PROJECT DESCRIPTION

„ The unique fungal lysine biosynthesis enzymes: new targets for antifungal agents?”,

Scientific purpose

The main scientific purpose of this project is to verify the hypothesis that enzymes catalyzing reactions of L-lysine biosynthesis in *Candida albicans* may become new targets for antifungal chemotherapy. Usefulness of these unique fungal enzymes as molecular bases for new drugs discovery will be assessed by testing virulence and survival of *C. albicans* mutant cells, lacking selected genes encoding enzymes of α-aminoadipate pathway (AAP).

In the parallel studies, isolation, characterisation and crystallization attempts of AAP enzymes will be carried out. Information gathered from 3D structure data obtained by X-ray diffraction measurements and kinetic analysis will be used as basis for rational design of inhibitors with potential use for antifungal chemotherapy and/or as fungicides.

The importance of project / existing state of knowledge

Systemic infections caused by human pathogenic fungi in immunocompromized patients continue to be one of the important clinical problems. Number of patients increase, both because of AIDS epidemic, as well as the consequences of applying therapies that cause weakening of the immune system (such as steroid therapy, the use of immunosuppressants in transplant patients). Systemic fungal infections are caused mainly by species belonging to *Aspergillus* and *Candida* [1]. Fungi are also known as one of the most common infectious agents causing nosocomial infections. Among human fungal pathogens, *Candida albicans* plays a dominant role. This opportunistic yeast is responsible for ~ 8% of all hospital-acquired microbial infections.

Efficacious antifungal chemotherapeutic should be characterized by fungicidal but not fungistatic mechanism of action, the widest possible spectrum of activity, the lowest mammalian toxicity and the minimum capacity to induce specific and/or multi-drug resistance. The antifungal agents currently available for the treatment of systemic fungal infections include: a polyene macrolide antibiotic amphotericin B and its lipid formulations, 5-fluorocytosine, inhibitors of ergosterol biosynthesis: fluconazole, voriconazole and itraconazole and an inhibitor of glucan synthase, caspofungin. None of these drugs meets all of the above conditions. Over the past ten years only two new antifungal drugs has been introduced into medical practice, namely voriconazole and caspofungin. In addition, only the latter has an unique, previously unexploited molecular target, an enzyme participating in biosyntheis of the crucial component of fungal cell wall. The continuing increase in the incidence of fungal infections together with the gradual rise in microbial resistance to antifungal drugs highlights the need to find novel compounds with divergent mechanisms of action.

Promising group of potential antifungal drugs are compounds known as anti-metabolites. Chemicals belonging to this group inhibit the biosynthesis of specific biomacromolecules, targeting enzymes involved in these processes as structural analogues of enzyme substrates or reaction intermediates. One of them, 5-fluorocytosine, a nucleoside analoque, is used in clinical

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treatment in combination with amphotericin B. Another compound, L-proline analogue known as BAY-108888, is in the Phase I clinical studies [3]. Compounds having the character of anti-metabolites, and therefore by definition, structurally similar to intermediates or end products of primary metabolic pathways, are poor substrates for membrane proteins exporting xenobiotics, presence of which determines the fungal multidrug resistance. What is more, some antifungal antimetabolites paradoxically show increased activity against multidrug-resistant fungal cells, compared to the sensitive cells [4]. These facts stimulate search for novel antifungals among the compounds of this nature.

Selective inhibitors of enzymes involved in biosynthesis of amino acids, which are essential for humans may become useful antifungal agents. Among this group of compounds some of these possibilities had already been examined, and results obtained should be regarded as promising. Azoxybacilin, an aliphatic amino acid with an azoxy side-chain, isolated from *B. cereus,* inhibits expression of the gene encoding an enzyme involved in the biosynthesis of L-methionine and has a broad spectrum of antifungal activity. [5]., Another compound from this group is 5-hydroxy-4-oxo-L-norvaline, known as RI-331. This is an effective inhibitor of homoserine dehydrogenase involved in biosynthesis of amino acids belonging to the aspartate family [6]. Both compounds show high antifungal *in vitro* and *in vivo* activity and low toxicity.

Taking into account all the factors mentioned above we have decided to start our research with the enzymes of lysine biosynthesis pathway in fungi as potential molecular targets for antifungal chemotherapy. L-Lysine is an essential amino acid for humans, while bacteria, plants and fungi have developed pathways of own lysine biosynthesis. There are two versions of L-lysine biosynthetic pathways: diaminopimelic acid pathway, which is characteristic for bacteria, plants and lower fungi and α-aminoadipate patway (AAP) for higher fungi: *Ascomycetes* and *Basidomycetes*. These two generas include almost all human pathogenic fungi. Enzymes that catalyze the reaction of the above mentioned first pathway are considered promising molecular targets for antibacterial chemotherapy [7], while the second pathway could be a source of new targets for antifungal chemotherapy [2]. Furthermore, inhibitors of enzymes of that pathway could be possibly used as fungicides in agriculture.

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α-Aminoadipate pathway consists of eight stages (Fig. 1) and it can be divided into two phases.

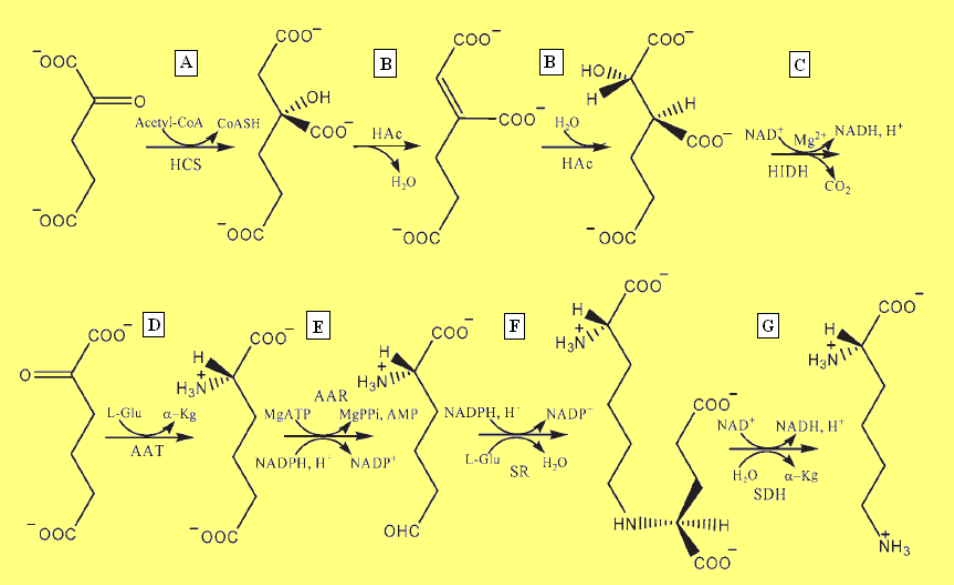

Fig 1. The enzymes of the lysine AAP biosynthetic pathway are as follows. **(A)** Homocitrate synthase (HCS; EC 4.1.3.21). **(B)** Homoaconitase (HAc; EC 4.2.1.36). **(C)** Homoisocitrate dehydrogenase (HIDH; EC 1.1.1.87). **(D)** α- Aminoadipate aminotransferase (AAT; EC 2.6.1.39). **(E)** α-Aminoadipate reductase (AAR; EC 1.2.1.31). **(F)** Saccharopine reductase (SR; EC 1.5.1.10). **(G)** Saccharopine dehydrogenase (SDH; EC 1.2.1.31).

Reactions A–C are similar to the three Krebs cycle reactions (starting with the condensation of oxaloacetate with acetyl-CoA and finishing with α-ketoglutarate synthesis). Despite some analogies, first three reactions are unique for the α -aminoadipate pathway and very specific for higher fungi. HCS catalyzes the first and committed step in the pathway, is highly regulated to economize the use of resources, and its reaction is thought to be the rate-limiting step in the pathway. The second phase starts from the transamination leading to the creation of α - aminoadipate (D). Next (E-G) transformation finally lead to the creation of L-lysine. D-G stages from the second phase are considered to be reversal reaction of L-lysine biodegradation pathway although some of the enzymes catalyzing biosynthetic reactions are different from the enzymes catalysing reverse catabolic reactions.

Homocitric synthase (HCS), homoaconitase (HA) and homoisocitric dehydrogenase (HICD) catalyzing biosynthetic reactions present only in fungal cells and having no counterparts in mammalian cells, are the most obvious candidates for the molecular targets. It is also possible that the enzymes catalysing biosynthetic reactions D – G (Fig. 1) are somewhat different from the enzymes catalysing reverse catabolic reactions present in mammalian cells and thus can be also considered target candidates.

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In 1985 Shepherd provided some evidence that lysine auxotrophic mutants of *Candida albicans* were not capable of causing disseminated candidiasis. [8]. Recent studies indicate that mutant cells of pathogenic fungi *Aspergillus fumigatus* with *lysF* gene deletion, encoding homoaconitase (stage B in Fig.1) leads to attenuated virulence in a low-dose mouse infection model of invasive aspergillosis [9]. These results suggests that *in vivo* the concentration of L-lysine and / or peptides containing the amino acid present in the mammal blood serum, are not high enough for fungal cells to supplement the deficit of L-lysine caused by blocking of its own biosynthesis. It means that the selective inhibition of the enzyme(s) of the □-aminoadipate pathway may control the growth of fungal pathogens *in vivo*.

To test this hypothesis Zabriskie and Jackson prepared amide analogs of saccharopine and tested them as inhibitors of the commercially available saccharopine dehydrogenase from *S. cerevisiae*. Compounds were found to be quite effective inhibitors, however, showed no antifungal activity [10]. Another results suggests that phenyl analoques of (R)-homocitrate and (2R, 2S) – homoisocitrate show antifungal activity *in vitro* against *Aspergillus nidulans* [11]. Both of these, quite recent publications, are the only reports describing inhibitors of fungi lysine biosynthesis, synthesised in the purpose of testing for antifungal activity.

Results obtained so far in our laboratory show that the disruption of both genes encoding homocitrate synthase (double null *lys21*Δ/*lys22*Δ mutant of *C. albicans* lacked homocitrate synthase activity) (stage A in Fig. 1) does not affect virulence of *C. albicans* in the disseminated infection model [12]. This conclusion may be confronted with the previous reports of other authors who demonstrated avirulence of lysine auxotrophs of human pathogenic fungi. *Candida albicans* strains auxotrophic for L-lysine obtained by random mutagenesis were found to have reduced virulence following intravenous inoculation [8], but it is not clear if lysine biosynthesis was the only pathway affected. In light of our present results, it seems possible that there were other, undetected mutations affecting virulence. Mixed-inoculum infection experiments revealed that the growth of lysine-auxotrophic *A. nidulans lysA* strains deficient in saccharopine dehydrogenase was significantly slower than that of the prototrophic strain in the lungs of neutropenic mice. However, no effect was observed in the survival of mice inoculated with the auxotrophic mutant strain alone [13]. Finally, deletion of the *Aspergillus fumigatus* gene encoding homoaconitase led to the attenuated virulence in a lung tissue of mice infected intranasally [14]. Interestingly, the same phenomenon was demonstrated for *Cryptococcus neoformans* auxotrophic for L-methionine due to the targeted disruption of homoserine transacetylase, in mouse inhalation model [15]. It seems likely therefore, that the avirulence of mutant fungal pathogens auxotrophic for a particular amino acid, demonstrated previously by other authors in the models of pulmonary fungal infections, might be due to the possible low content of that amino acid in respiratory track tissues, lower than that present in the bloodstream. It may be especially low at the surface of lung air vesicles, where the inhaled fungal spores or vegetative cells must adhere at the onset of pulmonary infection. Schöbel *et al.* also obtained very similar results for *Aspergillus fumigatus* mutant cells lacking HCS activity. The mutant was avirulent when injected intravenously, but its virulence was strongly attenuated in the murine model of bronchopulmonary aspergillosis [16].

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Studies performed so far by us and other research groups has not clearly answered the question whether enzymes of the AAP should be considered promising targets for chemotherapy of fungal infections. This issue is addressed in the present project. We propose a series of experiments aimed at getting the definite conclusions.

- ***Establishing the consequences of the lack of activity of selected AAP enzymes for the virulence and survival of C. albicans cells.***
- ***Crystallization of selected AAP enzymes from C. albicans, X-ray diffraction measurements, construction of structural models and analysis.***

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