Aspergillus oryzae produces compounds inhibiting cholesterol biosynthesis downstream of dihydrolanosterol

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Abstract

The formation of cholesterol synthesis inhibiting molecules by five different strains of the koji mold Aspergillus oryzae was studied. After growing these strains on a complex liquid medium we found in crude organic phase extracts and specific fractions there from compounds inhibiting cholesterol synthesis in human hepatic T9A4 cells in vitro at enzyme sites downstream of dihydrolanosterol. This was evidenced by using different radioactively labeled precursors, namely acetate, mevalonate, 24,25-dihydro-[24,25-3H2]-lanosterol or [3-3H]-lathosterol.© 2004 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

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1. Introduction

Statins, such as monacolin J and lovastatin, are secondary metabolites produced by filamentous fungi of the species Aspergillus terreus and Monascus ruber [1]. This family of compounds has become one of the most successful cholesterol lowering drugs with a world wide distribution to millions of patients [2]. Only a single A. oryzae strain has been reported to produce low amounts of lovastatin [3,4]. The site in the biosynthetic pathway of cholesterol at which statins inhibit is the enzyme HMG-CoA reductase [1,5,6], thus reducing mevalonate synthesis and potentially all of its metabolic products. Since mevalonate is a common precursor for all isoprenoids such as sterols, ubiquinone, dolichols and isopentenyl adenine, blocking its formation may induce undesirable side effects [7–10]. The objective of this work was to screen A. oryzae strains, which traditionally are used for Koji food fermentations, for compounds inhibiting cholesterol synthesis and in particular to test if these compounds affect cholesterol biosynthesis downstream of mevalonate.
2. Materials and methods

2.1. Strains, media and metabolite extraction

*Aspergillus oryzae* strains were cultivated in shake flask in complex liquid medium at 220 rpm and at 28 °C, as described earlier [11]. After 280 h of fermentation 200 mL of culture broth was macerated for 2 h with 200 mL of methanol solution (80% v/v). The solution was passed through a filter paper and the filtrate was evaporated under vacuum at 30 °C to remove the solvent. The dry extract was dissolved in water, acidified to pH 3 with HCl and extracted several times with ethyl acetate. The crude extract was weighed after evaporation of the organic phase and subsequently resuspended in methanol.

2.2. Crude extract fractionation

Crude extracts were prepurified by vacuum liquid chromatography using silica gel (Kieselgel 60, Merck). The extract was fractionated by stepwise elution with hexane/ethyl acetate/methanol mixtures. Selected fractions were further separated by high-performance liquid chromatography (HPLC) using a Nucleosil 100-5 C18 column (250 × 4 mm) (Macherey & Nagel) with a post column (Lichrospher 100 RP-18 (Merck)). The mobile phase consisted of a mixture of 33 mM H3PO4 in water and acetonitrile (9:1 v/v).

2.3. Chemicals

Lovastatin (activity enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase [HMG-CoA reductase; mevalonate :NADP+ oxidoreductase (CoA-acylating), EC 1.1.1.34]) was purchased from Merck.

Radio labeled 24,25-Dihydro-[24,25-3H2]-lanosterol or [3-3H]-lathosterol were synthesized and purchased from Amersham Pharmacia Biotech (Cardiff, UK).

2.4. Mass spectrometrical analysis

2.4.1. GC–MS

Fractions were collected for biological activity determination. Active fractions were further analyzed by GC–MS (HP 5890 GC combined with a Finnigan MAT 8430 mass spectrometer). The fused silica capillary column employed was a J&W Sci. DB-5 (30 m × 0.32 mm, 0.25 µm film thickness). The carrier gas was helium (150 kPa) and the temperature program was 60 °C (1 min), 30 °C/min to 270 °C, 10 °C/min to 320 °C. The splitless injector was heated at 250 °C and the transfer line temperature at 280 °C. Mass spectra were obtained in the EI mode at 70 eV from 20 to 800 Da. The samples were injected before and after trimethylsilyl derivatization performed with a mixture of pyridine and BSTFA (1/3, v/v) heated 1 h at 100 °C.

2.4.2. HPLC-MS

Analyses were carried out using either a Micromass Quattro-LC mass spectrometer connected to a Waters HPLC system consisting of a 757 autosampler, a 600-MS pump with system controller and a type 486-MS UV detector. The HPLC column used was a Nucleosil 100 5-C18, 250 × 4 mm. Solvent A was water containing 0.1% trifluoroacetic acid (TFA), B was acetonitrile containing 0.1% TFA. Using a flow rate of 1 mL/min the separation started either in isocratic mode (10% A, 90% B) or with a linear gradient from 90% A and 10% B (5 min), reaching 10% A and 90% B in 25 min and continued with an isocratic run for 5 minutes. A flow rate of 1 mL/min was used with a 1/10 post column splitter admitting 0.1 mL/min into the mass spectrometers. Both MS worked with an electrospray interface set at a voltage of 4 kV. Mass spectra were acquired from 100 to 800 Da in positive mode.

2.5. In vitro activity

The human hepatic T9A4 cells [12] were grown in the serum-free LCM medium (Biofluids, Rockville, MD) under 3.5% CO2 at 37 °C. The cells were seeded in 24-well plates and incubated at confluency with 1mM 14C-acetate (1 mCi/mmol, Amersham), 14C-mevalonate, 24,25-dihydro-[24,25-3H2]-lanosterol or [3-3H]-lathosterol for 20 h in the absence (negative control) or in the presence of crude extract. Lovastatin served as positive control. Lipids were extracted twice by incubation with hexane/isopropanol (3:2) for 30 min at room temperature. The combined extracts were dried under N2, dissolved in hexane and subjected to high performance thin layer chromatography (Merek, Darmstadt, Germany) in a solvent mixture of hexane/diethyl ether/acetic acid (75:25:1). The incorporation of labeled precursor into cholesterol was detected with an instant imager (Camberra Packard, Zurich, Switzerland) and expressed as percent of the control experiment.

3. Results

3.1. Screening of *A. oryzae* strains inhibiting cholesterol biosynthesis

Crude extracts were prepared from cultures of several *A. oryzae* strains from the Nestlé Culture Collection [11]. The organic phase and the aqueous phase were each tested for in vitro inhibition of cholesterol synthesis in the presence of different metabolic precursors. The aqueous phase of the fungal culture extracts showed no effect on cholesterol synthesis. By contrast, the organic phase of extracts from strains *A. oryzae* CNCM I-2526, CNCM I-2527, CNCM I-2528, CNCM I-2533 and CNCM
I-2534 displayed inhibition of cholesterol synthesis. None of the extracts tested modified the protein content of the cells, as determined by the Bradford protein assay method (data not shown), indicating the absence of toxicity and cell growth inhibition effects. Table 1 shows that the addition of crude extracts in the range between 37–50 μg/mL reduced incorporation of acetate into cholesterol in the range between 36% and 54%. By comparison, addition of lovastatin to a final concentration of 0.5 μg/mL reduced cholesterol synthesis by 50%. Neither lovastatin nor monacolin J (statins) were detected in these extracts by HPLC-MS (data not shown). Moreover, cholesterol biosynthesis was strongly inhibited when mevalonate was given as precursor, (Table 1) which indicates that at least one point of inhibition is localized downstream of HMGCoa reductase. In conclusion, the five strains of A. oryzae from the Nestlé Culture Collection listed in Table 1 produced one or several molecules inhibiting cholesterol synthesis, but these compounds clearly do not belong to the class of statins.

3.2. Inhibition localized downstream of dihydrolanosterol

Strain A. oryzae CNCM I-2526 was further investigated as it showed a strong inhibition of cholesterol synthesis using mevalonate as precursor (Table 1). After cultivation in a bioreactor in complex medium [13], the whole culture broth was macerated for 2 h with an equal volume of methanol (80% v/v). This extract was passed through a filter paper and was evaporated and extracted several times with ethyl acetate (same protocol as for crude extract preparation).

The extract (5 g) of this fermentation broth again displayed inhibition of cholesterol synthesis. The crude extract was fractionated by vacuum liquid chromatography by stepwise elution with hexane/ethyl acetate/methanol mixtures in ratios 100:0:0, 95:5:0, 50:50:0, 0:100:0 and 0:0:100 v/v. Each elution fraction was collected and total dry matter was quantified as it showed a strong inhibition of cholesterol synthesis. The crude extract and several fractions when using this acetate and 100% methanol were the most active (Table 2).

Pathway intermediates downstream of mevalonate, lanosterol and lathosterol, were tested to determine more accurately the potential point(s) of inhibition within the cholesterol synthesis pathway. Each fraction was tested for inhibitory activity with the human hepatic T9A4 cells in the presence of labeled precursors in the cholesterol synthesis, namely 24,25-dihydro-[24,25-3H2]-lanosterol and [3-3H]-lathosterol [14,15], respectively. Firstly, the assimilation of dihydrolanosterol and lathosterol by the cells was confirmed by measuring the absorption of the respective radioactively labeled derivatives (data not shown). Secondly, addition of all these fractions (except for the 95:5:0, 80:20:0 hexane/ethyl acetate/methanol fractions) resulted in inhibition of cholesterol biosynthesis when 24,25-dihydro [24,25-3H2]-lanosterol was used as labeled precursor. When [3-3H]-lathosterol was given as precursor, no inhibition was observed (Table 2). The point of inhibition of cholesterol synthesis in presence of this active compound could therefore be localized between lanosterol and lathosterol.

The fraction eluting with 100% ethyl acetate (more active, ID50 = 8 ± 0.6) (Table 2) was separated by HPLC. The eluate collected from 18.5 to 19.5 min showed an inhibitory activity and was further analyzed by GC–MS. On the basis of the typical fragmentation pattern of the molecules,sterol analogues were detected in this fraction but only stigmasterol and stigmastanol could specifically be identified.

4. Discussion

The two demethylation reactions occurring between lanosterol and lathosterol are catalyzed by a cytochrome P-450 dependent enzyme. The first step, 14α-demethylation of lanosterol, results in the removal of a single methyl group, and during the second step (4-demethylation of demethylanosterol) two methyl groups are removed. Cholesterol biosynthesis was inhibited by crude extract and several fractions when using this labelled intermediary compound 24,25-Dihydro [24,25-3H2]-lanosterol, but was not inhibited at all when [3-3H]-lathosterol was given as precursor (Table 2). This shows that the active molecule(s) of A. oryzae CNCM I-2526 inhibited the conversion of lanosterol to lathosterol. This also excludes the class of squalestatins or viridiofungins which inhibit squalene synthase [16,17] (being located upstream of lanosterol) as the active compounds from strain CNCM I-2526. The inhibitory activity was distributed over several fractions (Table 2) and

<table>
<thead>
<tr>
<th>Strain</th>
<th>Final concentration of crude extract in vitro assay (μg/mL)</th>
<th>Incorporation of 14C-acetate</th>
<th>Incorporation of 14C-mevalonate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNCM 1-2526</td>
<td>38</td>
<td>40 ± 3</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>CNCM 1-2527</td>
<td>50</td>
<td>45 ± 6</td>
<td>34 ± 7</td>
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<td>39 ± 7</td>
<td>36 ± 5</td>
</tr>
<tr>
<td>CNCM 1-2534</td>
<td>40</td>
<td>54 ± 2</td>
<td>58 ± 5</td>
</tr>
</tbody>
</table>

* Values are average of triplicate assays, with standard deviation (SEM) indicated.
the presence of this activity within fractions of different physico-chemical properties, acting on the same conversion step, suggested the existence of several active molecules. The overall results to this point suggested the presence of this activity within fractions of different sources such as peanut, sunflower, soybean and olive oils [18] and are reported to inhibit cholesterol absorption [19–21]. Stigmasterol decreases HMG-CoA reductase gene expression in CaCo-2 cells [22]. In vitro tests with hepatic cells T9A4 confirmed that stigmasterol (purified from Sigma) inhibited the synthesis of cholesterol from 14C-acetate [23] whereas stigmastanol was not active. However, only a small fraction of the biological activity measured in the A. oryzae strains could be attributed to the amount and the specific activity of stigmasterol. Thus other active molecules remain unknown and need to be identified. Among these active metabolites, sterol analogues are likely candidates to be the compounds that reduce the level of 14-demethyl lanosterol and/or zymosterol intermediate synthesis by competitive inhibition [14,15]. The data presented in this study demonstrated the existence of several inhibitory molecules of cholesterol synthesis produced by A. oryzae active downstream of lanosterol. Further investigations are needed to test whether or not these molecules could form the basis of new cholesterol synthesis inhibiting agents.

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References


