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*Review*

# Glucosamine-6-phosphate synthase, a novel target for antifungal agents. Molecular modelling studies in drug design⋆

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**Fungal infections are a growing problem in contemporary medicine, yet only a few antifungal agents are used in clinical practice. In our laboratory we proposed the enzyme L-glutamine:D-fructose-6-phosphate amidotransferase (EC 2.6.1.16) as a new target for antifungals. The structure of this enzyme consists of two domains, N-terminal and C-terminal ones, catalysing glutamine hydrolysis and sugar-phosphate isomerisation, respectively. In our laboratory a series of potent selective inhibitors of GlcN-6-P synthase have been designed and synthesised. One group of these compounds, including the most studied $N^3$-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid (FMDP), behave like glutamine analogs acting as active-site-directed inactivators, blocking the N-terminal, glutamine-binding domain of the enzyme. The second group of GlcN-6-P synthase inhibitors mimic the transition state of the reaction taking place in the C-terminal sugar isomerising domain. Surprisingly, in spite of the fact that glutamine is the source of nitrogen for a number of enzymes it turned out that the glutamine analogue FMDP and its derivatives are selective against GlcN-6-P synthase and they do not block other enzymes, even belonging to the same family of glutamine amidotransferases. Our molecular modelling studies of this phenomenon revealed that even within the family of related enzymes substantial differences may exist in the geometry of the active site. In the case of the glutamine amidotransferase family the glutamine binding site of GlcN-6-P synthase fits a different region of the glutamine conformational space than other amidotransferases. Detailed analysis of the interaction pattern for the best known, so far, inhibitor of the sugar isomerising domain, namely 2-amino-2-deoxy-D-glucitol-6-phosphate (ADGP), allowed us to suggest changes in the structure of the inhibitor that should improve the interaction pattern. The novel ligand was designed and synthesised. Biological experiments confirmed our predictions. The new compound named ADMP is a much better inhibitor of glucosamine-6-phosphate synthase than ADGP.**

**Keywords**: glucosamine-6-phosphate synthase, glutamine amidotransferases, fungal infections, molecular modelling, drug design

Systemic fungal infections are a growing problem in contemporary medicine, yet only a few antifungal agents are used in clinical practice. Thus, searching not only for improved versions of existing drugs but also for new drug targets has become an urgent need. Unfortunately fungi and humans are both eukaryotic organisms and achieving selective toxicity against only one of them, fungi, is not a trivial task. One important qualitative difference between the fungal and mammalian cells is the presence of a cell wall in the former. In consequence, enzymes of pathways leading to the formation of the cell wall are potential targets for antifungal drugs.

In our laboratory we proposed one of these enzymes, namely L-glutamine:D-fructose-6-phosphate amidotransferase, known under the trivial name of glucosamine-6-phosphate synthase (EC 2.6.1.16), as such new target for antifungals (Chmara *et al.*, 1984a; 1985; Milewski *et al.*, 1988). This protein is a complex enzyme. It catalyses a complex reaction involving ammonia transfer from L-glutamine

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**Abbreviations**: ADGP, 2-amino-2-deoxy-D-glucitol-6-phosphate; ADMP, 2-amino-2-deoxy-D-mannitol-6-phosphate; AO5P, arabinose oxime 5-phosphate; BrAcDP, $N^3$-bromoacetyl-L-2,3-diaminopropanoic acid; DON, 6-diazo-5-oxo-L-norleucine; EADP, $N^3$-D-*trans*-epoxysuccinamoyl-L-2,3-diaminopropanoic acid; FMDP, $N^3$-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid; Fru-6-P, fructose-6-phosphate; GlcN-6-P, glucosamine-6-phosphate; MEP, molecular electrostatic potential; UDP-GlcNAc, uridine diphosphoglucose-*N*-acetyl-D-glucosamine.

to Fru-6-P, followed by isomerisation of the formed fructosamine-6-phosphate to glucosamine-6-phosphate (Fig. 1). This reaction is the first step of the pathway leading to the formation of UDP-GlcNAc, a product that is present in all types of organisms, but is used by these organisms in different ways. In fungi and bacteria it is used to build macromolecules important for the cell wall assembly, such as chitin and mannoproteins in fungi and peptidoglycan in bacteria. In mammals, UDP-GlcNAc is utilised for biosynthesis of glycoproteins and mucopolysaccharides.

In spite of the fact that glucosamine-6-phosphate synthase is present in all kinds of cells, it may be exploited as a target for potential antifungal drugs and selective toxicity can be achieved (Chmara *et al.*, 1984b). Obviously, glucosamine-6-phosphate, the product of this enzyme, is indispensable for fungi as well as for human cells, yet the consequences of its deficiency in both species are very different. It has been shown that even a short-time inactivation of GlcN-6-P synthase in fungal cells is lethal for the pathogen (it induces morphological changes, agglutination and lysis), while in mammals depletion of the aminosugar pool for a short time is not lethal, because of the much longer lifespan of mammalian cells, long half lifetime of GlcN-6-P synthase, and rapid expression of the mammalian gene encoding the enzyme (Bates *et al.*, 1966; Chmara & Borowski, 1986; Milewski *et al.*, 1986).

The native form of the bacterial enzyme is a homodimer with amino acids of both subunits participating in the buildup of the sugar-isomerising active site. The enzyme of eukaryotic origin is a homotetramer but the complete spatial arrangement of the subunits is not known yet. A single enzyme subunit contains two domains: the N-terminal and the C-terminal one, catalysing glutamine hydrolysis and sugar-phosphate isomerisation, respectively. The structures of these domains overexpressed and crystallised separately were known first, while the crystal structure of the complete GlcN-6-P synthase of bacterial origin has been solved only recently (Isupov *et al.*, 1996; Teplyakov, 1998; Teplyakov *et al.*, 2001).

So far several GlcN-6-P inhibitors have been reported, of both natural and synthetic origin, exhibiting fungicidal activity. Most of them are glutamine analogs acting as active site-directed alkylating agents. Obviously, to have any practical use as potential antifungal drugs the inhibitors should be specific against GlcN-6-P synthase (Fig. 2).

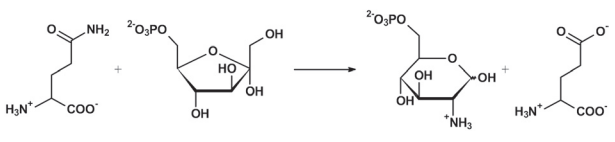

**Figure 1. Reaction catalysed by glucosamine-6-phosphate synthase.**

Tetaine was the first recognised selective inhibitor of GlcN-6-P synthase (Chmara *et al.*, 1984b; Milewski *et al.*, 1986). Later on a series of potent selective inhibitors of GlcN-6-P synthase have been designed and synthesised in our laboratory (Chmara *et al.*, 1984a; Andruszkiewicz *et al.*, 1986; 2000; Milewski *et al.*, 1992). The most studied of these compounds, namely $N^3$-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid (FMDP), a glutamine analog, acts as an active-site-directed inactivator, blocking the N-terminal, a glutamine binding domain of the enzyme. FMDP inactivates the enzyme due to formation of a covalent bond with the sulfhydryl group of its N-terminal cysteine residue by Michael addition reaction (Chmara *et al.*, 1985; Badet *et al.*, 1988; Kucharczyk *et al.*, 1990). The Cys1 residue is crucial for the enzyme activity since it serves as a catalytic nucleophile in the reaction of ammonia liberation from glutamine. Surprisingly, in spite of the fact that glutamine is the source of nitrogen for a number of enzymes, it turned out that FMDP and some of its derivatives exhibit selectivity against GlcN-6-P synthase and that these inhibitors do not block other enzymes, even belonging to the same family of so called glutamine amidotransferases (Chmara *et al.*, 1984b) (this type of selectivity is prerequisite for the lytic effect). This observation suggested that there may be some differences in the geometries of the GlcN-6-P synthase glutamine-binding site and the respective sites in other amidotransferases. Since high specificity is the critical factor in rational drug design, elucidation of the mechanism of the selective action of these inhibitors has become very important for the design of a novel group of antifungal agents — inhibitors of GlcN-6-P synthase.

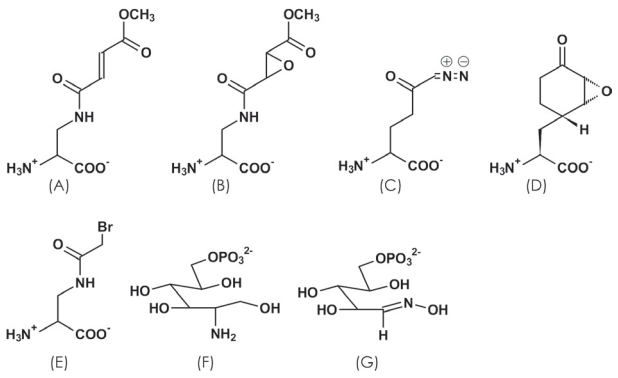

**Figure 2. Structures of selected inhibitors of glucosamine-6-phosphate synthase.**
Inhibitors of the glutamine binding site: FMDP (A), EADP (B), DON (C), anticapsin (D), BrAcDP (E). Inhibitors of the Fru-6-P binding site: ADGP (F), AO5P (G).

## THEORETICAL STUDIES

Over the years GlcN-6-P synthase has gained bad reputation due to its instability. As a consequence of this unfavourable property causing definite problems with purification and experimental studies of the enzyme, its tertiary structure remained unknown for a long time. At this point only experiments on model systems and molecular modelling could help investigate the mechanism of the catalysis and inhibition by specific GlcN-6-P synthase inhibitors (Tempczyk *et al.,* 1989; 1992; Tarnowska *et al.,* 1992).

## STUDIES ON THE GLUTAMINE BINDING SITE

On the basis of a model reaction of FMDP with a decapeptide identical with the N-terminal part of the enzyme, the conclusion was drawn that the enzyme inactivation begins with a Michael-type addition of the enzyme N-terminal cysteine thiol group to the FMDP double bond and then proceeds *via* intramolecular cyclisation incorporating also the free amino group of Cys1. Consequently, the enzyme becomes irreversibly inactivated (Badet *et al.,* 1988; Kucharczyk *et al.,* 1990). The details of this mechanism were also studied by means of computational chemistry methods. Quantum chemical semiempirical calculations and molecular mechanics conformational analysis were applied to study the energetics and the kinetic possibility of the formation of various stereoisomers of the cyclisation products. The results of these theoretical studies were in agreement with the experiments and allowed the details of the inactivation to be explained (Tarnowska *et al.,* 1992). However, those results still did not clarify the reasons for the observed FMDP selectivity and specificity.

Since the crystal structure of neither glucosamine-6-phosphate synthase nor of any of the other glutamine amidotransferases was known, direct analysis and comparison of the geometries of active sites of these enzymes was not possible. Thus the active analogue approach was used to get some insight into the origins of FMDP selectivity. Structure-activity relationship experiments revealed that the alpha amino group as well as the carboxyl moiety are important for the ligand to be recognised by the enzyme as its substrate (Chmara *et al.,* 1985; Andruszkiewicz *et al.,* 1993). Additionally, in the structure of the inhibitor some active electrophilic center at a proper position is also required for the enzyme alkylation and inactivation to take place. A group of well known and characterised, selective and nonselective, GlcN-6-P synthase inhibitors was chosen for the calculations. For each of these inhibitors the set of the three functional groups mentioned was selected as the reference points and then a thorough conformational analysis by the constrained search algorithm was performed. The result of the analysis revealed that molecules of all these inhibitors and glutamine, the enzyme's natural substrate, share some limited range of their conformational space (Wojciechowski *et al.,* 1995). They are all able to achieve a conformation with a similar spatial arrangement of the three mentioned reference groups. This obviously suggests that this arrangement resembles the geometry of the active site and it is the conformation that every ligand has to achieve to fit into the catalytic site of GlcN-6-P synthase and inactivate the enzyme. On the other hand, comparison of the common conformational space generated for the set of selective inhibitors, with the common conformational space of glutamine and its analogues known as nonselective inhibitors of various enzymes revealed that the former forms a "cap" on top of the later and covers only its most distant region. This is especially so when the distance between the carboxyl group and the electrophilic center is considered. For the glucosamine-6-phosphate synthase inhibitors this distance reaches above 6 Å. This particular arrangement of the reference groups we called the pharmacophore geometry of the GlcN-6-P synthase selective inhibitor. Each new molecule designed should follow this geometrical restrictions to behave as a selective inhibitor of glucosamine-6-phosphate synthase. This conclusion was also supported by some interesting experimental results. For one of the molecules considered, anticapsin, two diastereoisomers differing only in the configuration of C$\gamma$ atom were checked. When we superimposed these two molecules and all other inhibitors in the geometry resembling the pharmacophore geometry it turned out that all molecules fit very well not only with respect to the three pharmacophore reference points but also their backbones occupy a similar volume (Fig. 3). Only the molecule of anticapsin with the *R* configuration of the C$\gamma$ atom sticks out of the entire group. Biochemical experiments revealed that this molecule does not exhibit any significant activity against GlcN-6-P synthase. Only the *S* form acts as a potent inhibitor of this enzyme (Crossley & Stamford, 1993).

Obviously not only a structure of the molecule is important for the recognition by a particular enzyme, but also its distribution of the molecular electrostatic potential (MEP). By means of the University of Houston Brownian Dynamics program (UHBD) (Madura *et al.,* 1995) we calculated the distribution of the molecular electrostatic potential around the inhibitors considered and it turned out that it is very similar to the distribution of MEP around the glutamine itself, especially when the comparison was performed for molecules superimposed in the

GlcN-6-P synthase selective inhibitor pharmacophore geometry (Wojciechowski *et al.,* 2001)[1]. This way, despite the fact that some of the inhibitors are structurally very different from glutamine, they all look very similar when not only the structure but primarily the MEP distribution is compared (Fig. 4).

A more thorough analysis of the ligand–receptor interactions for GlcN-6-P synthase became possible when recently the structure of the enzyme of bacterial origin was solved (Fig. 5). On the basis of this crystal structure and a set of known inhibitors the details of the enzyme ligand interactions were studied by a series of docking experiments, followed by molecular dynamics calculations of the resulting complexes. The geometries of the enzyme–inhibitor complexes were modelled by means of the AutoDock software (Morris *et al.,* 1998) using the Lamarckian Genetic Algorithm. For the molecular dynamics simulations the Discover (Biosym, 1995) and GROMACS (Lindahl *et al.,* 2001) packages were used.

The analysis of the geometry of an FMDP molecule docked into the glucosamine-6-phosphate synthase glutamine-binding site revealed that the inhibitor docks in the extended conformation and that its conformation inside the enzyme binding pocket indeed resembles well the previously calculated geometry of the pharmacophore of GlcN-6-P synthase selective inhibitor (Wojciechowski, 1996). The complex obtained by docking was subsequently used as a starting conformation for the 5 ns molecular dynamics calculations. It turned out that the geometry of FMDP is "frozen" in the binding pocket due to numerous strong and stable hydrogen bonds formed between the amido moiety of the inhibitor and the Gly99 and Thr76 residues present in the binding site and well conserved in GlcN-6-P synthase sequences from various organisms (Smith *et al.,* 1996). Interestingly, this amido moiety present in the inhibitor and important for its activity is not present at this position in the glutamine molecule. Moreover, due to the presence of the neighbouring flat fumaroyl part the entire inhibitor molecule behaves very much like a rigid structure and is compelled to maintain its extended conformation (Fig. 6). Additionally, molecular dynamics calculations also revealed that the $\alpha$-amino group, which participates in numerous stable hydrogen bonds to a number of residues including Asp123, His97 and Thr76, serves as the "anchor" mostly responsible for keeping the inhibitor in the binding pocket. The second anchoring group is the already mentioned amido moiety while — interestingly — the carboxy group seems to be more mobile inside the binding pocket. This group does not participate in such a large number of stable hydrogen bonds as the previous two groups (Wojciechowski, 1996).

## STUDIES OF SUGAR BINDING SITE

Another group of GlcN-6-P synthase inhibitors mimic the transition state of the reaction taking place in the enzyme's C-terminal sugar isomerising domain. By definition the transition state analogs of the catalysed reaction should be already selective against this enzyme. One of such inhibitors, 2-amino-2-deoxy-D-glucitol-6-phosphate (ADGP), is an analog of the putative transition state of the catalysed sugar conversion reaction and it was known as the strongest inhibitor of this domain to date (Fig. 2F). When the structure of the complete bacterial enzyme was solved, it became possible to also include the sugar-binding domain in the process of rational design of new GlcN-6-P synthase selective inhibitors. One of the available crystal structures contains ADGP molecule bound in the glucosamine-6-phosphate synthase sugar isomerising site (Teplyakov *et al.,* 1999). A detailed analysis of the interaction pattern for this complex leads us to the conclusion that minor changes in the structure of the inhibitor should enhance the interactions and improve the inhibitory properties of the molecule. Particularly, it is suggested that the carboxyl moiety of Glu488 residue and His504 participate in the sugar ring opening and the proton transfers occurring during regular catalysis, when Fru-6-P is the ligand (Teplyakov *et al.,* 1999; 2002). According to our analysis, by modifying the structure of the ADGP inhibitor these two groups could also be used as extra interaction centers anchoring the ligand in the binding pocket. Additionally we carried out a computational docking experiment of the designed molecule, followed by molecular dynamics calculations of the resulting complex. A similar procedure was applied to the unmodified ADGP molecule itself. Comparison of both trajectories showed that the modified ligand, called now ADMP, should indeed exhibit stronger affinity for the sugar-binding site of GlcN-6-P synthase than its parent molecule, mostly due to the improved interactions with Glu488 (Milewski *et al.,* 2004)[2]. To verify this hypothesis in practice, the designed compound was synthesised and its inhibitory properties against the enzyme were checked. These biochemi-

[1]Wojciechowski M, Pawlowski M, Mazerski J, Borowski E (2001) *Abstracts of VIII International Symposium on Molecular Aspects of Chemotherapy*. Gdansk, Poland, Abstracts, pp 174.
[2]Milewski S, Janiak A, Nowak J, Wojciechowski M (2004) *29th FEBS Meeting*. Warsaw, Poland, Abstracts, *Eur J Biochem* **271** (Suppl. 1): P3.2–34.
[3]Janiak A, Wojciechowski M, Wakiec R, Milewska M, Hoffmann M, Melcer A, Nowak J, Liberek B, Wisniewski A, Milewski S (2004) *Fourth Multidisciplinary Conference on Drug Research*. Gdansk-Sobieszewo, Abstracts, P–115.

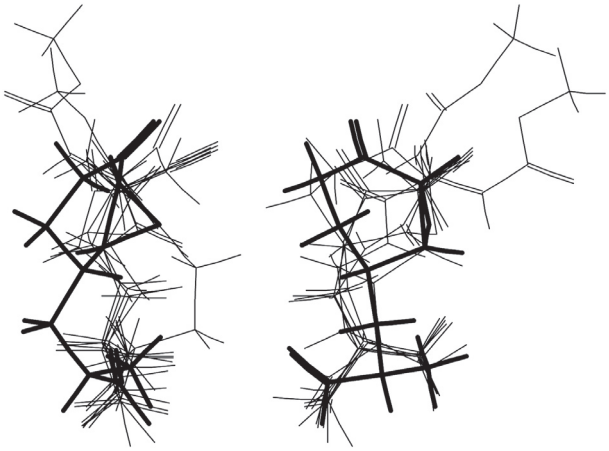

**Figure 3. Orthogonal view of the superposition of two diastereoisomers of anticapsin and other glutamine analogs — inhibitors of glutamine-binding site of GlcN-6-P synthase.**
The inactive isomer of anticapsin is drawn with the thicker line (Wojciechowski *et al.*, 1995).

cal experiments completely confirmed our theoretical predictions. This new molecule turned out to be a much better inhibitor of glucosamine-6-phosphate synthase than the lead ADGP molecule (Janiak *et al.*, 2004)[3].

## CONCLUSIONS

Our theoretical studies on inhibitors of glucosamine-6-phosphate synthase showed that selective inactivation of this enzyme is possible due to some differences between the geometries of the

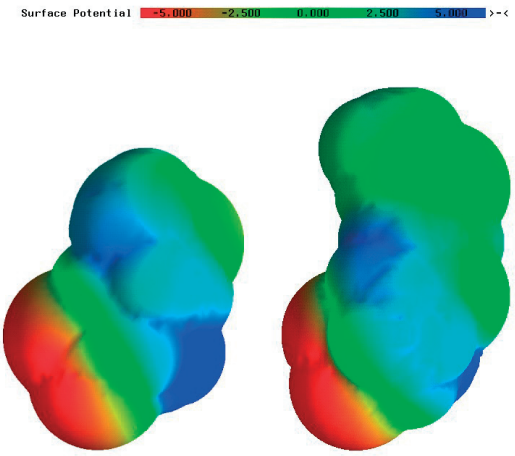


**Figure 4. Distribution of molecular electrostatic potential (MEP) on the solvent accessible surface of glutamine (left) and FMDP (right) (Wojciechowski *et al.*, 2001)[1].**
The pictures are prepared by means of the GRASP package (Nicholls *et al.*, 1991).

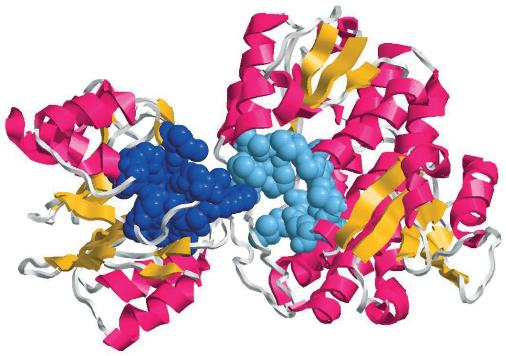

**Figure 5. Structure of bacterial glucosamine-6-phosphate synthase (PDB ID 1jxa).**
Amino-acids comprising the binding sites shown as spheres. Glutamine-binding site — blue spheres; Fru-6-P binding site — cyan spheres.

GlcN-6-P synthase glutamine-binding site and the glutamine-binding sites of other glutamine amidotransferases. Particularly the distance between the area of the binding site responsible for positioning of the ligand's carboxyl and the thiol group of the catalytic Cys1 residue seems to be longer in this enzyme than in other enzymes belonging to the family of amidotransferases.

As a more general conclusion we postulate that, similarly to the amidotransferases, within families of other related enzymes there may exist some differences in the geometry of the active sites.

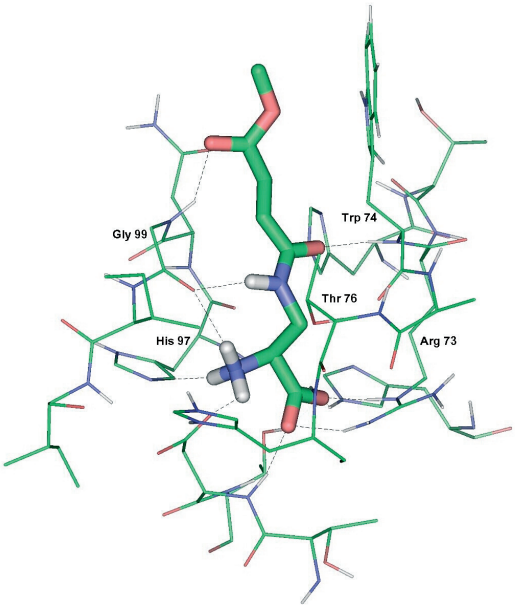


**Figure 6. Hydrogen bonds created by FMDP inside the glutamine-binding site of GlcN-6-P synthase.**
The result of docking of the inhibitor to the receptor (PDB ID 1gdo) by means of the AutoDock program (Morris *et al.*, 1998).

However, despite the fact that these differences are subtle, they can be studied by molecular modelling and utilised in the process of designing new inhibitors, selective only for one particular enzyme from the family. In the case of glutamine amidotransferases it turns out that although all these enzymes bind glutamine, the glutamine-binding site of glucosamine-6-phosphate synthase fits some different region of the glutamine conformational space than the sites of other amidotransferases.

The other, Fru-6-P-binding domain of GlcN-6-P synthase seems to be quite unexplored yet and our analysis including docking and molecular dynamics calculations proved to be effective in exploring the binding site properties and predicting the directions of modifications of existing inhibitors aimed at improving their enzyme affinity.

According to our studies glucosamine-6-phosphate synthase is a good and promising target for new antifungal agents and complete understanding of its catalysis and inhibition mechanisms may result in the development of an entirely new family of its inhibitors — potential antifungal drugs.

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