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Glycyrrhizic acid derivatives as influenza A/H1N1 virus inhibitors

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ABSTRACT

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.).[1,2] 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 persons have been died in Hong Kong in 1968 by the pandemic influenza A (H3N2).[3–5] The appearance of novel strain of influenza A in April 2009 ('swine flu') caused 162,380 cases and 1154 deaths in 168 countries,[6,7] and the World Health Organization (WHO) declared the pandemic influenza caused by a new strain of H1N1, which was identified as influenza virus formed as a result of a triple reassortment of swine, avian and human viruses and called influenza A/H1N1pdm09.[8–10]

Currently, two classes of 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.[11,12] 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.[13–16] At present an 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.[17,18]

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.[19,20]

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.[21–23] 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.[24,25] Preparation SNMC (Stronger neo-Minophagen Co.) containing GL was used for a long term chemotherapy of viral hepatitis B and C.[26] GL is attractive due its ability to stimulate γ-interferon production in vitro and in vivo, and low toxicity (LD_{50} 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.[27]

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.[28] Among GL derivatives new inhibitors of

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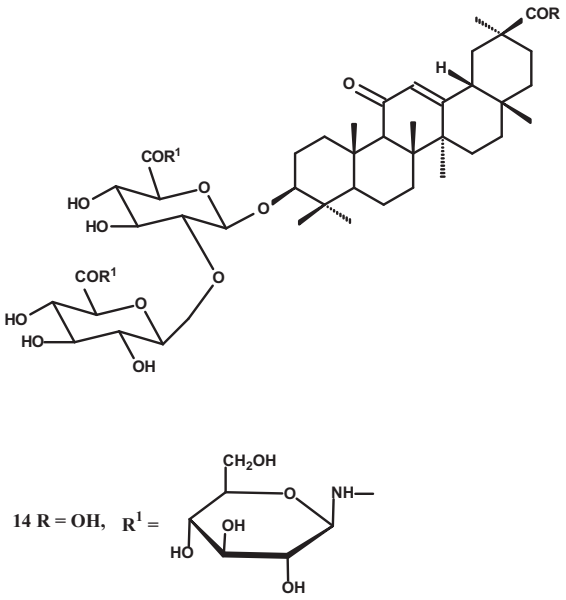
SARS-associated coronavirus,[29] Epstein–Barr virus,[30] and anti-HIV agents[31,32] were found. Structure–activity relationships study of GL derivatives as influenza A/H1N1 virus inhibitors was not carried out still. This Letter is devoted to the synthesis and anti-viral activity evaluation of some GL 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[27] and had a purity 95 ± 1% according to HPLC. Experimental details are given in the References and notes.[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 1/3.5/3.5/3.5.[34] 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*-hydroxysucinimide (HOSu)—DCC and 2.0–2.5 molar ratio of reagents and at 0–5 °C as was reported previously.[31,36] 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 ^{13}C data. There are signals of free 30-COOH at 180.3–180.8 ppm as in the ^{13}C 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^{ε}-carbobenzoxy(*Z*)-lysine *tert*-butyl esters hydrochlorides.[39,40] *tert*-Butyl ester groups of protected conjugates **10** and **12** were deleted with 50% CF_3COOH in DCM and pure compounds **11** and **13** were isolated by CC on SL. GL conjugate with D-galactose amine (**14**) connected to the carbohydrate part of glycoside by CONH-bonds was synthesized as described before.[41]

Stereo isomeric 18α-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.[23] GL analog (**18**) was prepared by reduction of GL trimethyl ester with $NaBH_4$ according to method.[43] Glycoside (**19**) with reduced C^{11}=O group was synthesized by reduction of GA with $NaBH_4$ 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 microtetrazolium test (MTT)[47] and CTD_{50} (compound concentration required to reduce 50% cell viability) value was estimated for each compound.



1 R = R^1 = OH

2 R = R^1 = Phe-OMe

3 R = R^1 = Tyr-OMe

4 R = R^1 = Ile-OMe

5 R = OH, R^1 = Phe-OMe

6 R = OH, R^1 = Tyr-OMe

7 R = OH, R^1 = Val-OMe

8 R = OH, R^1 = Hys-OMe

9 R = OH, R^1 = Asp(OMe)-OMe

10 R = OH, R^1 = Cys(Bn)-OBu^t

11 R = OH, R^1 = Cys(Bn)-OH

12 R = OH, R^1 = Lys(Z)-OBu^t

13 R = OH, R^1 = Lys(Z)-OH

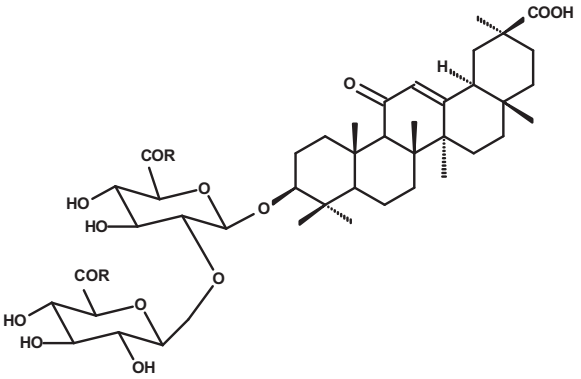
Bn = benzyl
Z = carbobenzoxy
Bu^t = tert-butyl

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 (ED_{50}) (concentration of compound that decreases the virus production two-fold comparing to control) was calculated. Selectivity index (SI) was calculated as relation of CTD_{50} to ED_{50}. 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 CTD_{50}) 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** (EC_{50} = 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).

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).

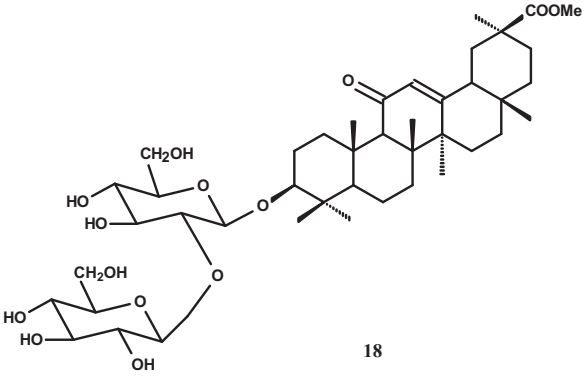


15 R = OH

16 R = Asp(OMe)-OMe

17 R = Asp(OMe)-OH

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 properties (compound **9**). Modification of glycoside with a long chaired diamine acid like *Z*-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).



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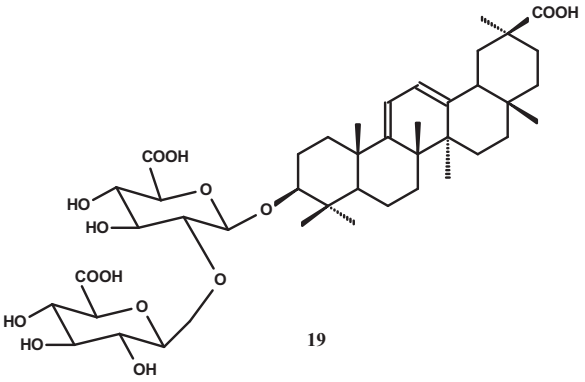
Stereo isomeric 18α-GL **15** was in 7.7 times more toxic to cells (CD_{50} = 47.5) than natural glycoside **1** (18β), and had a week anti-viral activity. Its derivatives **16** and **17** to be aspartic acid methyl esters conjugates were more cytotoxic and less active than the similar derivative of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



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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.[29] Presence of free 30-COOH group is important for anti-viral activity of GA conjugates with amino acids.

Previously, Utsunomiya et al.[50] 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.[51] Its mechanism of activity

Table 1
Antiviral activity of GL and derivatives against influenza virus A/H1N1/pdm09 in MDCK cells[a]

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

[a] The values of EC_{50} and CTD_{50} are mean of three different experiments, four parallels in each.

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.

Our results show that modification of GL is a perspective route in the search of new antivirals, and some of GL derivatives are potent as anti-influenza agents. But further studies are necessary to decipher the exact mechanism of anti-viral activity of GA derivatives.

Acknowledgments

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33. *Experimental:* IR spectra were recorded in mineral oil mulls on a Specord M-80 spectrophotometer. NMR spectra were recorded in CD_3OD with TMS internal standard on a Bruker AMX-300 spectrometer at operating frequency 300 (^1H) and 75.5 (^{13}C) MHz. Optical activity was measured in a 1-dm tube on a Perkin-Elmer 341 MC polarimeter. Thin layer chromatography (TLC) was performed on silica gel shits (Sorbfil plates, Sorbpolimer) using $CHCl_3$–MeOH–H_2O mixture (45:10:1, v %). Spots were detected by H_2SO_4 solution (5%) in EtOH with subsequent heating at 110–120 °C for 2–3 min. Column chromatography was carried out over silica gel KSK SG (50–150 fraction, dry classification) (Sorbpolimer) by using a gradient mixture $CHCl_3$–MeOH–H_2O (400:10:1 → 50:10:1, v %). The HPLC analysis were performed on a DuPont chromatograph with an absorbance detector at 254 nm over a Jupiter 5 μ C18 reversed-phase column with mobile phase (MP) MeOH–H_2O–AcOH (60:35:5, v %) for GA and over a Chromsil CN column with MP phosphate buffer (pH 5.17):CH_3CN:dioxane (210:15:10, v %) for 18α-GA at flow rate 1 mL/min (sample concentration 2 mg/mL). We used commercially available DCC, DEC, HOBt, HOSu (Aldrich), L-amino acids and their derivatives (Reanal). All solvents and NEM were distilled.
34. *General method for synthesis of compounds* ***2–4****.* *N*-hydroxybenzotriazole (3.5 mmol), 1-(3-dimethylaminopropyl)-3-ehylcarbodiimide hydrochloride (3.5 mmol) were added to GA (1 mmol) solution in 15–20 mL DMF-pyridine mixture (1:1) at 0–5 °C and mixed for 1 h at 0–5 °C and 5–6 h at rt. Then amino acid methyl ester hydrochloride (3.5 mmol), and *N*-ethylmorpholine (5–6 mmol) were added to a mixture and stored at rt for a night. Then a reaction mixture was diluted with cold water, acidified with citric acid (pH ~3–4), and a precipitate was filtered off, washed with water, and dried. Analytical samples were received by precipitation from ethanol–water mixtures as powders. All of the target compounds were pure according TLC.
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38. *Compound* ***5****.* Yield 55.6%; $[\alpha]_D^{20}$ +62° (*c* 0.04, EtOH). IR spectrum (*ν*, cm^{-1}): 3600–3200, 1729, 1662, 1616, 1538, 1521. ^{13}C NMR (CD_3OD) *δ* (ppm): 40.3 (C1), 27.4 (C2), 90.7 (C3), 40.7 (C4), 56.4 (C5), 18.5 (C6), 33.8 (C7), 46.8 (C8), 63.1 (C9), 38.1 (C10), 202.6 (C11), 128.2 (C12), 171.2 (C13), 44.6 (C14), 27.4 (C15), 27.6 (C16), 33.0 (C17), 42.5 (C19), 44.9 (C20), 32.0 (C21), 39.0 (C22), 28.4 (C23), 17.1 (C24), 17.4 (C25), 19.3 (C26), 23.9 (C27), 28.8 (C28), 29.2 (C29), 180.4 (C30), 104.7 (C1′), 81.0 (C2′), 75.9 (C3′), 73.4 (C4′), 77.3 (C5′), 172.3 (C6′), 104.9 (C1″), 75.4 (C2″), 75.8 (C3″), 73.5 (C4″), 77.9 (C5″), 172.3 (C6″); 2Phe-OMe: 173.0, 172.9, 137.6, 130.5–128.9, 54.9, 54.5, 53.00, 52.9. Found: N, 2.3%, calcd for $C_{62}H_{84}O_{18}N_2$: N, 2.4%.
Compound ***6****,* yield 56.5%; $[\alpha]_D^{20}$ +55° (*c* 0.04, MeOH); IR (*ν*, cm^{-1}): 3600–3200, 1734, 1665, 1615, 1597, 1516. ^{13}C NMR (CD_3OD) *δ* (ppm): 40.6 (C1), 27.2 (C2), 90.6 (C3), 40.6 (C4), 56.4 (C5), 18.4 (C6), 33.8 (C7), 46.7 (C8), 63.1 (C9), 38.0 (C10), 202.6 (C11), 128.9 (C12), 171.2 (C13), 44.6 (C14), 27.3 (C15), 27.6 (C16), 32.9 (C17), 48.2(C18), 42.4 (C19), 44.9 (C20), 32.0 (C21), 39.0 (C22), 28.5 (C23), 17.1 (C24), 17.3 (C25), 19.3 (C26), 24.0 (C27), 28.8 (C28), 29.2 (C29), 180.3 (C30), 104.5 (C1′), 81.0 (C2′), 76.1 (C3′), 73.3 (C4′), 77.1 (C5′), 172.3 (C6′), 104.8 (C1″), 75.2 (C2″), 75.7 (C3″), 73.3 (C4″), 77.8 (C5″), 172.3 (C6″); 2Tyr-OMe: 172.9, 172.8, 157.5, 131.5–127.9, 116.4, 62.6, 61.7, 54.8, 54.8, 53.0, 52.9. Found: N, 2.2%, calcd for $C_{62}H_{84}N_2O_{20}$: N, 2.4%.
Compound ***7****:* yield 58.8%; $[\alpha]_D^{20}$ +52° (*c* 0.04, MeOH). IR (*ν*, cm^{-1}): 3600–3200, 1740, 1669, 1540. ^{13}C NMR (CD_3OD) *δ* (ppm): 40.5 (C1), 27.4 (C2), 90.6 (C3), 40.2 (C4), 56.7 (C5), 18.0 (C6), 44.3 (C8), 63.4 (C9), 202.9 (C11), 129.2 (C12), 171.7 (C13), 46.8 (C14), 28.7 (C16), 45.2 (C20), 31.9 (C21), 39.3 (C22), 29.0 (C23), 17.2 (C24), 17.5 (C25), 19.2 (C26), 24.1 (C27), 29.2 (C28), 29.5 (C29), 180.8 (C30), 105.2 (C1′), 81.8 (C2′), 76.3 (C3′), 73.7 (C4′), 78.0 (C5′), 171.7 (C6′), 105.2 (C1″), 76.1 (C2″), 76.5 (C3″), 73.6 (C4″), 77.3 (C5″), 171.7 (C6″); 2Val-OMe: 173.4, 173.2, 59.3, 58.7, 53.0, 52.9, 32.6, 32.5, 19.7, 19.6. Found: N, 2.7%, calcd for $C_{54}H_{84}N_2O_{18}$: N, 2.7%.
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45. *Viruses and cells:* Experiments were performed with influenza virus A/California/07/09/H1N1/pdm09 from the collection of viral strains from Center for Disease Control and Prevention (CDC, Atlanta) in MDCK cells (ATCC CCL 34).
46. *Cytoxicity assay:* Test compounds were dissolved in DMSO to 5 mg/mL, and the serial twofold dilutions (1000–4 μ/mL) were prepared in Eagle's minimal essential medium (MEM). MDCK cells were seeded in 96-wells plates and cultivated in MEM with addition of 5% fetal calf serum. After the cell monolayer formation the cells were washed by serum-free MEM, the dilutions of test compounds were applied to the MDCK cells and incubated for 48 h at 37 °C. After being incubated, the cells were washed twice with PBS and number of viable cells was evaluated by a microtetrazolium test (MTT). Briefly, 3-(4,5-dimethyltiazolyl-2)-2,5-diphenyltetrazolium bromide (ICN Biochemicals Inc., Aurora, Ohio, 0.5 mg/mL, 0.1 mL per well) was added with subsequent incubation of plates at 37 °C in 5% CO_2 for 60 min. The colored deposit was dissolved in 100 μL of DMSO. The plates were swirled gently and left in the dark at RT for 30 min. Optical density was measured by using a

spectrophotometer (Victor 1420, Perkin Elmer, Finland) at wavelength 535 nm. Based on this data, the CTD_{50} (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 (ID_{50}). 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 CTD_{50} to ED_{50}) were calculated.
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