Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 2639–2642

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Multisubstrate Analogue Inhibitors of Glucosamine-6-phosphate Synthase from *Candida albicans*

Sridar V. Chittur*,† and Robert K. Griffith

Department of Basic Pharmaceutical Sciences, School of Pharmacy, West Virginia University, Morgantown, WV 26505, USA

Received 21 September 2001; accepted 14 June 2002

Abstract—Compounds **1**–**6** were designed as multisubstrate inhibitors of glucosamine synthase. These compounds are also useful probes for measuring the distances between the two active sites in the multidomain protein. Our data suggest that the two binding pockets are in close proximity to each other.

Inhibition of the chitin biosynthetic enzyme, glucosamine-6-phospate synthase (EC.2.6.1.16: L-glutamine:D-fructose 6-phosphate amidotransferase, GFAT) is a promising approach to the development of novel antifungal agents targeting the cell wall.[1–3] This enzyme is a multidomain protein belonging to the N-terminal nucleophile (Ntn) family of glutamine amidotransferases and crystal structures for individual domains have been published.[4–6] The best known inhibitors of GFAT possess reactive functionalities that challenge their clinical utility and illustrate the need for new molecules.[1,7–16] In this study we present compounds **1**–**6** that possess structural moieties for binding to both active sites on the enzyme.

Badet et al. synthesized and studied a series of bisubstrate analogues against glucosamine synthase from *Escherichia coli*.[17] However, none of these compounds exhibited significant inhibition up to 3 mM. This was attributed to the high flexibility of these analogues preventing correct orientation required for binding, and conformational changes that may be occurring in the protein on binding of its substrates.

The search for new potent antifungal drugs has intensified in the last decade. The advent of AIDS as well as novel procedures in organ transplantation and cancer chemotherapy has created new populations of patients prone to secondary fungal infections. The toxicities and/or poor bioavailabilities associated with current antifungal agents only compound this problem. Newer strains of fungi have also been isolated clinically, which show resistance to current drugs. These factors have prompted a search for unexplored targets in these organisms that may be exploited to develop novel antifungal agents.

The experimental antifungal agents, N^3-(4-methoxyfumaroyl)-(*S*)-2,3-diaminopropanoic acid (FMDP), nikkomycins and polyoxins, all of which target the fungal cell wall, require incorporation into peptides for inhibition of intact cells.[14,18–23] The use of peptide carriers facilitates transport of the warhead into the target cells. Though this complicates their formulation into oral dosage forms, current developments in pharmaceutical formulation may still facilitate their use clinically. All the compounds investigated in this study also need peptide carriers for transport but they serve as leads for more potent agents.

Structures of a related Ntn amidotransferase PRPP amidotransfaerase (GPATase) indicate that activation of this enzyme results in the formation of a 20-Å tunnel that connects the two active sites.[24,25] This tunnel is created by the ordering of a flexible loop in the C-terminal domain, which closes over the space between the active sites, effectively sequestering both sites from bulk solvent. Similar tunnels are known to exist in the Triad amidotransferases like carbamoyl phosphate synthase (CPS), guanosine monophosphate synthetase (GMPS) and anthranilate synthase (AS).[26–28] Though it would

*Corresponding author. Tel.: +1-518-591-8322; fax: +1-518-525-2799; e-mail: schittur@myomatrix.com
†Current address. Myomatrix Therapeutics, DB224, One University Place, Rensselear, NY 12144, USA.

0960-894X/02/$ - see front matter
PII: S0960-894X(02)00510-3

be reasonable to expect such a tunnel to exist in GFAT, a crystal structure of the complete multidomain protein is not available. Hence, we decided on a chemical probe approach to determine this interdomain distance. Two possible orientations for substrate binding exist at the active site such that the bound fructose-6-phosphate and glutamine are either parallel or orthogonal to each other. Based on the observation that glucosamine synthase does not accept molecular ammonia as a nitrogen source for amide transfer, it would be necessary for the glutamine amide and C-2 of bound fructosamine to be in close proximity. Both orientations satisfy this requirement for efficient transfer of nascent ammonia. Unlike any other amidotransferase, glucosamine synthase does not accept exogenous ammonia as its nitrogen source.[29] Hence it is possible that these domains may be close to each other in the native quaternary structure of the enzyme. Inhibitory properties of compounds AP3 (**1**), AP4 (**2**), AP5 (**3**), AP6 (**4**), AP7 (**5**), and CGS19755 (**6**) (Fig. 1) would help to establish the orientation of substrate binding and also the approximate distance between the phosphate binding site and the amino acid binding site.

Candida albicans or *Saccharomyces cerevisiae* was cultured in 2-L batches in YEPD medium at 37 °C. Cells were harvested from an overnight culture by centrifugation (3000 × *g*, 15 min) at 4 °C and washed twice with distilled water. A typical 2-L batch yielded 60 g of cells. The cells were suspended in Buffer A (50 mM potassium phosphate (pH 6.5), 1 mM dithiotreitol (DTT), 1 mM EDTA, 0.5 M sucrose) such that the mixture contained 1 part packed wet cells to 2 parts of the homogenization medium. The cell suspension was homogenized in a Bead-Beater (Biospec Ltd) chamber using pre-chilled glass beads (0.5 mm) for 30 s intervals with 1 min cooling on ice for a total homogenization time of 3 min. The crushed cell suspension was pipetted off using a sterile pipette and then centrifuged (10,000 × *g*, 15 min) at 4 °C. The supernatant (cell-free extract) Buffer A extract was assayed for protein content using the commercial bradford assay kit (Bio-Rad). Proteins in the buffer A extract were salted out over 20 min by addition of 1.5 mL of protamine sulfate (1% w/v) to every 75 mg of protein in the buffer A extract with vigorous stirring. Thereafter, the suspension was centrifuged (10,000 × *g*, 15 min) at 4 °C, the precipitate was washed twice with buffer A and then mixed with 30 mL of buffer B (0.1 M pyrophosphate (pH 6.5), 1 mM DTT, 1 mM EDTA, 0.5 M sucrose). This suspension was stirred for 4–5 h at 4 °C followed by centrifugation (10,000 × *g*, 15 min). The supernatant after centrifugation, termed the pyrophosphate extract (buffer B extract) was then concentrated to one third its volume using an Amicon model 1080 ultrafiltration cell and a Diaflo XM50 membrane (cut-off limit 50,000 MW) at 4 °C. This concentrated buffer B extract was used for the activity assays and inhibition assays.

Compounds **1**–**5** were purchased from Research Biochemicals International (Natick, MA) while compound **6** was a generous gift from the Pharmaceuticals Division, CIBA-GEIGY Corporation (Summit, NJ).

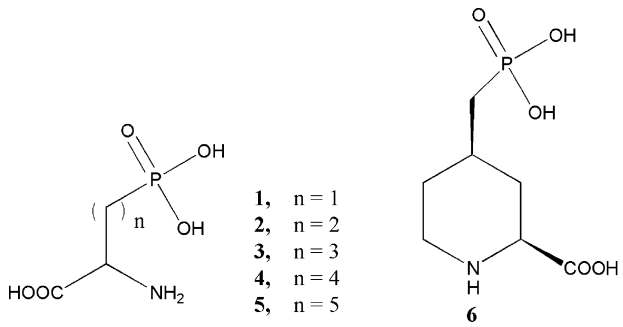

Figure 1. Multisubstrate analogue inhibitors of glucosamine synthase.

The protocol for the glucosamine synthase activity assay is a modified Elson–Morgan procedure as reported by Roseman et al.[30] Briefly, a 0.5 mL reaction mixture containing 15 mM D-fructose-6-phosphate, 10 mM L-glutamine in incubation buffer (25 mM potassium phosphate buffer (pH 6.5), 1 mM DTT, 1 mM EDTA) and 1 mg enzyme was incubated for 30 min at 30 °C. Reactions were terminated in a boiling water bath for 3 min to denature the enzyme. The protein precipitate was removed by centrifugation (10,000 rpm, 3 min) and a 0.4 mL aliquot of the supernatant was used to assay for glucosamine-6-phosphate. To the supernatant was added 50 μL of saturated bicarbonate, stirred followed by addition of 50 μL of freshly prepared cold 5% acetic anhydride. The reaction tubes were stirred vigorously for 3 min and then transferred to a boiling water bath for 3 min to destroy any excess acetic anhydride. After cooling to room temperature, 100 μL of sodium borate was added, the contents mixed for 3 min and heated for 3 min in a boiling water bath. After this mixture was cooled to room temperature, 3 mL of diluted Erlich's reagent was added to each tube and the tubes then incubated for 20 min at 37 °C. The absorbance at 585 nm was measured. Glucosamine standards were included along with the complete reaction mixtures that were boiled at zero time. A unit of activity is defined as the amount of enzyme (mg) catalyzing the synthesis of 1.0 μmol of glucosamine-6-phosphate per 30 min.

From the data for 50% inhibitory concentration IC_{50} shown in Table 1, was found to be the most potent inhibitor of glucosamine synthase from *C. albicans*. The correlation between the distance from the α-carbon of the amino acid to the phosphonate phosphorus (C_α-P

Table 1. Inhibitory potencies for compounds **1**–**6** against *C. albicans* GFAT

Compd	IC_{50}, μM[a]	Cα-P distance (Å)[b]
1	0.01	2.87
2	10	4.25
3	210	5.38
4	250	6.73
5	270	7.94
6	0.4	4.83

[a]Values are average of three experiments, errors were calculated to be not more than 10% in all cases.
[b]Cα-P indicates distance between the carbon alpha to the carboxylic acid, and the phosphonate phosphorus. These distances were calculated using the minimizer supplied in Alchemy III.

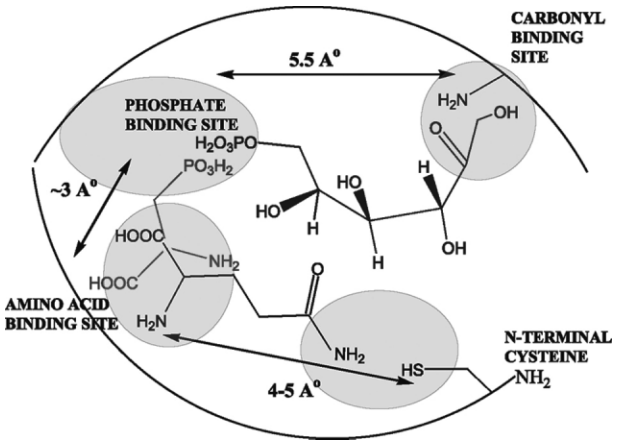

Figure 2. A two-dimensional map of the active sites in GFAT.

distance) indicates that the shorter the chain length, the better the inhibitory activity of the compound. Also since **6**, a structural analogue of **3**, showed a higher potency than **2**, it may be assumed that the rigid nature of the molecule facilitates better binding by **6**. This is also in keeping with the finding that the glutamine-binding site can tolerate bulky groups, and in our case, the hydrophobic nature of the piperidine ring enhances binding to this site. Our data also indicates that the distance between the phosphate binding site and the amino acid binding site is ≈3 Å in *C. albicans* GFAT.

Recently, a study using anhydro-1,2-hexitol 6-phosphates suggested that the two substrate binding domains in *E. coli* GFAT were in close proximity.[31] This, along with data obtained from studies reported by Milewski et al. and Badet et al. indicates that the binding sites in the enzyme are as shown in Figure 2.[12,32] Synthesis of future multisubstrate inhibitors that could also possibly bind to the Lys residue through Schiff base formation may help determination of distances between the Lys in the isomerase active site and the glutaminase active site.

The fact that racemic **1** showed a 3-fold higher potency than FMDP in *C. albicans* is exciting. However **1** did not inhibit glucosamine synthase isolated from *S. cerevisiae* at concentrations <10 mM. This is not surprising, since significant differences exist between the enzyme proteins from *S. cerevisiae* and *C. albicans*.[33] The *Candida* protein is larger in size than the *Saccharomyces* enzyme and sequence alignments indicate regions that are highly different between both species. Glucosamine synthase from *Candida* was also more sensitive to FMDP and UDP-N-acetyl glucosamine than the *Saccharomyces* enzyme.[34]

The unstable nature of the partially purified enzyme extract did not allow us to determine a K_i^{app} for these compounds. Hence, it cannot be stated whether the inhibition is truly multisubstrate or selectively competitive with respect to either substrate. A recently published purification protocol affords a stable homogeneously purified enzyme from the recently developed clone.[35] Further experiments with this enzyme would allow a thorough characterization of the inhibition.

Compound **1** was found to be the most potent inhibitor in the series for the *Candida* enzyme with **5** being the least potent. This finding is supported by Badet's results wherein he reported that the longer compounds have IC_{50}s >3 mM. The finding that racemic **1** was a inhibitor in the nanomolar range and approximately three orders more potent than FMDP the best known inhibitor of glucosamine synthase highlights this class of compounds as useful probes of GFAT activity. The data from these studies stimulates further characterization of the aminophosphonate class of molecules for future antifungal drug design.

Acknowledgements

This work was made possible by NIH Grant AI31193 to R.K.G.

References and Notes

1. Andruszkiewicz, R.; Jedrzejczak, R.; Zieniawa, T.; Wojciechowski, M.; Borowski, E. *J. Enzyme Inhib.* **2000**, *15*, 429.
2. Borowski, E. *Farmaco* **2000**, *55*, 206.
3. Le Camus, C.; Chassagne, A.; Badet-Denisot, M. A.; Badet, B. *Tetrahedron Lett.* **1998**, *39*, 287.
4. Isupov, M. N.; Obmolova, G.; Butterworth, S.; Badet-Denisot, M. A.; Badet, B.; Polikarpov, I.; Littlechild, J. A.; Teplyakov, A. *Structure* **1996**, *4*, 801.
5. Teplyakov, A.; Obmolova, G.; Badet-Denisot, M. A.; Badet, B. *Protein Sci.* **1999**, *8*, 596.
6. Teplyakov, A.; Obmolova, G.; Badet-Denisot, M. A.; Badet, B.; Polikarpov, I. *Structure* **1998**, *6*, 1047.
7. Chmara, H.; Milewski, S.; Andruszkiewicz, R.; Mignini, F.; Borowski, E. *Microbiology-UK* **1998**, *144*, 1349.
8. Andruszkiewicz, R.; Polish, J. *Chem.* **1995**, *69*, 1615.
9. Andruszkiewicz, R.; Milewski, S.; Borowski, E. *J. Enzyme Inhib.* **1995**, *9*, 123.
10. Bearne, S. L.; Wolfenden, R. *Biochemistry* **1995**, *34*, 11515.
11. Andruszkiewicz, R.; Chmara, H.; Zieniawa, T.; Borowski, E. *Eur. J. Med. Chem.* **1994**, *29*, 61.
12. Andruszkiewicz, R.; Chmara, H.; Milewski, S.; Kasprzak, L.; Borowski, E.; Polish, J. *Chem.* **1993**, *67*, 673.
13. Milewski, S.; Chamara, H.; Andruszkiewicz, R.; Borowski, E. *Biochim. Biophys. Acta* **1992**, *1115*, 225.
14. Andruszkiewicz, R.; Chmara, H.; Milewski, S.; Zieniawa, T.; Borowski, E. *J. Med. Chem.* **1990**, *33*, 2755.
15. Andruszkiewicz, R.; Chmara, H.; Milewski, S.; Borowski, E. *J. Med. Chem.* **1987**, *30*, 1715.
16. Chmara, H.; Andruszkiewicz, R.; Borowski, E. *Biochem. Biophys. Res. Commun.* **1984**, *120*, 865.
17. Badet-Denisot, M. A.; Leriche, C.; Massiere, F.; Badet, B. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 815.
18. Mehta, R. J.; Kingsbury, W. D.; Valenta, J.; Actor, P. *Antimicrob. Agents Chemother.* **1984**, *25*, 373.
19. Becker, J. M., Naider, F. In *Peptide-Based Drug Design: Controlling Transport and Metabolism*; Taylor, M. D., Amidon, G. L., Eds., American Chemical Society: Washington, DC, 1995; pp 369–384.
20. McCarthy, P. J.; Newman, D. J.; Nisbet, L. J.; Kingsbury, W. D. *Antimicrob. Agents Chemother.* **1985**, *28*, 494.

21. McCarthy, P. J.; Troke, P. F.; Gull, K. *J. Gen. Micro.* **1985**, *131*, 775.
22. Naider, F., Becker, J. M. In *Curr. Top. Med. Mycol.*, McGinnis, M. R., Ed., Springer Verlag: New York, 1988; Vol. 2; pp 170–198.
23. Payne, J. W.; Shallow, D. A. *FEMS Microbiol. Lett.* **1985**, *28*, 55.
24. Kim, J. H.; Krahn, J. M.; Tomchick, D. R.; Smith, J. L.; Zalkin, H. *J. Biol. Chem.* **1996**, *271*, 15549.
25. Bera, A. K.; Chen, S.; Smith, J. L.; Zalkin, H. *J. Biol. Chem.* **1999**, *274*, 36498.
26. Thoden, J. B.; Holden, H. M.; Wesenberg, G.; Raushel, F. M.; Rayment, I. *Biochemistry* **1997**, *36*, 6305.
27. Tesmer, J. J. G.; Klem, T. J.; Deras, M. L.; Davisson, V. J.; Smith, J. L. *Nat. Struct. Biol.* **1996**, *3*, 74.
28. Knochel, T.; Ivens, A.; Hester, G.; Gonzalez, A.; Bauerle, R.; Wilmanns, M.; Kirschner, K.; Jansonius, J. N. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 9479.
29. Zalkin, H.; Smith, J. *Adv. Enzymol. Rel. Areas Mol. Biol.* **1998**, *72*, 87.
30. Roseman, S.; Daffner, I. *Anal. Chem.* **1956**, *28*, 1743.
31. Leriche, C.; Badet-Denisot, M. A.; Badet, B. *Eur. J. Biochem.* **1997**, *245*, 418.
32. Golinelli-Pimpaneau, B.; LeGoffic, F.; Badet, B. *J. Am. Chem. Soc.* **1989**, *111*, 3029.
33. Milewski, S.; Smith, R. J.; Brown, A. J. P.; Gooday, G. W. In *Chitin Enzymology*; Muzzarelli, R. A. A., Ed., Atec Edizioni: Italy, 1996; Vol. 2, pp 439–446.
34. Smith, R. J.; Milewski, S.; Brown, A. J. P.; Gooday, G. W. *J. Bacteriol.* **1996**, *178*, 2320.
35. Sachadyn, P.; Jedrzejczak, R.; Milewski, S.; Kur, J.; Borowski, E. *Protein Expr. Purif.* **2000**, *19*, 343.