Antiviral Activity of Glycyrrhizic Acid Derivatives against SARS–Coronavirus

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Glycyrrhizin (GL) was shown to inhibit SARS-coronavirus (SARS-CoV) replication in vitro. Here the anti-SARS-CoV activity of 15 GL derivatives was tested. The introduction of 2-acetamido-β-d-glucopyranosylamine into the glycoside chain of GL resulted in 10-fold increased anti-SARS-CoV activity compared to GL. Amides of GL and conjugates of GL with two amino acid residues and a free 30-COOH function presented up to 70-fold increased activity against SARS-CoV but also increased cytotoxicity resulting in decreased selectivity index.

Introduction

The most important bioactive compounds of licorice root (Glycyrrhiza radix) are the triterpene glycoside glycyrrhizic acid (glycyrrhizin, GL) and its aglycone 18β-glycyrrhetinic acid (GLA).1 Both compounds are reported to have antitumoral, antiinflammatory, and antiviral properties. GL is active against a broad spectrum of viruses, including herpes viruses,2,3 flaviviruses,4 and human immunodeficiency virus.5 GL was already used to treat patients with hepatitis C6 and upper respiratory tract infections.7 Recent studies have shown that GL is active against a broad range of viruses,4 and human immunodeficiency virus.5 GL was already used to treat patients with hepatitis C6 and upper respiratory tract infections.7

Recently, in a large screening of more than 10 000 compounds, two derivatives of GL were found to possess anti-SARS-CoV activity.10 Elongation of the GL carbohydrate fragments has already been shown to significantly affect the bioactivity of glycosides.11 Therefore, we tested the antiviral activity of 15 derivatives of glycyrrhizin to find more potent compounds against SARS-CoV.

Chemistry

Several derivatives (1–15) of GL were used for screening of anti-SARS-CoV activity. The GL conjugate 1 of 2-acetamido-β-d-glucopyranosylamine was synthesized by the coupling reaction of GL and N-acetyl-β-d-glucopyranosylamine as described previously.12 Among the derivatives 2–8 of GL (glycopeptides) were synthesized by using N,N′-dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (HOSu) or DCC–N-hydroxybenzotriazole (HOBT) as previously reported.13–16 The selective synthesis of compounds 2 and 3 containing two residues of S-benzyl-L-cysteine or glycyl-L-leucine in the carbohydrate part of the GL molecule was carried out by the activated ester method (DCC–HOSu) using tert-butyldicyclohexylamines.

Results and Discussion

We tested the anti-SARS-CoV activity of 15 GL derivatives (Chart 1). Seven derivatives inhibited SARS-CoV replication at lower concentrations compared to GL (Table 1). The introduction of N-acetylglucosamine into the glycoside chain of GL (1) increased the anti SARS-CoV activity about 9 times compared to GL. Compound 1 inhibited SARS-CoV replication at an EC50 of 40 μM. The cytotoxic concentration inhibiting 50% cell viability (CC50) was not reached for this compound in concentrations up to 3 000 μM. The resulting selectivity index (SI) is > 75. At a concentration of 500 μM of 1, no cytopathic effect (CPE) was detectable and the immunocytochemical staining showed > 99% suppression of viral antigen expression (Supporting Information). It is known that D-glucosamine is a main component of various glycoconjugates.20 This residue is usually N-acetylated and

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Chart 1

Table 1. Effects of GL and GL Derivatives on SARS-CoV Replication in Vero Cells

<table>
<thead>
<tr>
<th>compound</th>
<th>EC50, μM</th>
<th>CC50, μM</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL</td>
<td>365 ± 12</td>
<td>&gt;24000</td>
<td>&gt;65</td>
</tr>
<tr>
<td>GLA</td>
<td>&gt;20</td>
<td>20 ± 5</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>40 ± 13</td>
<td>&gt;3000</td>
<td>&gt;75</td>
</tr>
<tr>
<td>2</td>
<td>35 ± 7</td>
<td>1462 ± 50</td>
<td>41</td>
</tr>
<tr>
<td>3</td>
<td>139 ± 20</td>
<td>215 ± 18</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
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<tr>
<td>7</td>
<td>&gt;1000</td>
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</tr>
<tr>
<td>8</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>8 ± 2</td>
<td>44 ± 6</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td>50 ± 10</td>
<td>250 ± 19</td>
<td>5</td>
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<tr>
<td>11</td>
<td>5 ± 3</td>
<td>15 ± 3</td>
<td>3</td>
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<td>12</td>
<td>16 ± 1</td>
<td>66 ± 8</td>
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<td>&gt;1000</td>
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</tr>
<tr>
<td>15</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>-</td>
</tr>
</tbody>
</table>

a Concentration of compound inhibiting cytopathic effect to 50% of untreated cells. b Concentration of compound decreasing cell viability at 50% in confluent Vero cell cultures. c Ratio of CC50 to EC50. d Results represent mean value ± SD of three independent experiments.

Coronaviruses are highly glycosylated, especially in the spike proteins (S-protein), which project from the surface of the viral envelope. The S-protein has been shown to be important for viral entry into the cells by binding to cellular receptors. We speculate that viral entry is inhibited by binding of N-acetylglycosamine to the carbohydrates of the S-proteins. The \( \beta-D \)-glucuronopyranosyl-(1\(\rightarrow\)2)-\( \beta-D \)-glucuronopyranoside analogue 9 of GL with the changed carbohydrate part, heterocyclic amides 10 and 11 of GL, and the acyl hydrizide 12 were active against SARS-CoV with an EC50 ranging from 5 μM up to 50 μM. However, these compounds presented a high cytotoxicity compared to GL and derivatives 1 and 2, resulting in low SI ranging from 2 to 5. So, the chemical modification of GL by the introduction of CONH bonds into the GL molecule might increase its anti-SARS-CoV activity as well as cytotoxicity. The elongation of the GL carbohydrate part by the introduction of N-acetyl-\( \beta-D \)-glucopyranosylamine residues is most favorable for the intensification of antiviral activity because it increases the transporting properties of saponin molecules and changes their interaction with cellular receptors. We tested the anti-SARS-CoV activity of several GL glycopeptides. The L-Cys(SBn)-containing glycopeptide 2 and the Gly-Leu containing glycopeptide 3 were active against SARS-CoV. Compound 2 was 10-fold more active against SARS-CoV.

has the \( \beta \)-configuration of the glycoside bond. We suppose that the introduction of \( N \)-acetylglucosamine residues into the carbohydrate part of the GL molecule increases its hydrophilic properties. This might be important for the interaction of GL with viral proteins.
state after deprotection by hydrogenolysis over Pd/C (10%) in 75% CH₂COOH and purification by CC (67%).

**General Procedure for Deblocking Compounds 2–4.** tert-Butyl esters 2–4 (0.5 mmol) were treated with CF₃COOH (2 mL) in CH₂Cl₂ (2 mL) at 20–22 °C, the reaction mixture was evaporated, and the residue was purified by CC. Yields of target products were 50–53%.

β-g-Glucopyranosyl-(1→2)-β-g-gluco-pyranoside 9 of GLA methyl ester was synthesized by the reduction of GA tri-methyl ester (1 mmol) in aqueous MeOH (150 mL) with NaBH₄ (2 g) at 20–22 °C for 8 h and recrystallized from EtOH as described previously:17 mp 206–207 °C; [α]D₂₀ +57° (c 0.04; MeOH), lit.:18 mp 205–207 °C; [α]D₂₀ +55° (c 0.02; AcOH).

Bis-(6,6′-diazide) 13 of GLA methyl ester was synthesized from 9 with 57% yield as described in:17 [α]D₂₀ + 42° (c 0.02; MeOH), lit.:17 [α]D₂₀ + 40° (c 0.02; dioxane).

The GL amide 10 of 6-amino-2-thio-uracil was synthesized by the coupling of GA (1 mmol) in DMF–Py (5:1, 25 mL) with 6-amino-2-thiouracil (1.5 mmol) in the presence of DCC (1.5 mmol) at 50 °C. The target product was produced in a pure state by dilution of the reaction solution with cold water after removing of N,N′-dicyclohexylurea and reprecipitation from acid acetone. The yield was 45%: [α]D₂₀ + 25° (c 0.02; dioxane). UV, λmax (dioxane) 247 nm (lg ε 4.06).

The GA amide 11 of 5-aminoaracil was synthesized by using 5-aminoaracil (4 mmol) and DCC (3.4 mmol) with 80% yield as previously published.18

GA 30-methyl ester acyl hydrazide (12) was produced by reaction of GA trimethyl ester (2.5 mmol) with aqueous NH₂NH₂ (85%, 3 mL) in MeOH (50 mL) under reflux for 4 h and recrystallized from EtOH (the yield was 77%): mp 254–256 °C; [α]D₂₀ +49° (c 0.03; dioxane).

The 3-O-Hydrogen phthalate of GLA methyl ester 14 and the 3-O-hydrogen succinate 15 of 18,19-dehydro-GLA methyl ester were synthesized as published previously.19

**Cell Culture and Virus Preparation.** Vero cells were obtained from ATCC (African green monkey kidney; ATCC CCL81, Manassas, VA). Cells were incubated at 37 °C in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL of penicillin, and 100 μg/mL of streptomycin. SARS-CoV strain FFM1 was isolated from respiratory specimens of a SARS patient, admitted to the Infectious Diseases Department of Frankfurt University Hospital, (Frankfurt, Germany), and cultivated on Vero cells.20 SARS-CoV stocks used in the experiments were stored at −80 °C. Virus titers were determined as 50% tissue culture infectious dose (TCID₅₀/mL) in confluent cells in 96-well microtiter plates.21

**Immunocytochemical Staining of Viral Antigens and Visual CPE Assay.** Vero cells were seeded in 96-well plates. For virus adsorption, confluent Vero cells were incubated with SARS-CoV strain FFM1 at MOI 0.01 for 1 h, washed with PBS, and incubated in MEM supplemented with 2% FBS and different concentrations of the tested compounds. Virus infection was assessed by visually scoring the virus-induced CPE 72 h postinfection. The EC₅₀ was determined as concentration of compound required to inhibit the CPE effect to 50% of the control value. For immunocytochemical staining, cells were fixed 72 h postinfection in 60% MeOH/40% acetone for 15 min at −20 °C. Immunocytochemical staining was performed using human immune serum obtained from a SARS patient as described previously.8

**Determination of Cytotoxicity.** We assessed the cytotoxicity of the drugs by using a 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromid (MTT) assay as described previously.22 In brief, Vero cells were seeded in 96-well plates. After cells reached confluency, medium was removed and cells were incubated in MEM supplemented with 2% FBS containing different concentrations of the compounds. After incubation for 72 h, 25 μL of MTT solution was added and cells were incubated for additional 4 h at 37 °C before adding 100 μL of sodium docecyl sulfate–dimethylformamide (SDS/DMF) solution. After incubation for 12 h, the absorbance at 620/680 nm

**Experimental Section.**

**General Procedure for the Preparation of Glycopeptides 4–8.** Glucose containing glycopepti...
was determined using a multiwell ELISA reader. CC<sub>50</sub> was recorded as the concentration of a compound that reduced the viability of cells to 50% compared to the control. The SI was calculated as the ratio of CC<sub>50</sub> to EC<sub>50</sub>.

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Supporting Information Available: Immunocytochemical staining and visual CPE-Assay of SARS-CoV infected cells treated with different concentrations of the N-acetylglycosamine derivative 1 of glycyrrhizin. This material is available free of charge via the Internet at http://pubs.acs.org.

References