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Glycyrrhizic acid derivatives as influenza A/H1N1 virus inhibitors

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A R T I C L E   I N F O
Article history:
Received 24 November 2014
Revised 24 February 2015
Accepted 26 February 2015
Available online 7 March 2015

Keywords:
Glycyrrhizic acid
Derivatives
Analogs
Influenza A/H1N1 virus
Antivirals

A B S T R A C T
This Letter describes the synthesis and antiviral activity study of some glycyrrhizic acid (GL) derivatives against influenza A/H1N1/pdm09 virus in MDCK cells. Conjugation of GL with l-amino acids or their methyl esters, and amino sugar (D-galactose amine) dramatically changed its activity. The most active compounds were GL conjugates with aromatic amino acids methyl esters (phenylalanine and tyrosine) (SI = 61 and 38), and S-benzyl-cysteine (SI = 71). Thus modification of GL is a perspective route in the search of new antivirals, and some of GL derivatives are potent as anti-influenza A/H1N1 agents.

The search for new antiviral agents is one of the most important tasks of chemistry and medicine because of wide spread of socially dangerous viral infections such as HIV, hepatitis B and C, and the emergence of new viral infections ('avian flu' and 'swine flu', influenza A/H1N1, Ebola fever etc.). The pandemic of influenza A/H1N1 (Spanish flu) in 1918–1919 claimed 40 million lives, Asian influenza virus A/H2N2 in 1957 has led to death of 4 million persons, and 2 million people have died in Hong Kong in 1968 by the pandemic influenza A (H3N2). The appearance of novel strain of influenza A in April 2009 ('swine flu') caused 162,380 cases and 1154 deaths in 168 countries, and the World Health Organization (WHO) declared the pandemic influenza A (H1N1). The search for new antiviral drugs of adamantane structure—Amantadine and Rimantadine, and the neuraminidase inhibitors—Zanamivir/Relenza (GlaxoWellcome/Biota) and Oseltamivir/Tamiflu (Hoffman-La-Roche/Gilead) are used in medicine for the treatment of influenza, but both groups of compounds have drawbacks and are characterized by a viral resistance appearance. Numerous chemical compounds, which did not reach clinical trials belong to the different chemical classes exhibiting different levels of anti-influenza activity in vitro and in vivo, and are directed at different targets in the viral life cycle. At present any research studies targeting the search of new antivirals against influenza A virus are of high priority in drug development, and are oriented to the search of new compounds or modification of already known compounds-leaders with the proved antiviral activity.

One of the modern and innovative approaches in the search of new antivirals is an application of available natural compounds or plant metabolites with a new mechanism of antiviral activity. Natural compounds and their derivatives are promising new candidates for the treatment of viral, bacterial and fungal infections. Glycyrrhizic acid (GL) (1), a major triterpene glycoside isolated from Glycyrrhiza glabra L. (licorice) and Gl. uralensis Fisher roots, is the leading natural glycoside and promising scaffold for creation of new antiviral agents. To date GL is a principal plant derived metabolite suitable for the long-term treatment of HIV infection as it does not lead to emergence of drug resistance. Preparation SNMC (Stronger neo-Minophagen Co.) containing GL was used for a long term chemotherapy of viral hepatitis B and C. GL is attractive due its ability to stimulate γ-interferon production in vitro and in vivo, and low toxicity (LD50 5000 mg/kg). But GL is active as an antiviral agent in vivo in high doses and causes some side effects connecting with its structural similarity to corticosteroids, it may influence on a water–salt interchange, intensify of Na+-content retention and reduce K+-content in some patients. We reported previously that biological activity of GL could be improved by its chemical modifications and some semisynthetic GL derivatives were found as potent immune modulators and anti-viral agents. Among GL derivatives new inhibitors of
SARS-associated coronavirus,29 Epstein–Barr virus,30 and anti-HIV agents31,32 were found. Structure–activity relationships study of GL derivatives as influenza A/H1N1 virus inhibitors was not carried out still. This Letter is devoted to the synthesis and anti-viral activity evaluation of some GL derivatives and analogs against influenza A/H1N1/pdm09 virus in vitro.

GL 1 was isolated and purified from Gl uralensis Fisher roots collected in Siberia as was described previously.33 GL conjugates (2–4) were synthesized by condensation of glycoside 1 with l-amino acids methyl esters at room temperature (22–25°C) (rt) by using N-hydroxybenzotriazole (HOBt) and 1-((3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (DEC) in the molar ratio 1/3/5/5/3.5. Yields of target compounds were 90–92%. Analytical and spectral data of compounds 2–4 were similar to those received previously by using HOBt-N,N'-dicyclohexylcarbodiimide (DCC).35

GL conjugates (5–9) with free 30-COOH were prepared by the activated esters method by using N-hydroxsucinimide (HOSu)—DCC and 2.0–2.5 molar ratio of reagents and at 0–5°C as was reported previously.13,36 Target compounds were isolated by column chromatography (CC) on silica gel (SL) with 55–60% yields. Structures of new compounds 5–7 were confirmed by IR and NMR 13C data. There are signals of free 30-COOH at 180.3–180.8 ppm as in the 13C NMR GA.37 Analytical and spectral data for new compounds 5–7 are given in the References and notes.38 Analytical and spectral data for compounds 8 and 9 were similar to those synthesized before.23,31 Compounds (11) and (13) containing free amino acids have been synthesized previously by using HOSu—DCC and S-benzyl(Bn)-cysteine- or N-carbobenzoxy(Z)-lysine tert-butyl esters hydrochlorides.39,40 tert-Butyl ester groups of protected conjugates 10 and 12 were deleted with 50% CF3COOH in DCM and pure compounds 11 and 13 were isolated by CC on SL. GL conjugate with p-galactose amine (14) connected to the carbohydrate part of glycoside by CONH-bonds was synthesized as described before.41

Stereo isomeric 18x-GL (15) was produced by the alkaline isomerization of GL according to method.42 Its derivatives (16, 17) to be conjugates with methyl esters of l-aspartic acid were described previously.25 GL analog (18) was prepared by reduction of GL trimethyl ester with NaBH4 according to method.43 Glycoside (19) with reduced C11=O group was synthesized by reduction of GA with NaBH4 in 2-PrOH as described before.44

GL 1 and its derivatives and analogs 2–19 were evaluated for their inhibitory activity against pandemic influenza A/H1N1/pdm09 virus in the MDCK cells.45 Cytotoxicity of compounds was evaluated in MDCK cells by a cell viability assay.46 Number of viable cells was evaluated by a microtiterazolium test (MTT)47 and CTD50 (compound concentration required to reduce 50% cell viability) value was estimated for each compound.

Anti-viral activity of compounds was evaluated by quantification virus yield using the end-point dilution method.48 Rimantadine was used as a reference compound. Assay details are given in the References and notes.49 Anti-viral activity of the test compounds was evaluated by their ability to decrease the virus titer and 50% of the effective dose (ED50) (concentration of compound that decreases the virus production two-fold comparing to control) was calculated. Selectivity index (SI) was calculated as relation of CTD50 to ED50. The compounds having SI > 10 were considered active.

The results of experiments are shown in Table 1. As can be seen from the data presented, GL (95 ± 1% of purity) possessed low cytotoxicity (high value of CTD50) and did not show anti-viral activity against pandemic influenza A/H1N1 virus (SI = 1). Conjugates of GL containing three residues of amino acids methyl esters 2–4 were more active. The most active among them was compound 2 with three residues of phenylalanine methyl ester (SI = 28), compound 3 containing tyrosine methyl ester residues was less active (SI = 18). Modification of glycoside part of GL by introduction of amino acids methyl esters residues changed both cytotoxicity and anti-viral activity of GL derivatives. The most active GL conjugates with free C-30 COOH were compounds 5 (EC50 = 4.3 μM,
SI = 61) and 6 (EC$_{50}$ = 6.8 μM, SI = 38). Introduction of phenylalanine or tyrosine methyl ester residues into the carbohydrate part of GL almost did not change toxicity but potentiated anti-viral activity in 61 times for conjugate 5 and 38 times for compound 6. Conjugation of GL with S-benzyl-cysteine (compound 11) led to increase in anti-viral activity in 71 times (EC$_{50}$ = 3.5 μM, SI = 71). Compounds 5, 6 and 11 had more wide SI values than Rimantadine (in 12.2, 7.8, and 14.2 times, respectively).

Conjugation of GL with amino acids containing two COOH groups such as aspartic acid dimethyl ester led to the sharp increase of cytotoxicity, and does not confer virus-inhibiting property (compound 9). Modification of glycoside with a long chained diamine acid like L-lysine just slightly increased antiviral activity (compound 13). Introduction of heterocyclic amino acid as histidine (compound 8) almost did not influence to cytotoxicity but potentiated anti-viral activity as compared GL in 15 times.

Conjugation of GL with D-galactose amine (compound 14) led to significant increase of virus-inhibiting activity (SI = 36).

Stereo isomeric 18α-GL 15 was in 7.7 times more toxic to cells (CD$_{50}$ = 47.5) than natural glycoside 1 (18β), and had a week anti-viral activity. Its derivatives 16 and 17 to be aspartic acid methyl esters conjugates were more cytotoxic and less active than the similar derivative of GL 9.

Change of the carbohydrate part of GL to β-soforoside does not change the cytotoxicity of glycoside but reduces the EC$_{50}$ in ~5 times and just rises the anti-viral activity. GL analog 19 missing C$^{11}$=O group and containing 9(11),12(13)-diene system in the triterpene part was more toxic (in 17 times) for MDCK cells than natural glycoside, and had in 174 times less value of EC$_{50}$. But its anti-viral activity was moderate (SI = 10).

Thus according to our structure–activity study GL had no significant anti-viral activity against influenza A/H1N1/pdm09 virus, and two stereoisomers 1 and 15 differed substantially in their toxicity. Conjugation of GL with amino acids or their methyl esters, and amino sugar dramatically changed its activity. The most active compounds are conjugates of GL 5, 6, 11 and 14. Introduction of cysteine or phenylalanine moieties into the carbohydrate part of GA appeared to be the most efficient in terms of anti-viral activity in relation to influenza A/H1N1/pdm09 virus. This result is corresponding to our previous data concerning anti-viral activity of GL derivatives against SARS-CoV. Presence of free 30-COOH group is important for anti-viral activity of GA conjugates with amino acids. Previously, Utsunomiya et al. have demonstrated that due to GL ability to induce interferon-γ, it exerts strong protective activity on the model of lethal influenza infection in white mouse infected with as high virus dose as 10 LD$_{50}$. In influenza-infected human macrophages, its application resulted in dramatic decrease of production of pro-inflammatory cytokines. Its mechanism of activity

| Compounds | CD$_{50}$ (μM) | EC$_{50}$ (μM) | SI |
|-----------|---------------|---------------|----|
| 1         | 364.6         | 364.6         | 1  |
| 2         | 133.2         | 4.8           | 28 |
| 3         | 159.5         | 8.7           | 18 |
| 4         | 16.4          | 2.6           | 6  |
| 5         | 262           | 4.3           | 61 |
| 6         | 254.8         | 6.8           | 38 |
| 7         | 29.1          | 5.7           | 5  |
| 8         | 298.1         | 20.4          | 15 |
| 9         | 37.3          | 26.6          | 1  |
| 11        | 248.1         | 3.5           | 71 |
| 13        | 154.7         | 54.6          | 3  |
| 14        | 259.4         | 7.2           | 36 |
| 15        | 47.5          | 15.2          | 3  |
| 16        | 38.0          | 9.9           | 4  |
| 17        | 58.3          | 4.7           | 12 |
| 18        | 370.8         | 68.1          | 5  |
| 19        | 21.4          | 2.1           | 10 |
| Rimantadine | 334.0       | 66.7          | 5  |

* The values of EC$_{50}$ and CD$_{50}$ are mean of three different experiments, four parallels in each.
solution (5%) in EtOH is supposed to be linked with decreasing of membrane fluidity, that is necessary for the fusion of viral envelope with cell membrane in the course of viral life cycle. Further studies are necessary to decipher the exact mechanism of anti-viral activity of GL derivatives.

Acknowledgments

This work was financially supported by the Russian Science Foundation (grant 14-03-01307).

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Experimental: IR spectra were recorded in mineral oil mulls on a Spectrophotometer. NMR spectra were recorded in CD3OD with TMS internal standard on a Bruker AMX-300 spectrometer at operating frequency 300 (1) and 75.5 (2) MHz. Optical activity was measured in a 1-dm tube on a Perkin-Elmer 341 CM polarimeter. This later chromatography (TLC) was performed on silica gel plates (Merck, Germany) 60 F254 (45:10:1, v:v). Spots were detected by H2SO4 solution (3%) in EtOH with subsequent heating at 110–120 ºC for 2–3 min. Column chromatography was carried out on silica gel KSK SC (50–150 fraction, dry classification) (Schoopil...
spectrophotometer (Victor 1420, Perkin Elmer, Finland) at wavelength 535 nm. Based on this data, the CTD50 (compound concentration required to reduce 50% cell viability) value was estimated for each compound.

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49. Antiviral assay: The serial twofold dilutions of compounds were prepared in MEM containing 2 mM arginine, 2 mM glutamine and 2 μg/mL trypsin and applied to MDCK cells. After being incubated for 1 h at 37 °C the cells were inoculated with influenza virus in a dose 1–10^6 of 50% infecting dose (ID50). Cells that were not treated by the compounds were used as control. After cultivation for 48 h at 36 °C the hemagglutination assay was carried out as follows. Supernatant (100 μL) was transferred into round-bottom wells and mixed with 100 μL of 1% suspension of chicken erythrocytes followed by 1 h incubation at rt. The virus titer was considered as a reciprocal to the final dilution of the inoculum able to cause a positive hemagglutination in 50% of wells and expressed in 50% infecting doses. Anti-viral activity of test compounds was evaluated by their ability to decrease the virus titer. Based on the results, 50% of the effective dose (concentration of compound which decreases virus production two-fold comparing to control) and selectivity index (SI, relation of CTD50 to ED50) were calculated.

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