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Glycyrrhizic acid derivatives as Dengue virus inhibitors

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ABSTRACT

Dengue virus (DENV) is one of the most geographically distributed pathogenic flaviviruses transmitted by mosquitoes Aedes sps. In this study, the structure-antiviral activity relationships of Glycyrrhizic acid (GL) derivatives was evaluated by the inhibitory assays on the cytopathic effect (CPE) and viral infectivity of DENV type 2 (DENV2) in Vero E6 cells. GL (96% purity) had a low cytotoxicity to Vero E6 cells, inhibited DENV2-induced CPE, and reduced the DENV-2 infectivity with the IC50 of 8.1 μM. Conjugation of GL with amino acids or their methyl esters and the introduction of aromatic acylhydrazide residues into the carbohydrate part strongly influenced on the antiviral activity. Among compounds tested GL conjugates with isocyanic 13 and 11-amino-decanic acid 17 were found as potent anti-DENV2 inhibitors (IC50 1.2–1.3 μM). Therefore, modification of GL is a perspective way in the search of new antivirals against DENV2 infection.
GL inhibits a number of DNA and RNA viruses (Vaccinia, New Castle, Vesicular stomatitis, varicella-zoster, Herpes simplex type 1, Herpes B), influenza viruses, cytomegaloviruses, hepatitis B and C. GL is used clinically for the treatment of chronic viral hepatitis B and C in Japan and China (SNMC preparations, Compound Glycyrrhizin Ingecpion Injection, Compound Glycyrrhizin Ingecpion Tablets). GL inhibits the reproduction of HIV-1 in the culture of MT-4 cells and is suggested for long-term therapy for HIV-infected patients in combination with other anti-HIV drugs. GL also affects other cellular factors, such as protein kinase II, casein kinase II and transcription factors (activating protein I and nuclear factor kB).

Chemical modification of GL is a promising way of obtaining new immune modulators and antiviral agents. Early we synthesized a series of amidic acid and dipeptide conjugates, which exceed the natural glycoside by anti-HIV activity. GL and its derivatives constitute the first group of substances that inhibit the Marburg virus, which causes acute hemorrhagic fever with a high level of lethality. The inhibitory effect has been found for GL derivatives in vitro against SARS-associated coronaviruses. Among GL derivatives inhibitors of the Epstein-Barr virus were found. Most recently, we detected inhibitory effects of GL derivatives in vitro against influenza A/H1N1 virus.

GL is also of interest as a scaffold for the preparation of potential inhibitors of pathogenic flaviviruses like DENV, since it is reported about the GL ability to inhibit DENV and yellow fever viruses at high non-toxic concentrations. The antiviral activity of GL and its derivatives is associated with the ability to potentiate Y-interferon production in vitro and in vivo. The stimulating effect of GL was noted on the secretion of IL-2, inducing the production of interferon by peripheral lymphocytes. The antiviral activity of GL derivatives against DENV was not studied earlier.

In this study, we report firstly about the antiviral activity of GL derivatives (2–21) (Fig. 1) to be conjugates with l- and d-amino acids and their methyl esters and aromatic acylhydrazides against DENV2 and the structure-antiviral activity regularities for those compounds. GL derivatives used in the study were divided into 3 groups. Group I (compounds 3–15) are GL conjugates containing three free amino acids or their methyl esters residues. Group II (compounds 16, 17) is presented by GL conjugates containing amino components in the carbohydrate part of glycose. Group III includes acyl hydrazide (18–21) with C30-methyl ester at the R position and residues at the R′ position in GL derivatives.

GL was produced from its mono ammonium salt according to Refs. and had a purity 96.0 ± 1.0% (HPLC). Trimethyl ester (2) was produced from GL by using diazomethane. GL conjugates with amino acids esters (3–12) were prepared by using N-hydroxybenzotriazole (HOBT), N,N′-dicyclohexylcarbodiimide (DCC) or 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (DECA) as we described previously. GL conjugates (10–12), containing α-amino acids methyl esters, were synthesized according to Ref. Conjugates (13–14) with free amino acids according to Refs. Compounds (16, 17) containing the residues of aspartic and 11-amino undecanoc acid methyl esters in the carbohydrate part of GL were produced by using N-hydroxysuccinimide (HOSu) and DCC. The analytical and spectral data of GL derivatives 2–17 were identical to the literature data, the purity of compounds was monitored by thin layer chromatography and high pressure liquid chromatography (HPLC) (%) of purity) (in the Supplementary Material, Supplemental Figs. 1-3).

To expand the library of GL derivatives for testing GL derivatives 18–21 were produced by the modification of GL with the introduction of aromatic acyl hydrazides residues in the carbohydrate part. General experimental details, analytical and spectral data (IR and C) for new compounds (18–21) are given in the Supplementary data. Bis-hydrazide of GL was obtained from GL trimethyl ester 2 by reflux with hydrazine hydrate in methanol with 77% yield. The ester methyl group in the aglycone of compound 18 is retained, it was confirmed by the presence of C30 and OH shifts at 177.1 and 51.9 ppm in the C NMR spectrum. Aromatic acyl-hydrazides (19–21) were produced by the reaction of 18 with aromatic aldehydes in ethanol at reflux for 3 h and target compounds were isolated by column chromatography (CC) on silica gel (SG) with 72–74% yields. The structures of compounds 18–21 were confirmed by IR and C NMR spectrum. In the C NMR spectrum of compounds 18–21 the carbon atoms of the new CH=N bonds are detected at 146.2–148.6 ppm, and a set of signals of aromatic carbon atoms appears in the weak field region (131.9–115.5 ppm), C NMR spectrum of bis-(pyridine-4-carbal)-hydrazide 21 contains shifts of carbon atoms of pyridine residues in the weak field region (142.7–121.4 ppm). The elemental analysis of all compounds corresponded to the calculated data (see the Supplementary material).

The primary screening of cytotoxicity and antiviral activity of GL and a number of its derivatives (compounds 3–21) was performed in vitro against DENV2 (the strain 16681). Cytotoxicity and antiviral activity were investigated in Vero E6 cell culture in supporting Dulbecco modified medium (DMEM). An MTT cytotoxicity test for each compound was performed in a Vero E6 cell culture in 96-well plates, as described in our previous report. The cytotoxic (50%) concentration (CC50) was calculated according to cell survival rate of treated cells compared to mock cells using a computer program (John Spouge, NCBI, NIH). GL (96% purity) was used in the study of antiviral activity as a reference sample. It was established that for GL and its derivatives, the CC50 exceeded 100 μM (Table 1).

To determine the antiviral activity, a screening study of the cytopathic effect (CPE) reduction was initially conducted in Vero E6 cells infected with the DENV2 virus (MOI of 0.01) in the presence of 10 μM of the test compound. After 96 h of incubation, infected cells were photographed using reverse phase contrast microscopy. Microscopic photographs showed that GL and some of its derivatives at a concentration of 10 μM significantly reduce CPE in cells infected with DENV2. GL had low cytotoxicity to Vero E6 cells, concentration-dependently inhibited CPE and NS4B positive cells (70.5% inhibition).
The infectivity of virus was determined as quantitative analysis of the inhibitory effect of GL and its active derivatives dose-dependently in DENV2-induced CPE and infectivity was studied in a concentration-dependent manner (Table 1) proved the significant CPE reduction in the infected cells, post DENV-2 infection. The most active GL conjugates 6, 13, 15, and 17 (Table 1) showed the highest inhibitory activity on DENV2-induced CPE at the concentration of 10 μM (Fig. 2).

To assess the values of 50% inhibitory concentration (IC50), a quantitative analysis of the inhibitory effect of GL and its active derivatives on the infectivity of DENV-2 was carried out in vitro by the immunofluorescence method. The infectivity of virus was determined as the ratio of NS4B-positive cells to the total number of cells stained with DAPI (4',6-diamidino-2-phenylindole). This analysis has demonstrated that GL and its active derivatives (6, 13, 15, and 17) reduced the number of infected DENV-2 cells (viral NS4B-positive cells) in a dose-dependent form. For example, the rates of NS4B-positive cells were 26.9%, 37.2%, 29.4%, and 1.6% in 0.1, 1 and 10 μM compound 13-treated infected cells, respectively (Fig. 3). Meanwhile, the inhibitory rate on DENV-2 was 20.2%, and 95.7% by 1 and 10 μM compound 13, respectively. In addition, human lung epithelial A549 cells were used to examine the antiviral activity of GL derivative 13 against DENV2 (Supplemental Fig. 4), in which GL derivative 13 exhibited the potent anti-DENV2 activity in a cell-type independent manner. GL derivatives 13 and 15, containing free amino acids residues (Ile-OH) and (Tyr-OH), and 17 with two long chain amino acid fragments, were found as highly active against anti-DENV2 (IC50 values of 1.3–2.2 μM), as significantly more potent than GL (IC50 of 8.1 μM) (Table 1). In addition, compounds 13 and 17 exhibited the higher therapeutic index (TI, TI = CC50/IC50) than 70, which was 6-fold greater than that of GL. Tyrosine containing conjugate 15 was less active than 13 and 17.

Among group I GL derivatives, conjugates 3–5 and 7–12 with amino acid methyl esters at the R and R’ positions were less active than GL against DENV2. Only conjugate 6 containing Met-OMe fragments showed a significant inhibition on the in vitro replication of DENV2 and its IC50 (4.5 μM) was less than for GL. The anti-DENV activity of conjugate 6 could be linked with the introduction of Met-OMe at the R position of triterpene part, in which an S-methyl thioether side chain of methionine might form NH–S H-bonds with the residues of DENV2 target protein. Conjugates 13 and 15 containing Ile-OH and Tyr-OH at the R and R’ positions refined anti-DENV activity (IC50 of 1.3–2.2 μM), but conjugate 14 with Phe-OH dropped the anti-DENV activity. The finding inferred that the –OH group of tyrosine plays the important role in donating or accepting a hydrogen bond with the residues of viral target protein. A weak inhibitory activity on CPE test and NS4B-positive cells was found for compound 16 with a free triterpene COOH group and Asp(OMe)-OMe in the carbohydrate portion of GL. However, compound 17 containing 11-amino undecanoic acid methyl ester exhibited the potent efficacy on inhibiting the DENV2 replication (IC50 of 1.2 μM). Those results indicated that 11-amino undecanoic acid methyl ester at the R’ position in the carbohydrate part of GL had a more effective interaction with the antiviral target(s) than conjugate 16. GL derivatives of group III (18–21) lost the antiviral activity against DENV2. A weak inhibition (32.5%) of NS4B in DENV2-infected cells was found for compound 19 containing aromatic hydroxyl group. Therefore, the structure–antiviral activity study indicated that GL conjugates with three free amino acids (compounds 13, 15) and long chain amino acid residue in the carbohydrate part (compound 15) exhibited higher anti-DENV activity compared to group III derivatives. Thus, the presence of hydroxyl group at the R position of triterpene part or free carboxyl groups of amino acids are important for interacting with antiviral target(s). Meanwhile, GL derivatives 13, 15, and 17 are compounds with the significantly improved antiviral activity against DENV2 infection, suggesting that isoleucine, tyrosine, or 11-amino undecanoic acid methyl ester at the R’ position of the carbohydrate part also interconnected with the other part of active side of antiviral target(s).

We concluded that this study was the first report to display structure-antiviral activity relationships of GL derivatives, revealing that the modification of GL by the conjugation with amino acids and the introduction of aromatic acyl hydrazide residues into the carbohydrate part strongly influenced on the antiviral activity of GL against DENV2. Among GL derivatives tested, GL compounds 1325 and 1725 have been identified as potent DENV2 inhibitors. Thus, the conjugation of GL derivatives with amino acids is a perspective way to produce potent DENV2 inhibitors. The second way to potentiate GL activity is its modification with the introducing of long chain amino acids in the

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**Table 1**

Antiviral activity of GL derivatives against DENV2 in Vero E6 cells.

| Compounds | CPE (IC50/μM) | Inhibition of NS4B-positive cells (%) | Cytotoxicity to Vero cells (CC50, μM) | Reduction in virus-infected cells (IC50, μM) | TI (CC50/IC50) |
|-----------|---------------|-------------------------------------|-------------------------------------|-------------------------------------|----------------|
| 1         | + +           | 70.5                                | 100                                 | 8.1                                 | 12.3           |
| 3         | – –           | ND                                  | ND                                  | ND                                  | ND             |
| 4         | – –           | ND                                  | ND                                  | ND                                  | ND             |
| 5         | – –           | ND                                  | ND                                  | ND                                  | ND             |
| 6         | + +           | 66.3                                | 100                                 | 4.5                                 | 22.2           |
| 7         | – –           | ND                                  | ND                                  | ND                                  | ND             |
| 8         | – –           | ND                                  | ND                                  | ND                                  | ND             |
| 9         | +            | 53.1                                | ND                                  | ND                                  | ND             |
| 10        | – –           | ND                                  | ND                                  | ND                                  | ND             |
| 11        | – –           | ND                                  | ND                                  | ND                                  | ND             |
| 12        | – –           | ND                                  | ND                                  | ND                                  | ND             |
| 13        | + + +         | 95.7                                | 100                                 | 1.3                                 | 76.3           |
| 14        | – –           | ND                                  | ND                                  | ND                                  | ND             |
| 15        | + +           | 68.5                                | 100                                 | 2.2                                 | 46.5           |
| 16        | +            | 35.8                                | ND                                  | ND                                  | ND             |
| 17        | + + +         | 79.5                                | 100                                 | 1.2                                 | 80             |
| 18        | – –           | ND                                  | ND                                  | ND                                  | ND             |
| 19        | +            | 32.5                                | ND                                  | ND                                  | ND             |
| 20        | – –           | ND                                  | ND                                  | ND                                  | ND             |
| 21        | – –           | ND                                  | ND                                  | ND                                  | ND             |

ND – not detected.

*a Inhibitory rate = (1 – NS4B positive percentage in treated infected cells/NS4B positive percentage in mock-treated infected cells) * 100.

*b CC50, 50% cytotoxicity concentration determined by the survival rate of treated cells at different concentrations of each compound.

*c IC50, 50% inhibitory concentration calculated according to NS4B positive percentage in the presence of different concentrations of each compound.

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**Fig. 2.** Inhibition of DENV2-induced cytopathic effects by GL conjugate with isoleucine (Compound 13). Vero E6 cells were infected with DENV2 at a MOI of 0.01 and immediately treated with the indicated concentrations of compound 13. Images of DENV2-induced cytopathic effect were photographed 96 h post infection by phase-contrast microscopy.
carbohydrate part of the molecule keeping triterpene COOH group free. Highly active GL derivatives will be the subject of extensive research on antiviral activity and the study of the mechanisms of antiviral action against DENV2 by using docking studies and their interaction with DENV2 targets like NS2B-NS3 protease, NS3 helicase, and NS5 RNA-dependent RNA polymerase.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2019.126645.

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