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A convenient cellular assay for the identification of the molecular target of ergosterol biosynthesis inhibitors and quantification of their effects on total ergosterol biosynthesis

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ABSTRACT

Increasing resistance of clinically relevant fungi is causing major problems in anti-mycotic therapy. Particularly for immunosuppressed patients fungal infections are of concern and increasing resistance against clinically used antimycotic drugs is hampering successful treatment. In the search for new antifungals ergosterol biosynthesis still is the most prominent target. However, several pitfalls in the bioactivity testing of such substances remain. Two of the major drawbacks certainly are the membrane association of most enzymes participating in ergosterol biosynthesis, and the difficulty to selectively associate growth inhibitory effects with the target pathway (ergosterol biosynthesis). Here we describe a GC–MS based cellular assay for target identification and selective potency determination of test components. In the qualitative part of the assay GC–MS analysis of cell lysates allows target identification by analysis of the changes in the sterol pattern. The quantitative part of the assay makes use of ^{13}C-acetate feeding combined with GC–MS analysis allowing the selective quantification of a compound's effect on total ergosterol biosynthesis. The described cellular assay was analytically and biologically validated and used to characterize the novel ergosterol biosynthesis inhibitor JK-250.

1. Introduction

During the last decade novel therapies and medicines evolved allowing the successful treatment of severe diseases such as AIDS or cancer. However major pitfalls in many treatment regimens of immunosuppressed patients are co-infections by opportunistic pathogens [1,2]. Particularly local or systemic mycoses represent a major problem. Not only systemic mycoses are generally difficult to treat but furthermore treatment efficiency is additionally compromised by an increasing number of drug-resistant strains. Some of the most common opportunistic fungi, such as *Candida* and *Aspergillus* species, present an increasingly growing resistance to currently available antifungals out of different chemical classes [3–5]. The importance of this fact can be underlined by clinical studies not only showing the increasing development of drug resistance but also emphasizing its societal impact [6,7]. Hence medicinal chemists are striving to develop novel, potent antifungal drugs controlling the problem of resistance and offering novel options for treatment [8–10].

The mode of action common to the majority of antifungals is enzyme inhibition of the post-squalene part of ergosterol biosynthesis (Fig. 1) or an interaction with ergosterol in the plasma membrane (amphotericin B, nystatin) [3,4].

Clinically three different classes of ergosterol biosynthesis inhibitors are available: allylamines (naftifine, terbinafine) inhibiting squalene epoxidase [3,4], azoles (e.g. clotrimazole, fluconazole) inhibiting C14-demethylase (Fig. 1, enzyme A; see also Fig. 6 in Section 3.1) [3,4,11,12], and one morpholine (amorolfine) inhibiting both C14-reductase and $\Delta^{8,7}$-isomerase (Fig. 1, enzymes B and E; see also in Fig. 7 Section 3.1) [3,13,14]. Overall, ergosterol biosynthesis is accomplished by eight distinct enzymes involved in the conversion of lanosterol, the first sterol in this pathway, to ergosterol [15–18]. For most of the involved enzymes specific inhibitors are known, and different types of mechanisms are discussed for the cytotoxic effects of ergosterol biosynthesis inhibitors: on one hand the fluidity, permeability and functionality of the fungal cell membrane is decreased by the deficiency of ergosterol, on the other hand distinct accumulating ergosterol biosynthesis intermediates are toxic for the fungal cells [12,19–22].

In the course of developing novel enzyme inhibitors for distal ergosterol biosynthesis a major difficulty lies in the membrane association of the involved enzymes compromising isolation and compound testing [22,23]. As shown by others and ourselves, whole-cell screening systems combined with sterol pattern analysis can help overcoming this problem, and allow for the easy

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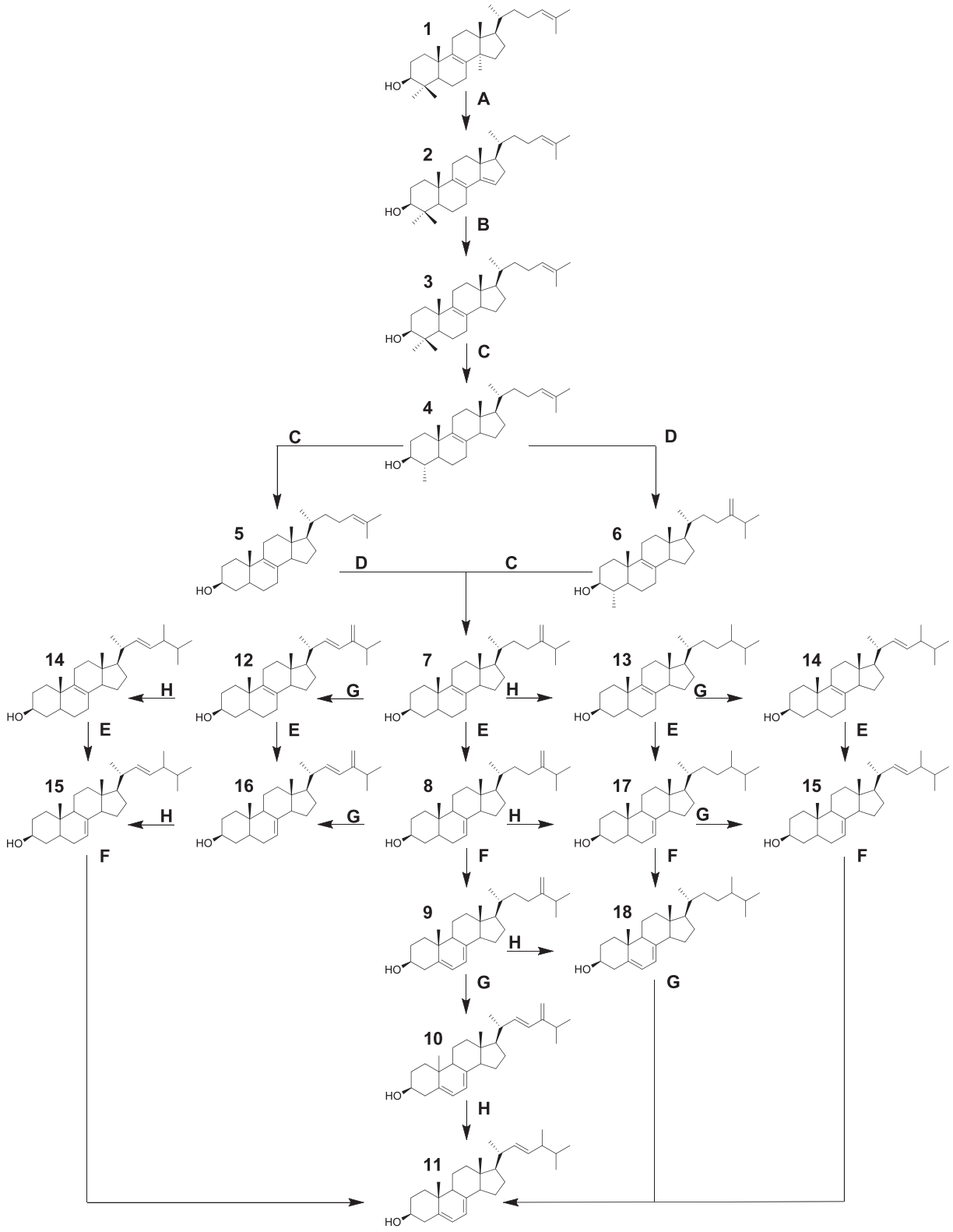

Fig. 1. Post-lanosterol part of ergosterol biosynthesis in untreated yeast cells. Enzymes: A sterol C14-demethylase, B sterol Δ^{14}-reductase, C sterol C4-demethylase, D sterol C24-methyltransferase, E sterol $\Delta^{8/7}$-isomerase, F sterol C5-desaturase, G sterol C22-desaturase, H sterol $\Delta^{24(28)}$-reductase. Sterols: **1** lanosterol, **2** 4,4-dimethylcholesta-8,14,24-trien-3β-ol, **3** 4,4-dimethylzymosterol, **4** 4-methylzymosterol, **5** zymosterol, **6** 4-methylfecosterol, **7** fecosterol, **8** episterol, **9** 5-dehydroepisterol, **10** 24(28)-dehydroergosterol, **11** ergosterol, **12** ergosta-8,22,24(28)-trien-3β-ol, **13** ergosta-8-en-3β-ol, **14** ergosta-8,22-dien-3β-ol, **15** 5,6-dihydroergosterol, **16** ergosta-7,22,24(28)-trien-3β-ol, **17** ergosta-7-en-3β-ol, **18** ergosta-5,7-dien-3β-ol.

identification of target enzymes of inhibitors in yeasts, plants, and human cells [12,24–30].

Here we present an advanced *in vitro* screening assay for distal ergosterol biosynthesis inhibitors which was developed along the