Bioorganic & Medicinal Chemistry Letters 30 (2020) 127518

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Structure-aided optimization of 3-*O*-β-chacotriosyl epiursolic acid derivatives as novel H5N1 virus entry inhibitors

Sumei Li[a,e], Xiuhua Jia[c,e], Hui Li[b], Yilu Ye[d], Xuesha Zhang[b], Yongfeng Gao[b], Guoqing Guo[a], Shuwen Liu[d,*], Gaopeng Song[b,*]

[a] Department of Human Anatomy, School of Medicine, Jinan University, Guangzhou 510632, China
[b] College of Materials and Energy, South China Agricultural University, Guangzhou 510642, China
[c] Department of Ophthalmology, The Third Affiliated Hospital, Sun Yat-sen University, Guangzhou 510630, China
[d] School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, China

ARTICLE INFO

Keywords:
3-Epiursolic acid saponins
Semi-synthesis
H5N1 entry inhibitors
Structure–activity relationships

ABSTRACT

It is urgent to develop new antiviral agents due to the continuous emergence of drug-resistant strains of influenza virus. Our earlier studies have identified that certain pentacyclic triterpene saponins with 3-O-β-chacotriosyl residue are novel H5N1 virus entry inhibitors. In the present study, a series of C-28 modified 3-*O*-β-chacotriosyl epiursolic acid derivatives via conjugation with different kinds of sides were synthesized, of which anti-H5N1 activities in A549 cells were evaluated *in vitro*. Among them, **10** exhibited strongest anti-H5N1 potency at the low-micromole level without cytotoxicity, surpassing the potency of ribavirin. Further mechanism studies of the lead compound **10** based on HI, SPR and molecular modeling revealed that these new 3-epiursolic acid saponins could bind tightly to the viral envelope HA protein, thus blocking the invasion of H5N1 viruses into host cells.

Highly pathogenic H5N1 influenza A virus (IAV) has resulted in a world-wide devastating effect on public health with a substantial mortality and morbidity since 1997.[1,2] However, there is no effective vaccine to prevent H5N1 virus up to now. Currently, the treatment of H5N1 influenza A infection only depends on a limited number of antiviral drugs including amantadine, rimantadine, zanamivir, oseltamivir and so on, which are able to inhibit two viral targets, namely M2 ion channel and neuraminidase (NA).[3,4] It is worth noting that prevention of H5N1 influenza infection by antiviral drugs presents a formidable challenge as the high variability within each segment of H5N1 virus leads to the virus developing resistance to the above existing antiviral drugs in the current clinical use.[5–7] Therefore, there is an urgent need to develop new antiviral agents with different mechanisms of action.

Hemagglutinin (HA) that is a mushroom-like glycoprotein on the surface of IAVs, plays a key role in the viral lifecycle.[8,9] It is well known that HA can bind to sialylglyconjugates on the surface of the host cells, leading to endocytosis of the virus and subsequently membrane fusion of the virus and the host cells.[10–12] Considering HA is essential for both host cell recognition and membrane fusion, it can be regarded as a potential target for antiviral therapy development. Encouragingly, human monoclonal antibodies can target HA and show broad spectrum antiviral activities against H1N1, H3N2, H5N1 and so on.[13,14] Besides antibodies, more and more IAVs entry inhibitors have been identified and developed as new antiviral agents[15–17], which are expected to have enormous value for the treatment of H5N1 influenza infections.

Pentacyclic triterpenes characterized by hydrophobic pentacyclic scaffolds, possess valuable inhibitory activities against human immunodeficiency virus (HIV), Ebola virus (EBOV), IAVs and hepatitis C virus (HCV) infection.[18–20] Further investigations indicate that these pentacyclic triterpenes are able to block viral entry into the host cells to inhibit viral infection.[21–24] Particularly, a number of pentacyclic triterpenes saponins with different saccharide chains have been obtained as novel IAVs entry inhibitors[25–27], which can inhibit IAVs entry into cells by either blocking virus attachment to host cell receptor or virus-cell membrane fusion. For example, **Y3** (Fig. 1), an analog of oleanic acid (OA), conjugated with acetylated galactose at C-17-COOH in OA, exhibited remarkable anti-IAVs potency *in vitro* and *in vivo* by interacting with HA.[25] Rollinger reported that several new oleanane-type triterpene saponins **Rg-1** and **Rg-2** (Fig. 1) comprising a branched or linear trisaccharide moiety at C-3-OH, were identified as potent anti-IAVs agents with IC_{50} values in the low nanomolar range.[26]

In our previous study, a new series of methyl 3-*O*-β-chacotriosyl ursolic acid (**1**, Fig. 2) and its derivatives displayed excellent inhibitory activity against H5N1 IAV entry based on an efficient HIV-based

* Corresponding authors.
E-mail addresses: liusw@smu.edu.cn (S. Liu), songgp1021@scau.edu.cn (G. Song).
[e] These authors contributed equally to this work.

https://doi.org/10.1016/j.bmcl.2020.127518
Received 15 July 2020; Received in revised form 21 August 2020; Accepted 21 August 2020
Available online 31 August 2020
0960-894X/

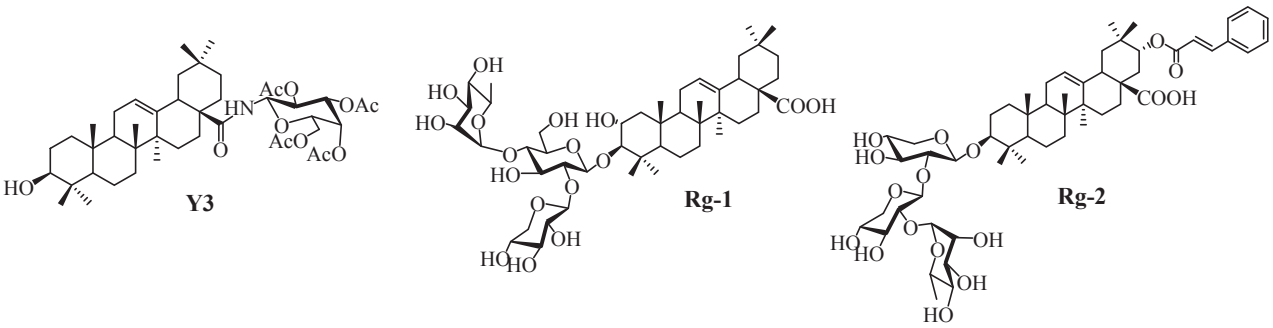


Fig. 1. Representative oleanane-type triterpene saponins Y3, Rg-1 and Rg-2 as IAV entry inhibitors.

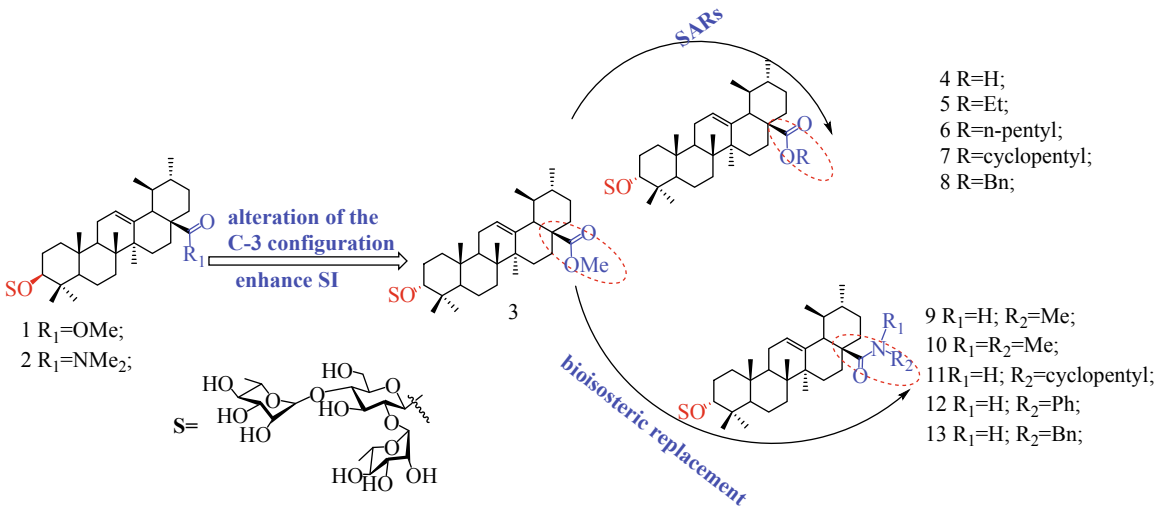


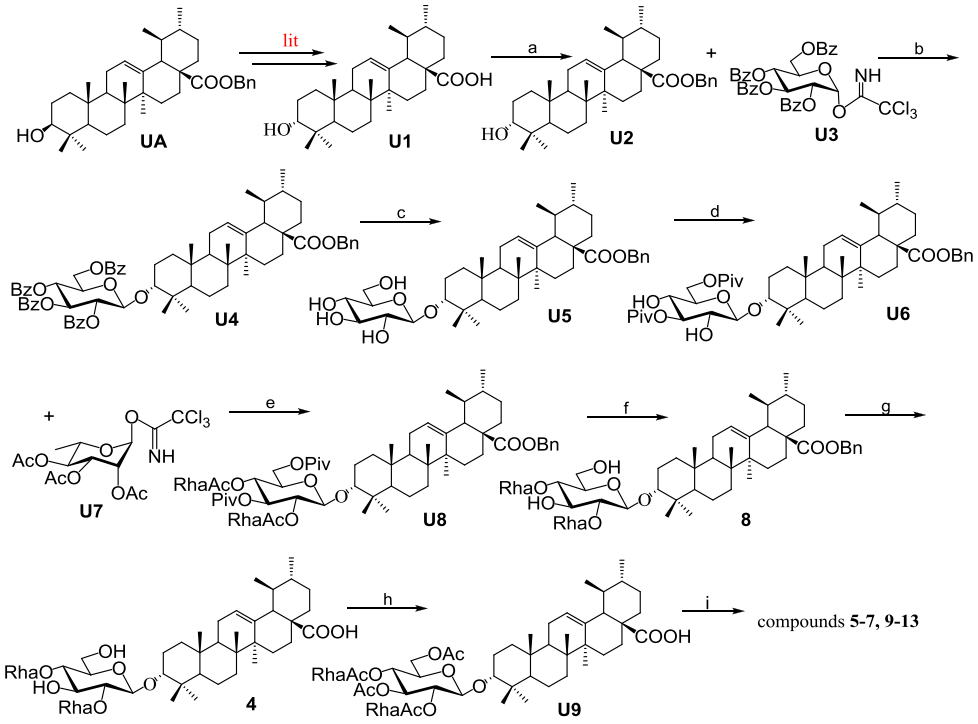
Fig 2. Structures of the lead compounds **1–3** and title saponins **4–13**.

pseudotyping system to screen a semisynthetic saponin library.[27] On the basis of SPR studies and docking predictions, **1** and its analogs were claimed to bind tightly to HA, disrupting the interaction of HA with the sialic acid receptor. Further structure–activity relationships (SARs) revealed that not only the chacotriosyl residue but also the ursolic acid (UA) aglycone moiety played a profound effect on anti-H5N1 activity, though several modifications were tolerated at certain positions. [27] Our prior studies on compound **1** suggested that the 17-position ($COOCH_3$ group) was preferred for further replacement with its bioisoster dimethylamide (**2**, Fig. 2), which was beneficial to improving the selective index. However, **2** still exhibited moderate cytotoxicity against MDCK cells with the CC_{50} value of 47 μM, which restricted its pharmaceutical use as a potential anti-H5N1 entry inhibitor. Moreover, both inhibition against H5N1 virus and mechanisms of action of **2** are still unclear despite increased selective index.

Encouragingly, we unexpectedly found that alteration of the C-3 configuration of methyl ursolate from 3β-OH to 3α-OH leading to methyl 3-*O*-β-chacotriosyl epiursolic acid (**3**, Fig. 2), could dramatically reduce cytotoxicity against MDCK cells. In view of the above results, as part of our persistent efforts toward the development of potential antiviral entry inhibitors derived from natural products[9–11,27], we designed and synthesized a series of 3-*O*-β-chacotriosyl epiursolic acid analogues **4–13** (Fig. 2) in cooperation with other chemical modifications at 17-COOH group, aiming to identify strongly potent H5N1 entry inhibitors with high selective index. And then, the present study determined whether the above 3-epiursolic acid saponins exert anti-H5N1 activity evaluated in cell-based assays and how they map out the potential mechanism of action for inhibition against H5N1 IAV virus.

The synthesis of title saponins **4–13** was outlined in Scheme 1. The important aglycone 3-epiursolic acid (**U1**) was obtained from the natural ursolic acid (UA) following the literature procedures[28], of which benzyl esterification furnished **U2**[28]. The resulting glycosylation of **U2** with the donor **U3** proceeded to yield **U4** in the presence of a strong Lewis acid TMSOTf. This process took advantage of the participation of the neighboring group in a stereoselective reaction at C-2 position in Glu to produce the complete β-stereoselectivity. Then all the Bz groups in Glu were deprotected with NaOMe in MeOH to give **U5**. Regioselective protection of the C-6-OH and C-3-OH in **U5** with pivaloyl (Piv) group was performed by treatment with pivaloyl chloride in pyridine at low temperature (−15 °C), giving rise to **U6**. Subsequent coupling of **U6** and the L-rhamnose donor **U7** by means of a similar TMSOTf-catalyzed glycosylation procedure afforded the trisaccharide glycoside **U8** with exclusive α-configuration at the new glycosidic linkage. Then both piv and Ac groups in **U8** were simultaneously deprotected under the basic conditions to yield the title saponin **8**. The free C-17 carboxyl group in **4** was prepared by hydrogenolysis of Bn group over 10% palladium/carbon. Subsequently, acetylation of **4** with acetic anhydride was performed to generate the important intermediate **U9**. Synthesis of the title saponins **5–7** and **9–13** was performed via an acyl chlorination with oxalyl chloride and subsequent coupling reaction, followed by removal of all the Ac groups using MeONa in MeOH.

The saponins **3–13** were tested for their inhibitory potency against wild-type A/ Duck/Guangdong/212/2004 H5N1 viruses using the cytopathic effect (CPE) reduction assay in A549 cells while the MTT screening was utilized to screen and exclude compounds with strong toxicity toward A549 cells.[9] As expected, little to no toxicity against A549 cells was observed when 3β-OH of UA was converted to 3α-OH, indicating the importance of the C-3 configuration of aglycone for the selectivity index. Among these title saponins, the example of the effect of compound **10** with strongest antiviral activity on CPE reduction was



Scheme 1. Reagents and conditions: (a) BnCl, K_2CO_3, DMF, 96%; (b) TMSOTf, CH_2Cl_2, 85%; (c) MeONa, CH_3OH, 93%; (d) PivCl, pyridine, 76%; (e) TMSOTf, CH_2Cl_2, 84% (f) NaOH, CH_3OH-THF-H_2O, 93%; (g) 10% Pd/C, H_2, CH_3OH-CH_2Cl_2, 84%; (h) Ac_2O, DMAP, pyridine, 87%; (h) (i) $(COCl)_2$, CH_2Cl_2 (ii) R-Br, K_2CO_3, DMF or R^1R^2NH, Et_3N, CH_2Cl_2; (iii) CH_3ONa, CH_3OH, 94% for **5**, 90% for **6**, 91% for **7**, 93% for **9**, 85% for **10**, 88% for **11**, 90% for **12**, 90% for **13**.

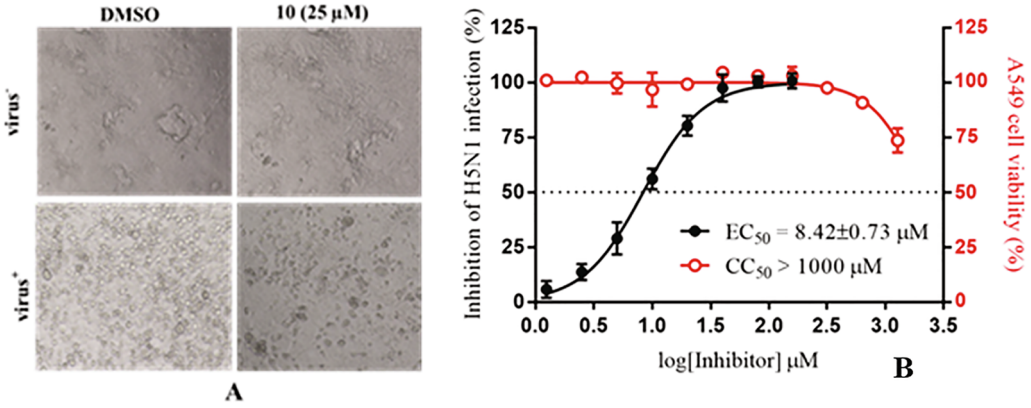


Fig 3. (A) Validation of the protection of A549 cells from influenza A/ Duck/Guangdong/212/2004 H5N1 by compound **10**. (B) Dose-response curve for potential compound **10** in the antiviral and cytotoxicity assay.

shown in Fig. 3A, demonstrating that these compounds could protect A549 cells from influenza virus H5N1-induced CPE compared with DMSO. In this manner, **10** displayed strong inhibition against H5N1 in A549 cells with an IC_{50} of 8.42 μM while exhibiting no cytotoxicity *in vitro* (Fig. 3B).

As shown in Table 1, the goal of this preliminary SARs was to investigate the significance of substitutions on the C-17-COOH group with respect to both anti-H5N1 activity and selective index. To confirm the advantage of substituents attached to the 17-COOH position of 3-epiursolic acid, **4** was synthesized to represent the minimal scaffold with no substituents attached. Saponins **5–8** were designed to replace the relatively small CH_3 moiety (**3**) for a much bulkier alkyl or aryl substituent to probe the effect of both length and size of side chains on antiviral activity. By observing the antiviral potential of **3–8**, it was noted that all the 3-epiursolic acid esters-chacotriose conjugates **3** and **5–8** exhibited substantially improved anti-H5N1 activity relative to **4**, suggesting that the introduced alkyl or aryl group might be directly involved in anti-IAV activity. Moreover, our rationale was that the lengthened side chains in C-28 position (**5** and **6**) could potentially

Table 1
Antiviral activity of compounds **3–13** against H5N1influenza A virus in A549 cells.

compound	IC_{50} (μM)	CC_{50} (μM)	SI
3	16.45 ± 0.58	363.42 ± 2.61	22.1
4	48.72 ± 1.83	282.75 ± 1.88	5.8
5	18.06 ± 0.52	374.63 ± 3.47	20.7
6	29.32 ± 1.45	488.60 ± 2.36	16.7
7	40.52 ± 3.26	523.72 ± 3.85	12.9
8	> 50	NT	NT
9	12.24 ± 0.60	> 1000	> 81.7
10	8.42 ± 0.36	> 1000	> 118.8
11	18.48 ± 1.22	> 1000	> 54.1
12	33.83 ± 1.61	> 1000	> 29.6
13	> 50	NT	NT
ribavirin	47.22 ± 1.05	129.65 ± 1.38	2.7

IC_{50}: 50% inhibitory concentration.
CC_{50}: 50% cellular cytotoxicity concentration.
SI: selectivity index as CC_{50}/IC_{50}.

extend the CH_3 moiety deeper into the unexplored side cavity, generating the additional hydrophobic interactions to enhance ligand affinity. However, this increase in steric bulk resulted in similar or a little loss of efficacy in spite of reduced cytotoxicity against A549 cells compared to **3**. Likewise, decoration of C-17-COOH with enlarged side chains (**7** and **8**) to exploit potential hydrophobic interactions also led to a little and even significant decreased antiviral activity. These results suggest that larger substituents sterically clashed inside that portion of the active site, of which introduction should be avoided. A similar trend was found for the bioisosteric surrogates **9–13** of 3-epiursolic acid ester since the methanamide product **9** was more active than other three monoamide analogs **11–13**. It was interesting to note that all the amide analogs showed marginal cytotoxicity toward A549 cells. These results demonstrate that amidation at the 17-COOH position was beneficial for improving the safety of these saponins while keeping their antiviral activity. With the failure of positively charged monoamide groups with different large substituent to produce antiviral agents, we therefore proceeded with exploration of small neutral N, *N*-disubstitution groups. Thus, the impact of N, *N*-disubstitution was determined with **10**, a disubstituted amide structure from **9**. Even more encouraging was that **10** had an increased both anti-H5N1 potency and selective index, possibly owing to optimal size and hydrophobic nature of dimethylamino group in C-28 position. These results highlight the significance of the short disubstituted amide residue in potential ligand affinity. Because of its good compromise between potency and selective index, **10** was considered for further studies of mechanism of action *in vitro*, which was chosen as the lead compound.

Next, we examined **10** could block H5N1 virus production most effectively at the virus entry step of cell infection based on a HA/HIV pseudotyped virus model while a VSVG/HIV pseudoviral was used as a specificity control to exclude inhibitory effect on post-entry infection of HIV backbone. As shown in Fig. 4A, 10 exhibited potential inhibitory activity against H5N1 (A/Tailand/Kan353/2004) with an IC_{50} of 6.29 ± 0.23 uM, but no cytotoxic activity against MDCK cells (Fig. 4B). Furthermore, we speculated that **10** could target H5N1 HA due to the fact that it not only had negligible effect on VSV-G enveloped pseudovirus (Fig. 4A) but also exhibited no significant inhibition on the activity of N1-typed neuraminidase (Fig. 4C).

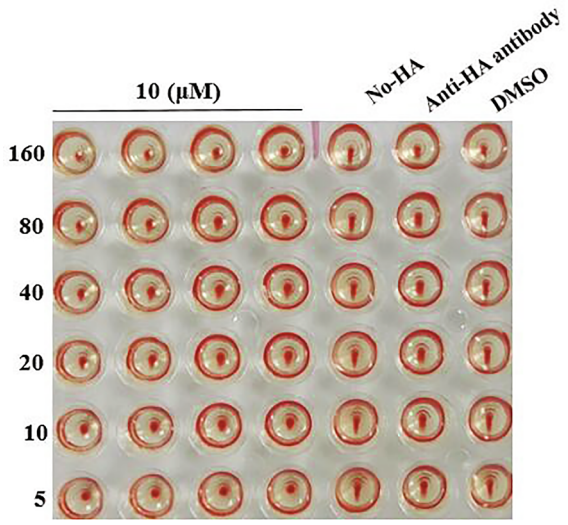


Fig. 5. Comparisons of the behaviors of **10** vs anti-HA antibody in inhibition of H5N1virus-induced aggregation of chicken erythrocytes.

It is influenza virus HA that plays a crucial role during viral invasion into host cells, which can bind to sialic acid on the surface of red blood cells (RBCs), leading to agglutination. Thus, a hemagglutination inhibition (HI) assay was performed to investigate whether the designed 3-*O*-β-chacotriosyl epiursolic acid derivatives blocked the ability of viral particles to target host cell receptors. It was observed that **10** could inhibit the binding of influenza virus H5N1 to RBCs in a concentration-dependent manner (Fig. 5) as anti-HA antibody do, indicating that these title saponins may target HA and block the interaction of H5 HA with sialic acid receptor in host cells.

To further investigate the interaction of the lead **10** with HA, we then characterized the affinity between HA and **10** based on a surface plasmon resonance (SPR) assay. A recombinant HA protein from virus strain A/Vietnam/1203/2004 (H5N1) was used as representative HA of

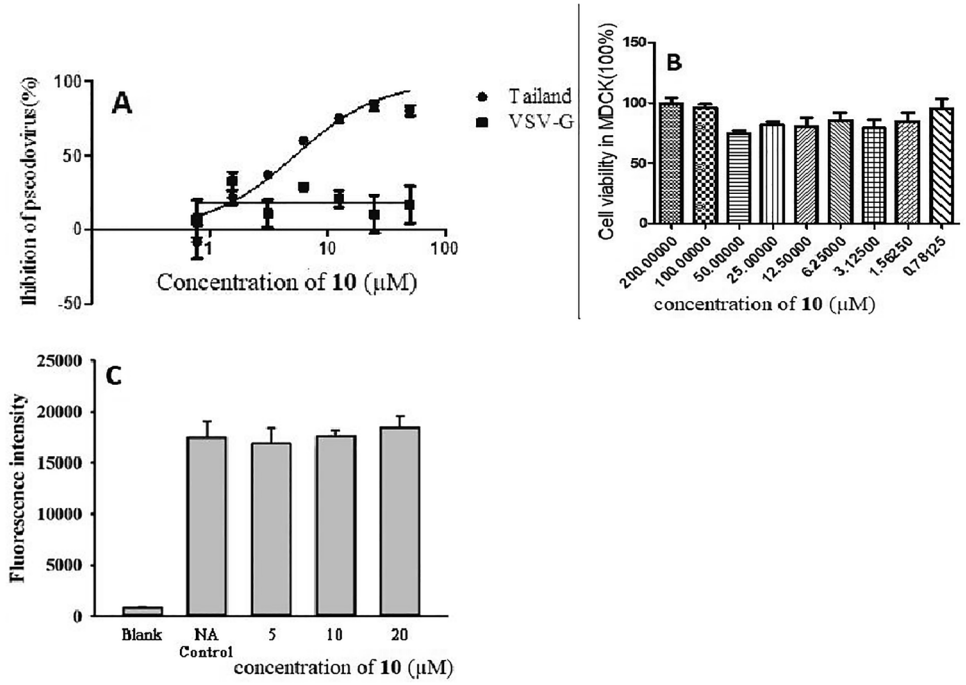


Fig. 4. (A) Compound **10** could inhibit H5N1 (A/Tailand/Kan353/2004) dose-dependently and did not inhibit VSVG pseudovirus. (B) **10** showed no cytotoxic activity against MDCK cells. (C) **10** did not inhibit neuraminidase activity.

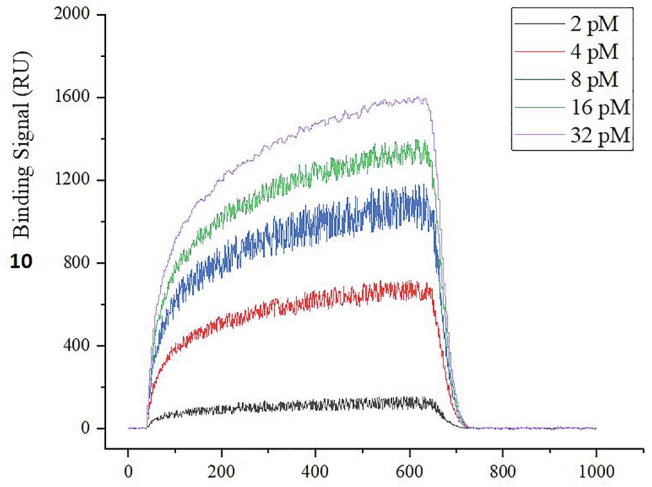


Fig. 6. Characterization of the affinity between **10** and H5N1 HA protein using surface plasmon resonance (SPR) analysis.

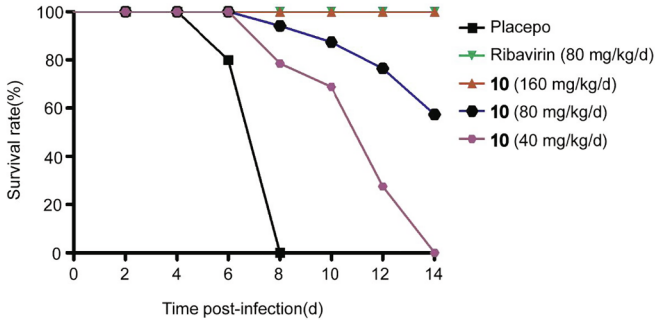


Fig. 8. Evaluation of the efficacy of **10** against influenza infection via intranasal administration. Balb/C mice, five in each group, were inoculated with $5LD_{50}$ of virus and intranasally administrated with **10** at dose 0, 40, 80 and 160 mg/Kg/d one daily for 5 days, starting 1 day before infection. The survival of infected mice was monitored daily for 14 days. Infected mice treated with ribavirin were used as positive control.

IAV H5N1 strains, due to the fact that the amino acid sequence identity of the full-length HA proteins between A/Vietnam/1203/2004 strain and A/Duck/Guangdong/99 (H5N1) strain used in above antiviral assay was over 97.0%.[11] As shown in Fig. 6, 10 could favorably interact with HA in a dose-dependent manner, and the equilibrium dissociation constant (K_D) of the interaction was 1.64 nM, indicating there was a strong affinity between **10** with H5N1 HA.

Here, to add further insights into the inhibition mechanism, we attempted to perform docking study of the selected compound **10** with the Surflex-Dock program of SYBYL 7.3. In this simulation, the HA1 sequence of A/ Duck/Guangdong/212/2004 H5N1 was identical to that of A/Vietnam/1194/2004, of which the neutral-pH crystal structure of the HA has been well defined recently.[9–11] Hence, **10** was docked into the influenza HA protein of H5N1 (Influenza strain A/Vietnam/1194/2004, PDB: 3ZP0).[29]

As shown in Fig. 7, the surflex-dock study indicated that **10** could occupy the binding pocket for sialic acid receptor, which shared a similar binding mode as other pentacyclic triterpenes analogs.[9] Specifically, the methyl moiety of 17-CON$(CH_3)_2$ and the 3-epiursolic acid scaffold formed multiple hydrophobic interactions with Leu133, Lys156, Lys193 and Leu194. More importantly, the chacotriosyl residue played a pivotal role in occupying sialyllactose core binding site. It is

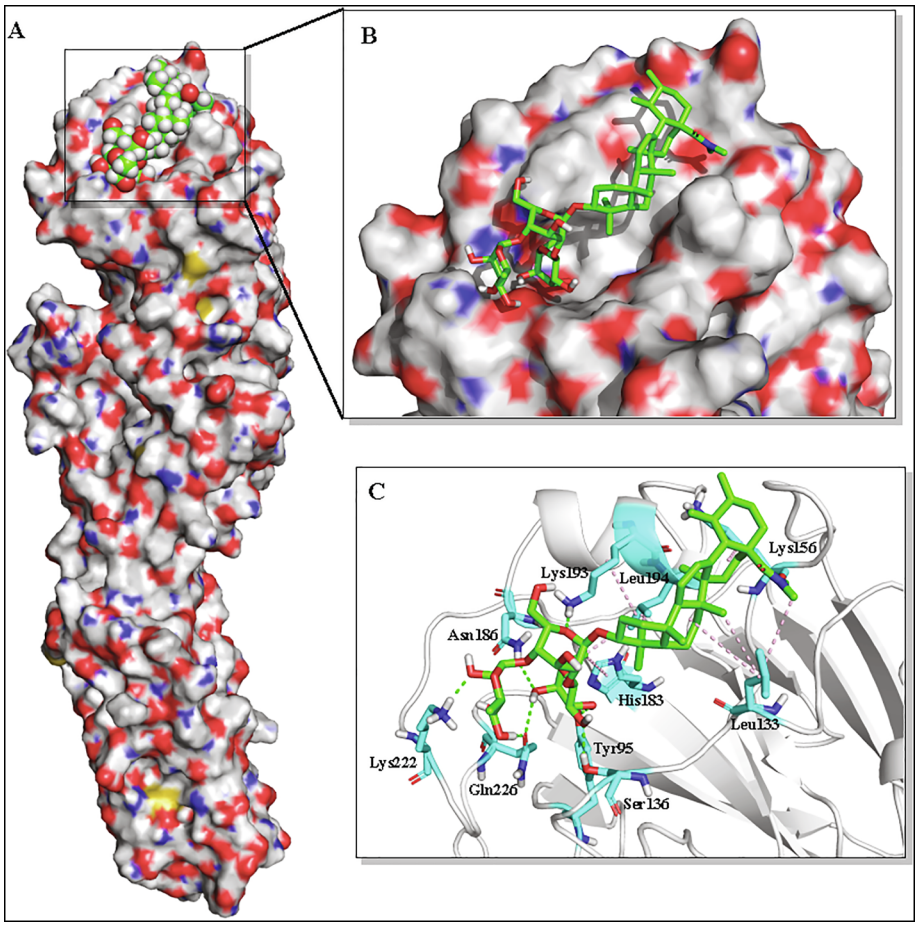


Fig. 7. Structural representative of compound **10** binding within hemagglutinin (Protein Data Bank: 3ZP0) according to surflex-dock calculation. (A) Overview of hemagglutinin. The binding pocket of sialic acid is highlighted in blue square. Protein and **10** were shown as white surface and yellow spheres, respectively. (B) Closer view of the binding pocket. Compound **10** was shown as yellow sticks. (C) 3D interaction plot green dashes indicate hydrogen bonds.

clear that residues in 130 loop, 190 helix and 220 loop were important for sialic acid binding. [25] It was found that C-6-OH of D-glucose formed hydrogen bond with Lys193 within 190 helix. Moreover, the C-2-OH and C-4-OH of the L-rhamnose moiety linked to C-2-OH of D-glucose formed stable hydrogen bonds with Ser136 within 130 loop and Gln226 within 220 loop, respectively. Interestingly, the L-rhamnose moiety linked to C-4-OH of D-glucose also exhibited great binding affinity with those critical residues in 220 loop, where the C-2-OH and C-4-OH of the L-rhamnose moiety formed stable hydrogen bonds with Lys222 and Gln226, respectively.

Finally, compound **10** was advanced to a mouse influenza A (Duck/Guangdong/212/2004 H5N1) model. As shown in Fig. 8, there was remarkable inhibition of the mortality for infected mice treated with **10** compared with the untreated mice. The protective effect of **10** on mice survival rate, was improved in a dose-dependent manner, though less effective than that provided by ribavirin. Taken together, these data indicate that **10** can protect mice from influenza virus H5N1 infection *in vivo*.

In the present study, we reported the synthesis and characterization of a series of 3-epiursolic acid saponins with 3-O-β-chacotriosyl residue generated from semisynthesis, and tested their anti-influenza A/Duck/Guangdong/99 (H5N1) virus activities in A549 cells. All the title pentacyclic triterpene saponins **4–13** showed no cytotoxicity to A549 cells, but showed certain antiviral activity with a potency ranging from moderate to potent. The subsequent SARs studies indicated that **10** showed the highest anti-H5N1 activity *in vitro* with IC_{50} at micromole level and significant antiviral potency *in vivo*. In particular, **10** not only inhibited virus-induced hemagglutination but also exhibited a strong affinity to HA with K_D value of 1.64 nM. These results suggested that the 3-epiursolic acid saponins might bind region of HA and interfere with influenza invasion by its interaction with the sialic acid receptor. Our study might establish the importance of 3-epiursolic acid derivatives for development of entry inhibitors of influenza viruses.

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.

Acknowledgements

This project was supported by the National Nature Science Foundation of China (31941019, 31872521); the Natural Science Foundation of Guangdong Province, China (2018A030313414); Science &Technology Project of Guangzhou, China (201804010457) and the special Project for prevention and control of SARS-CoV-2 from Educational Commission of Guangdong Province of China (2020KZDZX1039).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127518.

References

1. Meng WY, Yang QQ, Vrancken B, et al. *Emerg Microbes Infect.* 2019;8:823.
2. Zhong GX, Fan SF, Lopes TJS, et al. *Front Microbiol.* 2019;10:1411.
3. Zhu ZB, Li RM, Xiao GK, et al. *Eur J Med Chem.* 2012;57:211.
4. Zhao X, Li RF, Zhou Y, et al. *J Med Chem.* 2018;61:5187.
5. Bloom JD, Gong LI, Baltimore D. *Science.* 2010;328:1272.
6. Moscona A. *N Engl J Med.* 2005;353:2633.
7. Schade D, Kotthaus J, Riebling L, et al. *J Med Chem.* 2014;57:759.
8. Ekiert DC, Friesen RH, Bhabha G, et al. *Science.* 2011;333:843.
9. Liao YX, Chen LZ, Li SM, et al. *Bioorg Med Chem.* 2019;27:4048.
10. Li H, Chen LZ, Li SM, et al. Bioorg Med Chem Lett, 2019; 29: 2675.
11. Ye MD, Liao YX, Wu L, et al. *Viruses.* 2020;12:225.
12. Loregian A, Mercorelli B, Nannetti G, Compagnin C, Palù G. *Cell Mol Life Sci.* 2014;71:3659.
13. Whittle JRR, Zhang R, Khurana S, et al. *Proc Natl Acad Sci USA.* 2011;108:14216.
14. Laursen NS, Friesen RHE, Zhu XY, et al. *Science.* 2018;362:598.
15. Kadam RU, Wilson IA. *Proc Natl Acad Sci USA.* 2017;114:206.
16. Song GP, Shen XT, Li SM, et al. *Eur J Med Chem.* 2016;119:109.
17. van Dongen MJP, Kadam RU, Juraszek J, et al. *Science.* 2019;363:eaar6221.
18. Grishko VV, Galaiko NV, Tolmacheva IA, et al. *Eur J Med Chem.* 2014;83:601.
19. Wang C, Lu L, Na HY, et al. *J Med Chem.* 2014;54:7342.
20. Chen YY, Wang XC, Zhu YB, et al. *Mol Pharm.* 2020;17:2564.
21. Si LL, Meng K, Tian ZY, et al. *Sci Adv.* 2018;4:eaau8408.
22. Li HW, Li M, Xu RY, et al. *Eur J Med Chem.* 2019;163:560–568.
23. Shi YY, Si LL, Han X, et al. *Med Chem Commun.* 2017;8:1531.
24. Xiao SL, Tian ZY, Wang YF, Si LL, Zhang LH, Zhou DM. *Med Res Rev.* 2018;38:951.
25. Yu MR, Si LL, Wang YF, et al. *J Med Chem.* 2014;57:10058.
26. Mair CE, Grienke U, Wilhelm A, et al. *J Nat Prod.* 2018;81:515.
27. Song GP, Shen XT, Li SM, et al. *Eur J Med Chem.* 2015;93:431.
28. Wen XA, Sun HB, Liu J, et al. *J Med Chem.* 2008;51:3540.
29. Li SM, Jia XH, Shen XT, et al. *Bioorg Med Chem.* 2017;25:4384.