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 C[6] and upper respiratory tract infections.[7] GL was one of the first compounds found to be active against SARS-coronavirus (SARS-CoV) in vitro.[8] In Vero cells GL presents an effective concentration inhibiting 50% of virus growth (EC_{50}) of 365 μM against SARS-CoV. GL has also been used for treatment of SARS in patients.[9] Recently, in a large screening of more than 10 000 agents, two derivatives of GL were found to possess anti-SARS-CoV activity.[10] Elongation of the GL carbohydrate chain or introduction of amino acids or heterocyclic 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-glycopyranosylamine as described previously.[12] Amino acid 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 *S*-benzyl-L-cysteine or glycyl-L-valine *tert*-butyl esters as hydrochlorides in tetrahydrofuran (THF) in the presence of Et_3N. Glycopeptides **4** and **5** were prepared from GL and L-leucine *tert*-butyl or L-glutamic acid dibenzyl ester hydrochlorides by using DCC−HOBt in dioxane.[13,16] *tert*-Butyl ester groups were deblocked with CF_3COOH, and benzyl ester groups were removed by catalytic hydrogenolysis over 10% Pd/C in 75% CH_3COOH.[16] The β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside **9** of GLA methyl ester was synthesized by the reduction of GL trimethyl ester in aqueous MeOH with $NaBH_4$ at 20−22 °C, and its bis-6′,6″-diazide (**13**) was formed as previously described.[17] Heterocyclic amides **10** and **11** of GL were prepared by the reaction of GL with 5-aminouracyl and 6-amino-2-thiouracyl in the presence of DCC in dimethylformamide−pyridine (DMF−Py) mixtures.[18] GL 30-methyl ester diacyl hydrizide (**12**) was produced by the reaction of GL trimethyl ester with NH_2NH_2 (85%) in MeOH under reflux with the yield of 77%. 3-*O*-Acylates of GLA (**14**) and 18,19-dehydro-GLA (**15**) methyl esters were synthesized as described before.[19] All compounds synthesized were homogeneous according to thin-layer chromatography (TLC), and their structures were confirmed by IR, UV, and ^1H and ^{13}C NMR spectra. All derivatives were dissolved in DMSO and stored at 4 °C.

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*-acetylgycosamine 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 EC_{50} of 40 μM. The cytotoxic concentration inhibiting 50% cell viability (CC_{50}) was not reached for this compound in concentrations up to 3000 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

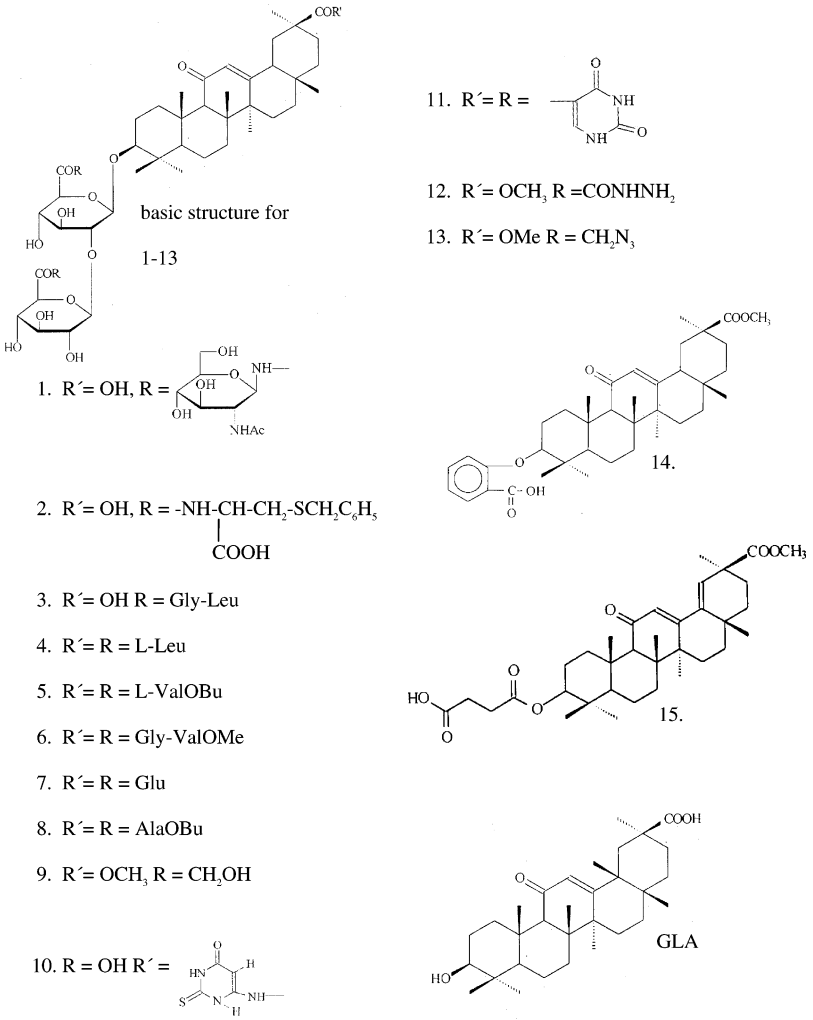

Table 1. Effects of GL and GL Derivatives on SARS-CoV Replication in Vero Cells

compound	EC_{50},[a] μM	CC_{50},[b] μM	SI[c]
GL	365 ± 12[d]	>24000	>65
GLA	>20	20 ± 5	-
1	40 ± 13	>3000	>75
2	35 ± 7	1462 ± 50	41
3	139 ± 20	215 ± 18	2
4	>1000	>1000	-
5	>1000	>1000	-
6	>1000	>1000	-
7	>1000	>1000	-
8	>1000	>1000	-
9	8 ± 2	44 ± 6	6
10	50 ± 10	250 ± 19	5
11	5 ± 3	15 ± 3	3
12	16 ± 1	66 ± 8	4
13	>1000	>1000	-
14	>1000	>1000	-
15	>1000	>1000	-

[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 CC_{50} to EC_{50}. [d] Results represent mean value ± SD of three independent experiments.

has the β-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. Coronaviruses are highly glycosylated, especially in the spike proteins (S-protein),[21] 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.[22] We speculate that viral entry is inhibited by binding of *N*-acetylglycosamine to the carbohydrates of the S-proteins. The β-D-glucuropyranosyl-(1→2)−β-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 EC_{50} 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-β-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.[12] 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

(EC_{50} 35 μM) as compared to GL, and the CC_{50} was 1462 μM, resulting in a SI of 41. The Gly-Leu-containing glycopeptide **3** presented an EC_{50} of 139 μM and a CC_{50} of 215 μM resulting in a SI of 2. In contrast, glycopeptides **4**−**8** did not present activity against SARS-CoV in concentrations up to 1 mM. A common feature of compounds **2** and **3** is the presence of a free carboxylic group at C30, while inactive glycopeptides **4**−**8** contain three amino acid or amino acids alkyl ester residues. This shows that the intact 30-COOH function of GL seems to be important for anti SARS-CoV activity of GL glycopeptides. After administration, GL is partly hydrolyzed to its aglycone, GLA, by glucuronidase.[23] In compounds where the two units of glucuronic acid were replaced with functional groups (**14**, **15**), no anti-SARS-CoV activity was observed. This indicates that the sugar moiety is essential for anti SARS-CoV activity. The importance of the glucuronic acid units for biologic activity of GL has also been shown for the antiinflammatory and thrombin inhibiting activities.[24,25] Taken together, our data demonstrates that modification of GL may lead to novel anti-SARS-CoV drugs with increased activity.

Experimental Section

TLC was carried out on Silufol plates (Silufol, Czech Republic) and Kieselgel F-60 plates (Merck, Germany). Spots of substances were detected by spraying with a 20% solution of phosphotungstic acid in ethanol and subsequent heating at 110−120 °C for 2−3 min. Column chromatography (CC) was carried out on silica gel L (40/100 μm) (Silufol, Czech Republic) by using $CHCl_3$−MeOH−H_2O mixtures 200:10:1, 100:10:1, 50:10:1 (a stepwise gradient, v/v). IR spectra were recorded on a Specord M 80 instrument; UV spectra were recorded on Specord M-40 spectrometers. ^{1}H and ^{13}C NMR spectra were measured on a Bruker AM-300 spectrometer with the working frequency of 300 and 75.5 MHz, respectively. Tetramethylsilane was used as an internal standard. Specific rotations were measured on a Perkin-Elmer 241 MC polarimeter in MeOH. Melting points were determined on a Boetius device.

All solvents were glass distilled and evaporated in a vacuum at 40−50 °C. L-Amino acid *tert*-butyl esters (Reanal, Hungary), *N*,*N*′-dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, *N*-hydroxytriazole, $NaBH_4$, NaN_3 (Aldrich, Germany), and glycyrrhizic acid (95 ± 2%) were used for synthesis.[26] The methyl ester of glycyl-L-leucine hydrochloride and *n*-butyl esters of l-alanine and L-valine hydrochlorides were prepared as previously published.[27] L-Glutamic acid dibenzyl ester hydrochloride was produced as described previously.[16]

The synthesis of the conjugate **1** of GA with 2-acetamido-2-deoxy-β-D-glucopyranosylamine was published previously:[12] $[\alpha]^{20}_D$ +80° (*c* 0.04; MeOH).

General Procedure for the Preparation of Glycopeptides 2 and 3. HOSu (4 mmol) and DCC (2.5 mmol) were added to a solution of GL (1 mmol) in THF (20 mL) and cooled to 0 °C. The reaction mixture was stirred for 2 h, and precipitated *N*,*N*′-dicyclohexylurea was filtered off. Then amino acid *tert*-butyl ester hydrochloride (2.5−3 mmol) and Et_3N (4−5 mmol) were added to the filtrate, and the reaction mixture was kept 1 h at 0 °C and 18−20 h at 20−22 °C, diluted with cold water, and acidified with citric acid to pH ∼ 4. The precipitate was filtered, washed with cold water, and dried. The resulting products were isolated in a homogeneous state by CC.

Glycopeptides **4**−**8** of GL were prepared by using DCC−HOSu or DCC−HOBt methods as described before.[13,14,16] In brief, 3−4 mmol of L-leucine *tert*-butyl ester, L-alanine or L-valine *n*-butyl esters, glycyl-L-valine methyl ester, and L-glutamic acid dibenzyl ester hydrochlorides were used as amino components. Glycopeptide **5** was obtained in a pure state after deprotection by hydrogenolysis over Pd/C (10%) in 75% CH_3COOH and purification by CC (67%).

General Procedure for Deblocking Compounds 2−4. *tert*-Butyl esters **2**−**4** (0.5 mmol) were treated with CF_3COOH (2 mL) in CH_2Cl_2 (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-D-Glucopyranosyl-(1→2)-β-D-glucopyranoside 9 of GLA methyl ester was synthesized by the reduction of GA trimethyl ester (1 mmol) in aqueous MeOH (150 mL) with $NaBH_4$ (2 g) at 20−22 °C for 8 h and recrystallized from EtOH as described previously:[17] mp 206−207 °C; $[\alpha]^{20}_D$ +57° (c 0.04; MeOH), lit.:[18] mp 205−207 °C; $[\alpha]^{20}_D$ +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] $[\alpha]^{20}_D$ +42 ° (*c* 0.02; MeOH), lit.:[17] $[\alpha]^{20}_D$ + 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 reprecipitated from acid acetone. The yield was 45%: $[\alpha]^{20}_D$ +25 ° (*c* 0.02; dioxane). UV, λ_{max} (dioxane) 247 nm (lg ϵ 4.06).

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

GA 30-methyl ester acyl hydrizide (12) was produced by reaction of GA trimethyl ester (2.5 mmol) with aqueous NH_2NH_2 (85%, 3 mL) in MeOH (50 mL) under reflux for 4 h and recrystallized from EtOH (the yield was 77%): mp 254−256 °C; $[\alpha]^{20}_D$ +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.[28] SARS-CoV stocks used in the experiments were stored at −80 °C. Virus titers were determined as 50% tissue culture infectious dose ($TCID_{50}$/mL) in confluent cells in 96-well microtiter plates.[28]

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 EC_{50} 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-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromid (MTT) assay as described previously.[29] 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 dodecyl sulfate−dimethylformamide (SDS/DMF) solution. After incubation for 12 h, the absorbance at 620/690 nm

was determined using a multiwell ELISA reader. CC_{50} 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_{50} to EC_{50}.

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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.

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