Synthesis of C(7) modified chrysin derivatives designing to inhibit \( \beta \)-ketoacyl-acyl carrier protein synthase III (FabH) as antibiotics

Huan-Qiu Li, Lei Shi, Qing-Shan Li, Peng-Gang Liu, Yin Luo, Jing Zhao, Hai-Liang Zhu *

State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing 210093, People’s Republic of China

ARTICLE INFO

Article history:
Received 1 July 2009
Revised 21 July 2009
Accepted 21 July 2009
Available online 25 July 2009

Keywords:
FabH
Antibiotics
Structure–activity relationship
Chrysin derivatives

ABSTRACT

As a naturally wide distributed flavone, chrysin exhibits numerous biological activities including anticancer, anti-inflammatory, and antimicrobials activities. \( \beta \)-Ketoacyl-acyl carrier protein synthase III (FabH) catalyzes the initial step of fatty acid biosynthesis via a type II fatty acid synthase in most bacteria.

The important role of this enzyme combined with its unique structural features and ubiquitous occurrence in bacteria has made it an attractive new target for the development of antibacterial agents. We first used a structure-based approach to develop 18 novel chrysin analogues that target FabH for the development of new antibiotics. Structure-based design methods were used for the expansion of the chrysin derivatives including molecular docking and SAR research. Based on the results, 5-hydroxy-2-phenyl-7-(2-(piperazin-1-yl)ethoxy)-4H-chromen-4-one (3g) showed the most potent antibacterial activity with MIC of 1.56–6.25 \( \mu \)g/mL against the test bacterial stains, and docking simulation was performed to position compound 3g into the Escherichia coli FabH active site to determine the probable binding conformation. The biological assays indicated that compound 3g is a potent inhibitor of E. coli FabH as antibiotics.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Although several classes of antibacterial agents are presently available, resistance in most of the pathogenic bacteria to these drugs constantly emerges. In order to prevent this serious medical problem, the elaboration of new types of antibacterial agents or the expansion of bioactivity of the previous drugs is a very important task.

Therefore, in recent years the research has been focused toward development of new antibacterial agents, which may act through novel target, surpassing the problem of acquired resistance.

A promising target is the fatty acid synthase (FAS) pathway in bacteria. Fatty acid biosynthesis (FAB) is an essential metabolic process for prokaryotic organisms and is required for cell viability and growth. Large multifunctional proteins termed type I fatty acid synthases (FAS I) catalyze these essential reactions in eukaryotes. In contrast, bacteria use multiple enzymes to accomplish the same goal and are referred to as type II, or dissociated, fatty acid synthases (FAS II). \( \beta \)-Ketoacyl-acyl carrier protein (ACP) synthase III, also known as FabH or KAS III, plays an essential and regulatory role in bacterial FAB. The enzyme initiates the fatty acid elongation cycles and is involved in the feedback regulation of the biosynthetic pathway via product inhibition. FabH catalyzes the condensation reaction between a CoA-attached acetyl group and an ACP-attached malonyl group, yielding acetoacetyl-ACP as its final product (Fig. 1). Two other condensing enzymes, FabB (KAS I) and FabF (KAS II), perform the chain elongation reactions in subsequent cycles leading to long-chain acyl ACP products.

While FabB and FabF are also condensing enzymes, FabH is structurally distinct. FabH proteins from both Gram-positive and Gram-negative bacteria are highly conserved at the sequence and structural level while there are no significantly homologous proteins in humans. Importantly, the residues that comprise the active site are essentially invariant in various bacterial FabH molecules. FabH represents a promising target for the design of novel antimicrobial drugs, since it regulates the fatty acid biosynthesis rate via an initiation pathway and its substrate specificity is a key factor in membrane fatty acid composition. These attributes suggest that small molecule inhibitors of FabH enzymatic activity could be potential development candidates leading to selective, non-toxic, and broad-spectrum antibacterials.

Chrysin (1,5,7-dihydroxyflavone, shown in Scheme 1), a flavonoid widely distributed in nature, has been reported to have many different biological activities such as anti-oxidant, anti-virus, anti-microbial, anti-inflammatory, and anti-tumor activities as well as antitumor effect. In the reported experiments, the efforts were centered mostly on the substitutions on the aromatic rings (either A or C) of the chrysin, or chrysin was linked with heterocyclic moieties separated by different carbon spacers. These derivatives, compared with their parent compound, displayed significant biological activity against a panel of susceptible and resistant Gram-positive and Gram-
negative organisms. Many flavonoids were reported to be good candidate KAS III inhibitors and may be utilized as effective antimicrobials. Thus, it would be valuable to explore the inhibitory action of novel chrysin derivatives with heterocyclic and alkyl amines linked by different carbon spacers. In this paper, we described the synthesis of two series of analogues of chrysin and the evaluation of these compounds as FabH inhibitors for the first time. The antibacterial activity against two Gram-negative bacterial strains, *Escherichia coli* and *Pseudomonas fluorescence*, and two Gram-positive bacterial strains, *Bacillus subtilis* and *Staphylococcus aureus*, of these analogues of chrysin were also been determined. Docking simulations were performed using the X-ray crystallographic structure of the FabH of *E. coli* in complex with an inhibitor to explore the binding modes of these compounds at the active site.

2. Results and discussion

2.1. Chemistry

The synthesis of compounds 2a–i and 3a–i was accomplished according to the general pathway illustrated in the Scheme 1. Compounds 1a and 1b were the key intermediates for synthesis of the compounds investigated. They were usually prepared from alkylation of 7-OH group by using 1,2- or 1,3-dihaloalkanes in the presence of bases such as NaOH or K₂CO₃ in anhydrous DMF. To increase the antibacterial properties of chrysin derivatives, chrysin derivatives in which the chrysin ring system is linked to the alkylamines by different spacers at C-7 position were investigated, with a view to modify their lipophilicity. The methods for alkylation of 1a and 1b were well documented. The literature survey indicated that a method reported by Liu et al is appropriate for N-alkylation of imidazole or benzimidazole, since the formation of quaternary imidazolium or benzimidazolium salts, as unwanted side product, was usually limited. Compounds in series 1 (2a–2i) contained a 2-carbon spacer in between chrysin and the substituent and compounds series 2 (3a–3i) contained a 3-carbon spacer in. All of the synthetic Compounds gave satisfactory analytical and spectroscopic data, which were in full accordance with their depicted structures.

2.2. Biological activity and molecular modeling

2.2.1. Antimicrobial activity

All the synthesized compounds (2a–i and 3a–i) were screened for antibacterial activity against two Gram-negative bacterial
strains: *E. coli* and *P. fluorescence* and two Gram-positive bacterial strains: *B. subtilis* and *S. aureus* by MTT method. The MICs (minimum inhibitory concentrations) of the compounds against these bacteria were presented in Table 1. Standard antibacterial agent kanamycin B was also screened under identical conditions for comparison. The results revealed that most of the synthesized compounds exhibited significant antibacterial activity. It was observed that many compounds exhibited interesting antibacterial activity displaying MIC values between 1.56 and 50.0 μg/ml.

Among compounds in 3a–3l, which contained a 3-carbon spacer, 3a, 3e, and 3g with N-heterocyclic rings at C-7 position displayed potent zone of antibacterial activities ranging from 1.56–25.0 μg/ml, exhibiting higher potencies than chrysin which was inactive. Especially compound 3g exhibited the most potent antibacterial activity with MIC of 1.56, 6.25, 6.25, and 3.13 μg/ml against *E. coli*, *P. fluorescence*, *B. subtilis*, and *S. aureus*, respectively, which was similar to the broad-spectrum antibiotic kanamycin B with corresponding MIC of 3.13, 3.13, 1.56, and 1.56 μg/ml. Compounds 3a and 3e also showed potent activity against the same strains. Other 3-carbon spacer derivatives with aliphatic chains substituent showed low inhibitory activity, this result disclosed that compounds with heterocyclic moieties at C-7 position of chrysin were more active than compounds with aliphatic chains. Compounds 2a–i which contain a 2-carbon spacer, displayed moderate antibacterial activities. The N-heterocyclic rings displaced compounds 2a, 2e, 2g, and 2l showed better antibacterial activity than other compounds, and the same comparison was also exhibited in compounds 3a–l. These results proved that the compounds with heterocyclic moieties at C-7 position of chrysin have a much higher activity than other species, although these compounds are not as good as kanamycin B which has an N-o-glucoside in the side chain in terms of broad-spectrum inhibition. A possible explanation for these results is that the lipophilicity of the chrysin derivatives and kanamycin B affected by their side chains played an important role for their antimicrobial activities.

### 2.2.2. *E. coli* FabH inhibitory activity

The *E. coli* FabH inhibitory potency of the chrysin derivatives (2a, 2b, 2c, 3a, 3e, and 3g) was examined and the results are summarized in Table 2. As shown in Table 2, most of the tested compounds displayed potent inhibitory activity. Among them, compounds 2b and 2c, with the alkyl amines at the C-7 position of chrysin had less inhibitory activity toward *E. coli* FabH. It suggested that a heterocyclic moiety at C-7 position of chrysin was essential for the FabH inhibitory activity. In addition to possessing antibacterial activity against both Gram-positive and Gram-negative bacteria, compound 3g with pyrrolidine moiety at C-7 position was also shown to be a good inhibitor of *E. coli* FabH, indicating that 3g may form a hydrophobic interaction of *E. coli* FabH. These biological assays indicated that compound 3g is a potent inhibitor of *E. coli* FabH as antibiotics.

Molecular docking of compound 3g and *E. coli* FabH was performed on the binding model based on the *E. coli* FabH–CoA complex structure (1HNJ.pdb). The 3D structure of *E. coli* FabH is shown in Figure 2. The FabH active site generally contains a catalytic triad tunnel consisting of Cys–His–Asn, which is conserved in various bacteria. This catalytic triad plays an important role in the regulation of chain elongation and substrate binding. Since the alkyl chain of CoA is broken by Cys of the catalytic triad of FabH, interactions between Cys and substrate appear to play an important role in substrate binding. Qiu et al. have refined three-dimensional structure of *E. coli* FabH in the presence and absence of malonyl-CoA by X-ray spectroscopy. Since malonyl moiety is degraded by *E. coli* FabH, molecular docking studies for FabH and malonyl-CoA was carried out to identify a plausible malonyl-binding mode. They found that in one of the binding modes appeared in the lower scored conformations, the malonyl carboxylate formed hydrogen bonds to the backbone nitrogen of Phe304. Enlightened by these facts, compound 3g with the most potent inhibitory activity was hit by pharmacophore map I mentioned above. The binding model of compound 3g and *E. coli* FabH is depicted in Figure 3. In the binding model, amino hydrogen of Asn247 forms hydrogen bond with 5-hydroxy of compound 3g. Pyrrolidine moiety at C-7 position may form a hydrophobic interaction with Asn274, Ile196, Phe157, and Met207 of *E. coli* FabH kinase.

### Table 1

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Gram-negative (μg/mL)</th>
<th>Gram-positive (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>E. coli</em></td>
<td><em>P. fluorescence</em></td>
</tr>
<tr>
<td>2a</td>
<td>12.5</td>
<td>6.25</td>
</tr>
<tr>
<td>2b</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>2c</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>2d</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>2e</td>
<td>12.5</td>
<td>25</td>
</tr>
<tr>
<td>2f</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>2g</td>
<td>6.25</td>
<td>12.5</td>
</tr>
<tr>
<td>2h</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>2l</td>
<td>12.5</td>
<td>25</td>
</tr>
<tr>
<td>3a</td>
<td>3.13</td>
<td>12.5</td>
</tr>
<tr>
<td>3b</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>3c</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>3d</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>3e</td>
<td>6.25</td>
<td>6.25</td>
</tr>
<tr>
<td>3f</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>3g</td>
<td>1.56</td>
<td>6.25</td>
</tr>
<tr>
<td>3h</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>3i</td>
<td>6.25</td>
<td>12.5</td>
</tr>
<tr>
<td>Kanamycin B</td>
<td>3.13</td>
<td>3.13</td>
</tr>
<tr>
<td>Chrysin</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th><em>E. coli</em> FabH (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td></td>
<td>11.2</td>
</tr>
<tr>
<td>2b</td>
<td>–(CH2CH3)3</td>
<td>70</td>
</tr>
<tr>
<td>2c</td>
<td>–NHCH2CH2CH3</td>
<td>54</td>
</tr>
<tr>
<td>3a</td>
<td></td>
<td>6.6</td>
</tr>
<tr>
<td>3e</td>
<td>Me</td>
<td>5.2</td>
</tr>
<tr>
<td>3g</td>
<td></td>
<td>3.1</td>
</tr>
</tbody>
</table>

Molecular docking of compound 3g and *E. coli* FabH was performed on the binding model based on the *E. coli* FabH–CoA complex structure (1HNJ.pdb). The 3D structure of *E. coli* FabH is shown in Figure 2. The FabH active site generally contains a catalytic triad tunnel consisting of Cys–His–Asn, which is conserved in various bacteria. This catalytic triad plays an important role in the regulation of chain elongation and substrate binding. Since the alkyl chain of CoA is broken by Cys of the catalytic triad of FabH, interactions between Cys and substrate appear to play an important role in substrate binding. Qiu et al. have refined three-dimensional structure of *E. coli* FabH in the presence and absence of malonyl-CoA by X-ray spectroscopy. Since malonyl moiety is degraded by *E. coli* FabH, molecular docking studies for FabH and malonyl-CoA was carried out to identify a plausible malonyl-binding mode. They found that in one of the binding modes appeared in the lower scored conformations, the malonyl carboxylate formed hydrogen bonds to the backbone nitrogen of Phe304. Enlightened by these facts, compound 3g with the most potent inhibitory activity was hit by pharmacophore map I mentioned above. The binding model of compound 3g and *E. coli* FabH is depicted in Figure 3. In the binding model, amino hydrogen of Asn247 forms hydrogen bond with 5-hydroxy of compound 3g. Pyrrolidine moiety at C-7 position may form a hydrophobic interaction with Asn274, Ile196, Phe157, and Met207 of *E. coli* FabH kinase.
t antibacterial and subtilis. Chrysin (>98 %) was purchased from ShanXi Huike Co., Ltd, Jiangsu, China and was used without further purification. All the $^1$H NMR spectra were recorded on a Bruker DRX 500 or DPX 300 model Spectrometer in DMSO-$_d_{6}$. Chemical shifts ($\delta$) for $^1$H NMR spectra were reported in parts per million to residual solvent protons. ESI-MS spectra were recorded on a Mariner System 5304 Mass spectrometer. Elemental analyses were performed on a CHN-O-Rapid instrument and were within ±0.4% of the theoretical values. Melting points were measured on a Boetius micromelting point apparatus.

4. General method for synthesis of compounds 2a–3i

4.2.5. 5-Hydroxy-7-(2-(4-methylpiperazin-1-yl)ethoxy)-2-phenyl-4H-chromen-4-one (2d)

Mp >300 °C; $^1$H NMR (DMSO-$d_{6}$): 2.28 (s, 3H), 2.36 (br s, 8H), 3.17 (t, $J = 5.5$ Hz, 2H), 4.12 (t, $J = 5.5$ Hz, 2H), 6.18 (s, 1H), 6.52 (s, 1H), 6.66 (s, 1H), 7.60 (t, $J = 7.5$ Hz, 3H), 7.84 (d, $J = 4.5$ Hz, 2H), 12.83 (s, 1H). ESI-MS $C_{22}H_{22}N_{2}O_{4} [M+H]^+$ 381.1. Anal. Calcd for $C_{22}H_{22}N_{2}O_{4}$: C, 69.46; H, 6.36; N, 7.36. Found: C, 69.69; H, 6.26; N, 7.48.

4.2.6. 7-(2-(Dibutylamino)ethoxy)-5-hydroxy-2-phenyl-4H-chromen-4-one (2f)

Mp 290–291 °C; $^1$H NMR (DMSO-$d_{6}$): 0.92 (t, $J = 7.1$ Hz, 6H), 1.27–1.31 (m, 4H), 1.37–1.39 (m, 4H), 2.94 (m, 4H), 3.11 (br s,
2H), 4.18 (t, J = 5.5 Hz, 2H), 6.42 (d, J = 2.0 Hz, 1H), 6.62 (d, J = 2.0 Hz, 1H), 6.70 (s, 1H), 7.48 (t, J = 7.5 Hz, 3H), 7.82 (d, J = 6.0 Hz, 2H), 12.85 (s, 1H). ESI-MS C26H33NO4 [M+H]+ 423.2. Anal. Calcd for C26H33NO4: C, 70.03; H, 6.64; N, 7.10. Found: C, 70.25; H, 6.43; N, 7.31.

4.2.14. 7-(3-(Dibutylamino)propoxy)-5-hydroxy-2-phenyl-4H-chromen-4-one (3f)

Mp 188–189 °C; 1H NMR (DMSO-d6): 0.90 (t, J = 7.1 Hz, 6H), 1.30–1.34 (m, 4H), 1.52–1.57 (m, 4H), 2.08–2.09 (m, 2H), 2.87 (t, J = 7.8 Hz, 2H), 3.11 (br s, 2H), 4.20 (d, J = 5.6 Hz, 2H), 6.42 (s, 1H), 6.66 (s, 1H), 6.78 (s, 1H), 7.52 (t, J = 7.5 Hz, 3H), 7.88 (d, J = 6.0 Hz, 2H), 12.70 (s, 1H). ESI-MS C26H33NO4 [M+H]+ 423.2. Anal. Calcd for C26H33NO4: C, 73.73; H, 7.85; N, 3.31. Found: C, 73.82; H, 7.60; N, 3.18.

4.2.15. 5-Hydroxy-2-phenyl-7-(3-(piperazin-1-yl)propoxy)-4H-chromen-4-one (3g)

Mp 89–91 °C; 1H NMR (DMSO-d6): 1.68 (br s, 4H), 1.86–1.91 (m, 2H), 2.43 (br s, 4H), 2.52 (t, J = 7.2 Hz, 2H), 4.13 (t, J = 6.25 Hz, 2H), 6.38 (s, 1H), 6.48 (s, 1H), 6.64 (s, 1H), 7.52 (t, J = 7.5 Hz, 3H), 8.40 (d, J = 6.0 Hz, 2H), 12.60 (s, 1H). ESI-MS C26H33NO4 [M+H]+ 366.1. Anal. Calcd for C26H33NO4: C, 72.31; H, 6.34; N, 3.83. Found: C, 72.52; H, 6.21; N, 3.57.

4.2.16. 8-Hydroxy-6-(3-(2-hydroxyethylamino)propoxy)-3-phenylphtalalen-1(4H)-one (3i)

Mp 114–116 °C; 1H NMR (DMSO-d6): 1.42 (t, J = 7.1 Hz, 2H), 2.36 (t, J = 7.1 Hz, 2H), 2.56–2.65 (m, 4H), 3.61 (s, 1H), 4.15 (t, J = 5.6 Hz, 2H), 6.30 (s, 1H), 6.54 (s, 1H), 6.94 (s, 1H), 7.52 (t, J = 6.5 Hz, 3H), 7.88 (d, J = 6.0 Hz, 2H), 12.44 (s, 1H). ESI-MS C26H33NO4 [M+H]+ 354.1. Anal. Calcd for C26H33NO4: C, 71.37; H, 6.56; N, 3.96. Found: C, 71.34; H, 6.51; N, 3.98.

4.3. Antibacterial activity

The antibacterial activity of the synthesized compounds was tested against E. coli, P. fluorescens, B. subtilis, and S. aureus using MH medium (Mueller-Hinton medium: casein hydrolysate 17.5 g, soluble starch 1.5 g, beef extract 1000 mL). The MICs (minimum inhibitory concentrations) of the test compounds were determined by a colorimetric method using the dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl trtrazolium bromide). A stock solution of the synthesized compound (100 µg/mL) in DMSO was prepared and graded quantities of the test compounds were incorporated in specified quantity of sterilized liquid MH medium. A specified quantity of the medium containing the compound was poured into microtitration plates. Suspension of the microorganism was prepared to contain approximately 10^5 cfu/mL and applied to microtitration plates with serially diluted compounds in DMSO to be tested and incubated at 37 °C for 24 h. After the MICs were visually determined, the MICs were visually determined on each of the microtitration plates, 50 µL of PBS (phosphate buffered saline 0.01 mol/L, pH 7.4: Na2HPO4 12H2O 2.9 g, KH2PO4 0.2 g, NaCl 8.0 g, KCl 0.2 g, distilled water 1000 mL) containing 2 mg of MTT/mL was added to each well. Incubation was continued at room temperature for 4–5 h. The content of each well was removed, and 100 µL of isopropanol containing 5% 1 mol/L HCl was added to extract the dye. After 12 h of incubation at room temperature, the optical density (OD) was measured with a microplate reader at 550 nm.

4.4. E. coli FabH purification and activity assay

Native E. coli FabH protein was overexpressed in E. coli DH10B cells using the pET30 vector and purified to homogeneity in three chromatographic steps (Q-Sepharose, MonoQ, and hydroxyapatite) at 4 °C. The selenomethionine-substituted protein was expressed in E. coli BL21(DE3) cells and purified in a similar way. Harvested cells containing FabH were lysed by sonication in 20 mM Tris, pH.
7.6, 5 mM imidazole, 0.5 M NaCl and centrifuged at 20,000 rpm for 30 min. The supernatant was applied to a Ni-NTA agarose column, washed, and eluted using a 5–500 mM imidazole gradient over 20 column volumes. Eluted protein was dialyzed against 20 mM Tris, pH 7.6, 1 mM DTT, and 100 mM NaCl. Purified FabD and FabHs were concentrated up to 2 mg/mL and stored at –80 °C in 20 mM Tris, pH 7.6, 100 mM NaCl, 1 mM DTT, and 20% glycerol for enzymatic assay.

In a final 20 μL reaction, 20 mM Na2HPO4, pH 7.0, 0.5 mM DTT, 10% ice-cold TCA and resuspended with 5 μL of 0.5 M NaOH. The pellet was washed with H2O was added to 15 μL. After 1 min incubation, a 2 μL mixture of 25 μM acetyl-CoA, 0.5 mM NADH, and 0.5 mM NADPH was added for FabH reaction for 25 min. The reaction was stopped by adding 20 μL of ice-cold 50% TCA, incubating for 5 min on ice, and centrifuging to pellet the protein. The pellet was washed with 10% ice-cold TCA and resuspended with 5 μL of 0.5 M NaOH. The incorporation of the 3H signal in the final product was read by liquid scintillation. When determining the inhibition constant (IC50), inhibitors were added from a concentrated DMSO stock such that the final concentration of DMSO did not exceed 2%.

4.5. Docking simulations

Molecular docking of compound 3g into the three-dimensional X-ray structure of E. coli FabH (PDB code: 1HNI) was carried out using the AUTODOCK software package (version 4.0) as implemented through the graphical user interface AutoDockTools (ADT 1.4.6).29

Acknowledgements

The work was financed by from National Natural Science Foundation of China (Project 30772627) and China Postdoctoral Science Foundation (Project 20080441043).

References and notes