lanes 4–6 compound 3 treated samples. Lanes 1 and 4, whole cell extracts; lanes 2 and 5, cytosolic fractions; lanes 3 and 6, membrane fractions. Protein marker molecular weights (kD) are indicated. * And # indicate proteins cross-reacting with the anti-Rho1p and anti-Ras antibodies, respectively.

of Ras1p, an FTase substrate, using a monoclonal antibody (Molecular Probes, panRAS) that recognises a sequence conserved in *C. albicans* Ras1p (data not shown). The localisation of the ~44 kD *C. albicans* Ras1p[15] to the membrane fraction was unaffected by either DMSO alone or compound **3** (Fig. 2). These results indicated that compound **3** specifically inhibited GGTase I in vivo and did not generally affect prenylation in *C. albicans* cells. It is interesting that compound **3** effects cell killing after 5 h of treatment. This observation could be explained if the geranylgeranylation of only newly synthesised Rho1p (i.e., that synthesised by the cell to compensate for the turn-over of Rho1p and/or depletion as a result of cell division) rather than pre-existing geranylgeranylated Rho1p, was inhibited. Further work is necessary to determine if this is the case, but in support of this idea, we have observed that a large proportion of tagged Rho1p expressed from a heterologous, regulatable promoter was localised to the cytosol in the presence, but not the absence, of compound **3**.

Taking these results together the apparent inhibition of GGTase I in vivo correlates with cidal activity of this compound.

References and Notes

1. Georgopapadakou, N. H.; Tkacz, J. S. *Trends Microbiol.* **1995**, *3*, 98.
2. (i) Stone, E. A.; Fung, H. B.; Kirschenbaum, H. L. *Clinical Therapeutics* **2002**, *24*, 351. (ii) Denning, D. W. *J. Antimicrobial Chemotherapy* **2002**, *49*, 889.
3. Kondoh, O.; Tachibana, Y.; Ohya, Y.; Arisawa, M.; Watanabe, T. *J. Bact.* **1997**, *179*, 7734.
4. Smith, S. E.; Csank, C.; Reyes, G.; Ghannoum, M. A.; Berlin, V. *FEMS Yeast Res.* **2002**, *2*, 103.
5. Cabib, E.; Drgonova, J.; Drgon, T. *Annu. Rev. Biochem.* **1998**, *67*, 307.
6. Omer, C. A.; Gibbs, J. B. *Mol Microbiol.* **1995**, *11*, 219.
7. *C. albicans* GGTase I is composed of 2 non-identical subunits, α and β. We cloned the genes encoding these subunits and to assess their requirement for *C. albicans* viability we employed 2 alternative but complementary approaches. The hemizapper or pop-in/pop-out method (Scherer, S.; Davis,

R.W. *Proc. Natl. Acad. Sci. U.S.A.*, **1979**, *76*, 4951) permitted a statistical analysis to determine whether a gene is essential. Using this approach we were unable to recover homozygous null mutants for either the α- or the β- subunit genes indicating that GGTase I is essential ($P > 0.995$). In addition, we placed the α- and β- subunit genes under the control of a regulatable promoter (see ref 4 for an example). Significant reduction of either of the GGTase I subunits resulted in a loss of cell viability, cell lysis and mislocalisation of geranylgeranyl transferase substrates (manuscripts in preparation).

8. Zhang, F. L.; Casey, P. J. *Annu. Rev. Biochem.* **1996**, *65*, 241.

9. Kinsbury, W. D.; Bochm, J. C.; Mehta, R. J.; Grappel, S. F. *J. Med. Chem.* **1983**, *26*, 1725.

10. Bergnes, G.; Berlin, V.; Come, J.; Kluge, A.; Murthi, K.; Pal, K.; U.S. Patent 6423519, 2002.

11. (i) Stirtan, W. G.; Poulter, C. D. *Biochem.* **1997**, *36*, 4552. (ii) Gomez, R.; Goodman, L. E.; Tripathy, S. K.; O'Rourke, E.; Manne, V.; Tamanoi, F. *Biochem. J.* **1993**, *289*, 25. (iii) Caplin, B. E.; Marshall, M. S. *Methods Enzymol.* **1995**, *250*, 51.

12. MIC and MFC determinations followed standard procedures using SC5314 *C. albicans* cells (Fonzi, W. A.; Irwin, M. Y. *Genetics*, **1993**, *134*, 717) except that YNBGlc medium was used: YNB w/o amino acids (Difco) supplemented with 1 μg/mL histidine, 2 μg/mL methionine, 2 μg/mL tryptophan, 200 μg/mL glutamine and 2% glucose (all obtained from Sigma). To determine the effect of high amino acid concentration on the MIC this medium was supplemented with an amino acid mixture at a final concentration of 1 mM (Sigma).

13. Kill curve methodology. A culture of SC5314 in YNBGlc medium was set up at 35 °C and shaken overnight at 220 rpm. The SC5314 cells were then washed, resuspended in fresh YNBGlc and counted. 2 flasks containing 100 mL YNBGlc were inoculated with 1×10^9 (1×10^7 cells/mL) SC5314 cells. Either compound **3** or vehicle (DMSO) was added to the cultures which were then incubated at 220 rpm, 35 °C. At various time points, a range of appropriate serial dilutions (10^{-5}–10^{-9}) of the cultures were made and 100 μL cells plated onto Sabourand plates. The plates were incubated at 35 °C for 48 h prior to counting.

14. Prenylation assay. A culture of SC5314 in YNBGlc medium was set up at 35 °C and shaken overnight at 220 rpm. The following morning the cell number was determined and cells were resuspended at 1×10^7 cells/mL in YNBGlc and treated with either compound 3 at 3 μg/mL or an equivalent volume of DMSO vehicle. Cells were harvested after 5 h exposure to compound, a time based on the killing kinetics of compound **3** (not shown). After the incubation, the cells were washed with 1 M sorbitol, transferred to 2 mL screwcap tubes, pelleted and frozen at −80 °C. Whole cell extracts were generated and 50–100 μL was subjected to high speed centrifugation (4 °C, 48,000 rpm for 1 h using a TI120.1 rotor in a Beckmann tabletop centrifuge) to resolve the membrane and cytosolic fractions. SDS.PAGE and western blotting analysis were then carried out.

15. Feng, Q.; Summers, E.; Guo, B.; Fink, G. *J. Bacteriol.* **1999**, *65*, 241.