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# Screening and verification of antiviral compounds against HSV-1 using a method based on a plaque inhibition assay

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## Abstract

**Background** Herpes simplex virus type 1 (HSV-1) infection is a common viral disease that mainly causes oral lesions, but can also cause genital lesions in some instances. Current treatments with nucleoside analogs are limited by the emergence of drug resistance. Therefore, novel anti-HSV-1 drugs are urgently needed.

**Methods** In this study, we screened a library of 2080 compounds for anti-HSV-1 activity using a plaque formation assay. We selected 11 potential inhibitors of HSV-1 and further evaluated their antiviral effects by plaque reduction assay and real-time polymerase chain reaction (qPCR).

**Results** Five compounds, namely ginsenoside Rd, brassinolide, rosamultin, 3'-hydroxy puerarin, and clinafloxacin HCl, showed potent anti-HSV-1 activity and completely suppressed plaque formation at a concentration of 10  $\mu$ M. Among them, clinafloxacin HCl, a fluoroquinolone antibiotic, exhibited a high selectivity index for HSV-1.

**Conclusions** Our findings suggest that these five compounds have potential antiviral properties against HSV-1 and may have different mechanisms of action. Further studies are warranted to elucidate the antiviral mechanisms of these compounds and to explore their therapeutic potential for HSV-1 infection.

**Keywords** Antiviral drug screening, HSV-1, Plaque formation, Plaque inhibition test

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## Introduction

In children, HSV-1 is a common cause of mucosal diseases, including orofacial infections, keratitis, and encephalitis, which can lead to morbidity and mortality [1, 2]. Keratitis caused by HSV-1 infection usually results in corneal scarring and loss of vision [3]. Disseminated HSV-1 infection in the newborn will lead to neurodevelopmental disorders [4]. Increasing evidence suggests that HSV-1 is becoming an important infectious risk factor for neonatal and immunocompromised patients [5–7]. Stress may increase the susceptibility to primary HSV-1 infection and reactivate the latent virus in neurons [8]. Acyclovir is the most effective antiviral drug for HSV infections, as it has high selectivity, bioavailability, and safety [9]. This drug belongs to the class of nucleoside analogues, which are widely used for treating HSV infection [10, 11]. Drug resistance is a major concern when treating HSV-1 infections with nucleoside analogs, such as acyclovir. These drugs cannot prevent HSV-1 from establishing latency in neurons, which poses a major clinical challenge [10]. Moreover, immunosuppressed patients who undergo prolonged treatment may develop acyclovir-resistant strains [12, 13].

Drug screening is a crucial approach for identifying novel antiviral inhibitors. Most antiviral drug screenings for HSV focused on a functional target of the viral particle. Virtual screening using molecular docking of inhibitors and cyclin-dependent kinase 2 (CDK2) has been reported [14]. Infected cell protein 0 (ICP0 protein) of HSV has been used to screen inhibitors blocking its E3 Ubiquitin ligase activity in an in vitro screening assay [15].

Previously, an improved plaque assay-based high-throughput antiviral drug screening method has been documented [16]. In the current study, an antiviral drug screening was performed using a HSV-1/Vero system based on an improved plaque assay, screening 11 potential inhibitors from a library of 2080 compounds. This method based on a single infectious viral particle can theoretically help discover novel antiviral inhibitors associated with different viral life cycle stages including attachment, entry, replication, maturation and release.

## Materials and methods

### Cell cultures and viruses

We cultured Vero cells (CCL-81, ATCC) and SK-N-SH cells (HTB11, ATCC) in DMEM supplemented with 10% fetal bovine serum. To obtain the HSV-1 strain GZ21P2, which is sensitive to acyclovir, we isolated it from a clinical sample and purified it by selecting a single plaque under agarose overlay medium. We then propagated the purified viral stock of GZ21P2 in Vero cells and assessed its titers using the plaque formation method.

### Drug preparation

A library of 2,080 unique compounds (Table S1) was provided to us by Selleck (Shanghai) in 30  $\mu$ L tubes of 10 mM DMSO. The verification compounds were dissolved in DMSO as per the manufacturer's instructions. Subsequently, stock solutions of 100 mM or lower were prepared and diluted with DMEM to obtain different concentrations.

### Drug screening

We screened a library of 10 mM compounds for anti-HSV-1 activity using Vero cells infected with HSV-1 strain GZ21P2. We added 1.6  $\mu$ L of each compound to the overlay medium containing 1.2% RC-591 (FMC Polymer, USA) in 96-well plates (two replicates per well). We used acyclovir (100  $\mu$ M) as a positive control and DMSO as a negative control (four wells each). The final concentration of DMSO in each well was 1% (v/v). After four days, we fixed and stained the cells with formaldehyde (8%) and neutral red (0.3%). We considered the experiment valid if the positive control wells had no plaques and the negative control wells had plaques. We selected the compounds that prevented plaque formation (intact cell monolayer) as potential inhibitors. We tested these inhibitors again at 100  $\mu$ M, 50  $\mu$ M, and 10  $\mu$ M in a second round using the same procedure. The drugs that completely inhibited plaque formation at 10  $\mu$ M were effective candidate drugs for the following inhibition effect verification. We followed our previously published method with minor modifications for this part [16].

### CCK-8 cytotoxicity assay

In brief, SN-N-SH cells were initially seeded into 96-well plates at a density of  $1 \times 10^4$  cells per well and incubated at 37 °C in a 5% CO<sub>2</sub> incubator. After a 24-hour incubation, 100  $\mu$ L of medium containing different concentration of drugs were added to each well, replacing the previous medium. We also used DMSO as a zero-concentration negative control. The final concentration of DMSO in each well was 0.1% (v/v). Following another 48-hour incubation period, the culture medium in each well was again replaced with 100  $\mu$ L of fresh medium containing 10  $\mu$ L of CCK-8 solution (Dojindo). After a further two-hour incubation, the plates were subjected to absorbance measurement at 450 nm using a microplate reader. The 50% cytotoxic concentration (CC<sub>50</sub>) of each compound was estimated by performing a linear regression analysis. We followed the manufacturer's protocol for the CCK-8 solution.

### Plaque reduction assay

To assess the anti-HSV-1 effect of these compounds against HSV-1, Vero cells were grown in 24-well plates ( $5 \times 10^4$  cells/well). After 24 h, the cells were infected with

HSV-1 at a multiplicity of infection (MOI) of 0.1 for 1 h, and then the supernatant was removed and replaced with fresh DMEM containing various concentrations of the compounds (DMSO as a negative control with zero drug concentration). The final concentration of DMSO in each well is 0.1% (v/v). After 24 h of incubation, the cells were lysed by three cycles of freeze-thaw, and the supernatant was collected by centrifugation at 5,000 rpm. The supernatant was serially diluted, and 60  $\mu$ L of the dilutions was added to Vero cell monolayers in 96-well plates. After 1 h of incubation at 37°C, an overlay medium (100  $\mu$ L) containing 1.2% RC-591 was added directly to each well. The cells were then fixed and stained as described in the [drug screening](#) section. The number of plaques was counted, and viral inhibition percentage was calculated using the following formula: viral inhibition (%) =  $[1 - (\text{number of plaques})_{\text{inhibitor}} / (\text{number of plaques})_{\text{control}}] \times 100$  [17]. The half-maximal inhibitory concentration (IC<sub>50</sub>) was determined using Graphpad Prism 8.0.

#### Quantification of viral DNA

To evaluate the antiviral effects of candidate compounds based on the viral DNA concentration, we performed the following steps. SN-N-SH cells seeded in 24-well plates a density of  $4 \times 10^4$  cells per well. The cells were pre-treated with the different concentrations of the candidate

compounds for 24 hours, followed by addition of viral stock (0.1 MOI) to each well for 1 hour to allow infection. Subsequently, the supernatant was removed and cells were cultured with drug-containing medium at 37°C for an additional 24 hours. DMSO was used as a negative control with zero drug concentration. The final concentration of DMSO in each well is 0.1% (v/v). The viral DNA was extracted from the supernatant using the TIANamp Virus DNA/RNA Kit (Tiangen) and analyzed by qPCR. The primer pairs targeting the pol gene of HSV-1 (PL 5'-ATCAACTTCGACTGGCCCTTC-3'; and PR, 5'-CCGTACATGTCGATGTTTACC-3') were previously described by Lakeman and Whitley [18].

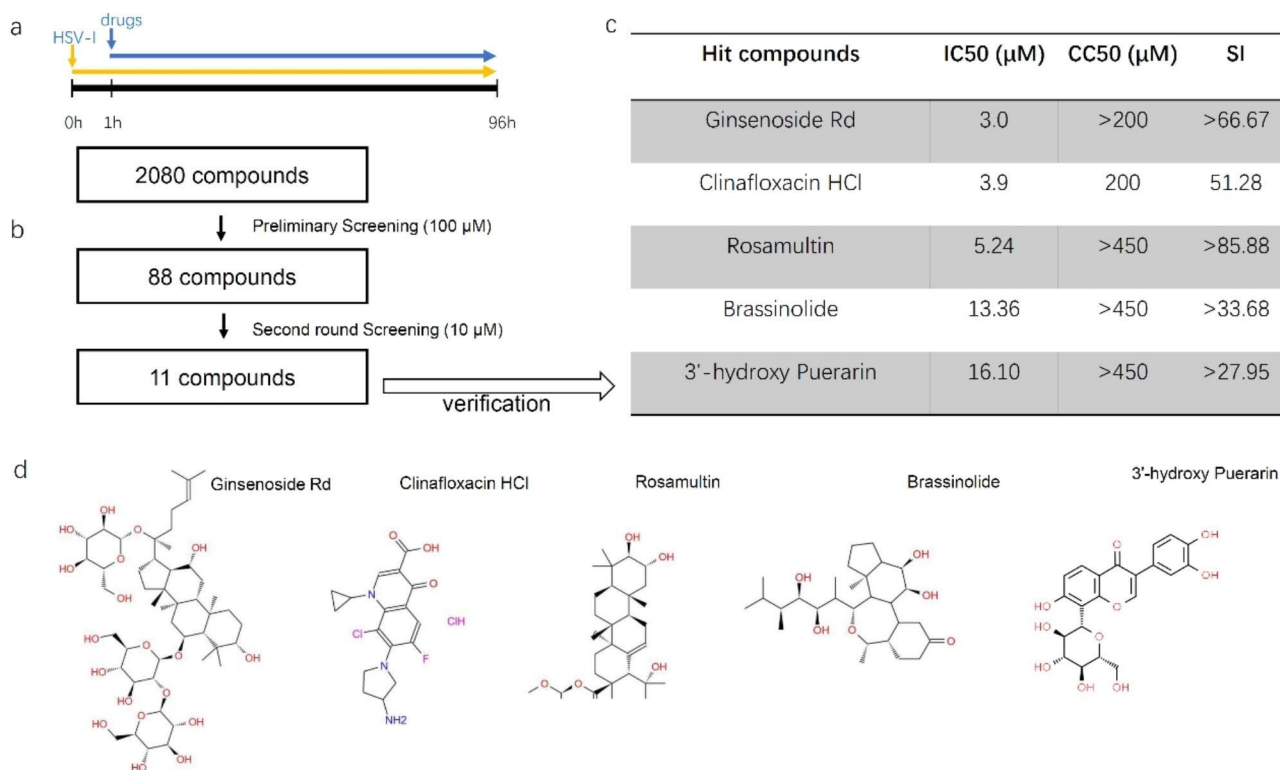
#### Statistical analysis

The data are expressed as the Mean  $\pm$  SD and analyzed using an unpaired Student's t-test. The level of significance was set at  $p < 0.05$ ,  $p < 0.01$ ,  $P < 0.001$  to determine the statistical significance of the results.

## Results

### Screening of a unique compound library for inhibitors of HSV-1

The diagram in Fig. 1 outlines the screening process used in this study (Fig. 1). In the initial screening phase, 88 compounds were evaluated at a concentration of 100  $\mu$ M.



**Fig. 1** Antiviral drug screening for inhibitors of HSV-1 from a 2080-compound library. (a) Timeline of drug screening. (b) Flowchart for drug screening. (c) Verification of five inhibitors. This result is based on plaque reduction effects of candidate inhibitors and their cytotoxicity against SK-N-SH cells. (d) Chemical structures of five candidate inhibitors

These compounds demonstrated complete inhibition of plaque formation, whereas the remaining compounds in the drug library did not exhibit this effect. Additionally, those compounds that exhibited cytotoxic activity against Vero cells completely caused cellular detachment at the bottom of wells in 96-well plates. From the initial screening, 11 candidate inhibitors were selected in the second round of screening. These compounds were found to completely suppress plaque formation at a concentration of 10  $\mu$ M (Fig. 1).

### Cytotoxicity

The majority of the 11 compounds evaluated in this study displayed negligible cytotoxicity against SK-N-SH cells, with the exception of ginsenoside Rd and cinafloxacin HCl, which exhibited cytotoxic effects at concentrations exceeding 200  $\mu$ M and 150  $\mu$ M, respectively. Interestingly, certain compounds appeared to stimulate SK-N-SH growth at specific concentrations (Fig. 2).

### Verification of candidate Drugs

To verify the antiviral activity of the 11 candidate drugs that were identified by our screening assay, we conducted the plaque reduction assay, which is widely regarded as the gold standard method in virology. Ginsenoside Rd, brassinolide, rosamultin, 3'-hydroxy puerarin and cinafloxacin HCl significantly inhibited virus production at the concentration of 100  $\mu$ M (Fig. 3a). The calculated selectivity index (SI,  $CC_{50}/IC_{50}$ ) were displayed in Fig. 1c. The plaque reduction assay of brassinolide was shown in Fig. S1.

To investigate the compounds' inhibitory effects on viral replication, relative quantification of viral DNA was performed. The results demonstrated that these compounds can dose-dependently reduce HSV-1 DNA, with ginsenoside Rd and Clinafloxacin HCl exhibiting significant inhibitory effects at a concentration of 5  $\mu$ M (Fig. 4).

### Discussion

The current treatment utilizing nucleoside analogs that interfere with viral polymerase is indeed effective at eliminating a sensitive HSV strain in patients with acute infections. However, these drugs are ineffective for latent infections due to their poor availability to the nervous system. A promising drug candidate, IM-250, a helicase-primase inhibitor with sufficient exposure to the target tissue, has been reported to be effective against latent neural HSV infections [19, 20]. Nevertheless, this novel anti-HSV inhibitor has yet to be approved for clinical use.

The 11 lead drug candidates identified in this study are from natural sources with the exception of cinafloxacin HCl, a synthetic antibiotic. These drugs exhibit a wide range of bioactivities, such as anti-inflammatory, anti-tumor and antibacterial effects. One of the most

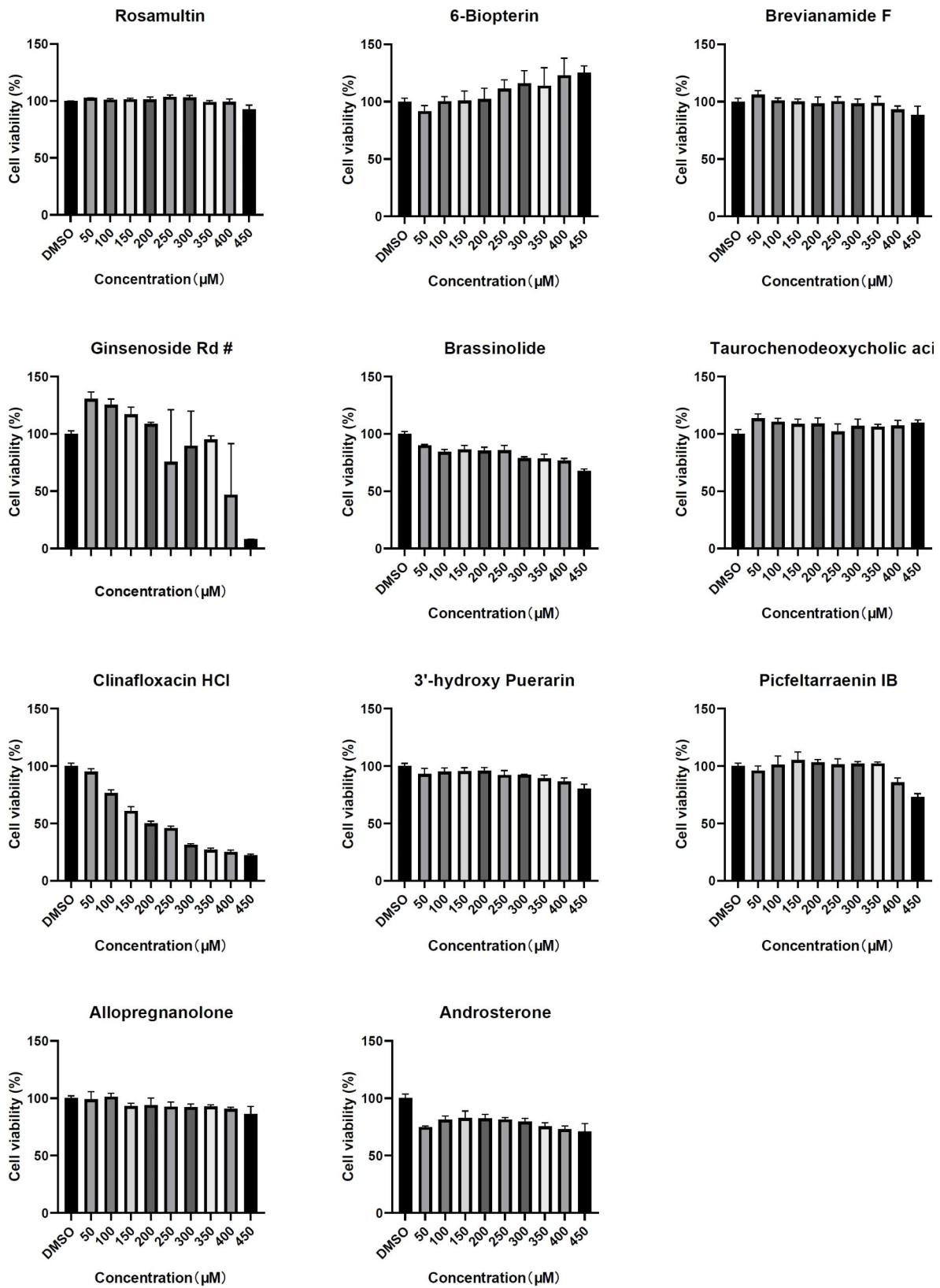
important inclusion criteria was that the drugs had not been reported to have anti-HSV-1 activity in published literature. Some may have been reported in patents, but there are no practical examples.

Brassinolide is a plant hormone well-known for its versatile roles in promoting cell elongation, plant growth, seed germination, and responses to stress [21]. Epibrassinolide, which differs slightly from brassinolide in molecular conformation, has proven to be pro-apoptotic to cancer cells through the Wnt signaling pathway [22]. Our results showed that brassinolide can significantly suppress the HSV-1 proliferation in SK-N-SH cells, which was confirmed by plaque reduction assay and real time PCR. In general, this is consistent with the description of two published patents [23, 24].

Ginsenoside is a compound isolated from the root of *Panax ginseng* Meyer, a traditional herbal medicine used in East Asian countries. Ginsenoside 20(R)-Rh2 was reported to possess inhibitory effects on the replication of mice and human gammaherpesviruses [25]. Ginsenoside 20(S)-Rg3 demonstrated inhibitory effects on both HSV-1 and HSV-2 [26]. Ginsenoside Rb1 could inhibit nerve cell apoptosis caused by HSV-1 infections. Ginsenoside Rd is also a multi-functional natural compound, effective in neurologic disorders, cardiovascular diseases and tumors [27–30]. This type of ginsenoside has not been reported to have significant antiviral activity against any virus [31]. In our experiment, Ginsenoside Rd can significantly inhibit the HSV-1 at a low concentration ( $IC_{50}=3.0$ ,  $SI>66.67$ ) in vitro, although it showed obvious cytotoxicity against SK-N-SH cells at concentrations higher than 200  $\mu$ M.

Rosamultin, isolated from *Rosa rugosa* root, has been reported to possess anti-hepatotoxic effects, protective effects against  $H_2O_2$ -induced oxidative stress, and anti-apoptosis in cardiomyocytes [32, 33]. Additionally, it was reported that rosamultin exhibits antiviral activity by inhibiting HIV-1 protease in vitro [34]. 3'-hydroxy puerarin, one of isoflavones isolated from the flowers of *Pueraria lobata*, has been identified as an inhibitor of lactate dehydrogenase [35]. Our study demonstrated that both compounds can inhibit HSV-1 at high concentrations, although no antiviral activity of these plant-derived compounds has been reported previously. Picfeltaarraenin IB, one of cucurbitacins isolated from *Picris fel-terrae*, showed no antiviral activity against HSV [36]. However, our results indicate that it can slightly inhibit the replication of HSV-1 in SK-N-SH cells at the concentration of 100  $\mu$ M (Fig. 3).

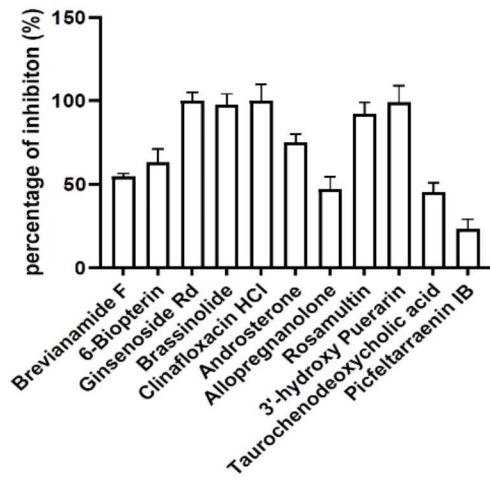
As a member of the fourth generation fluoroquinolones with broad antibacterial activity, cinafloxacin can inhibit both gram-negative and gram-positive bacteria by targeting DNA gyrase or topoisomerase [37]. To date, although fluoroquinolones such as enoxacin, ciprofloxacin,



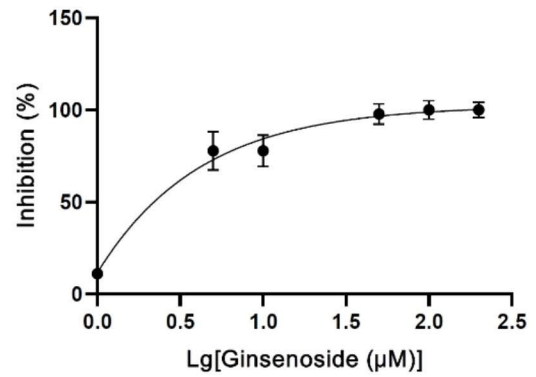
**Fig. 2** Cell viability of 11 candidate inhibitors of HSV-1. After adding candidate inhibitors with different concentrations, the viability of SK-N-SH cells was measured by a cell counting kit (CCK-8)



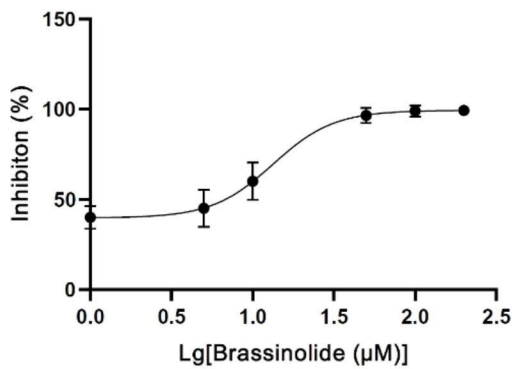
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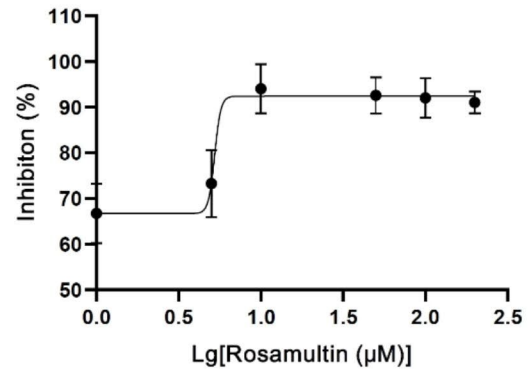
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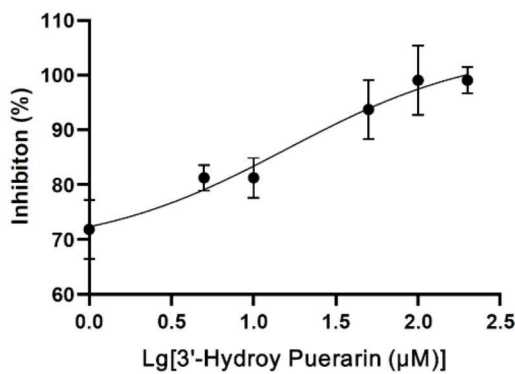
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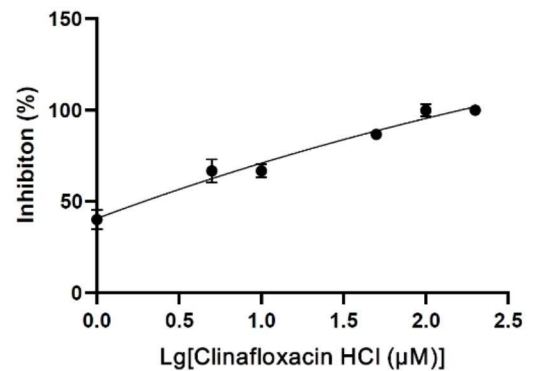
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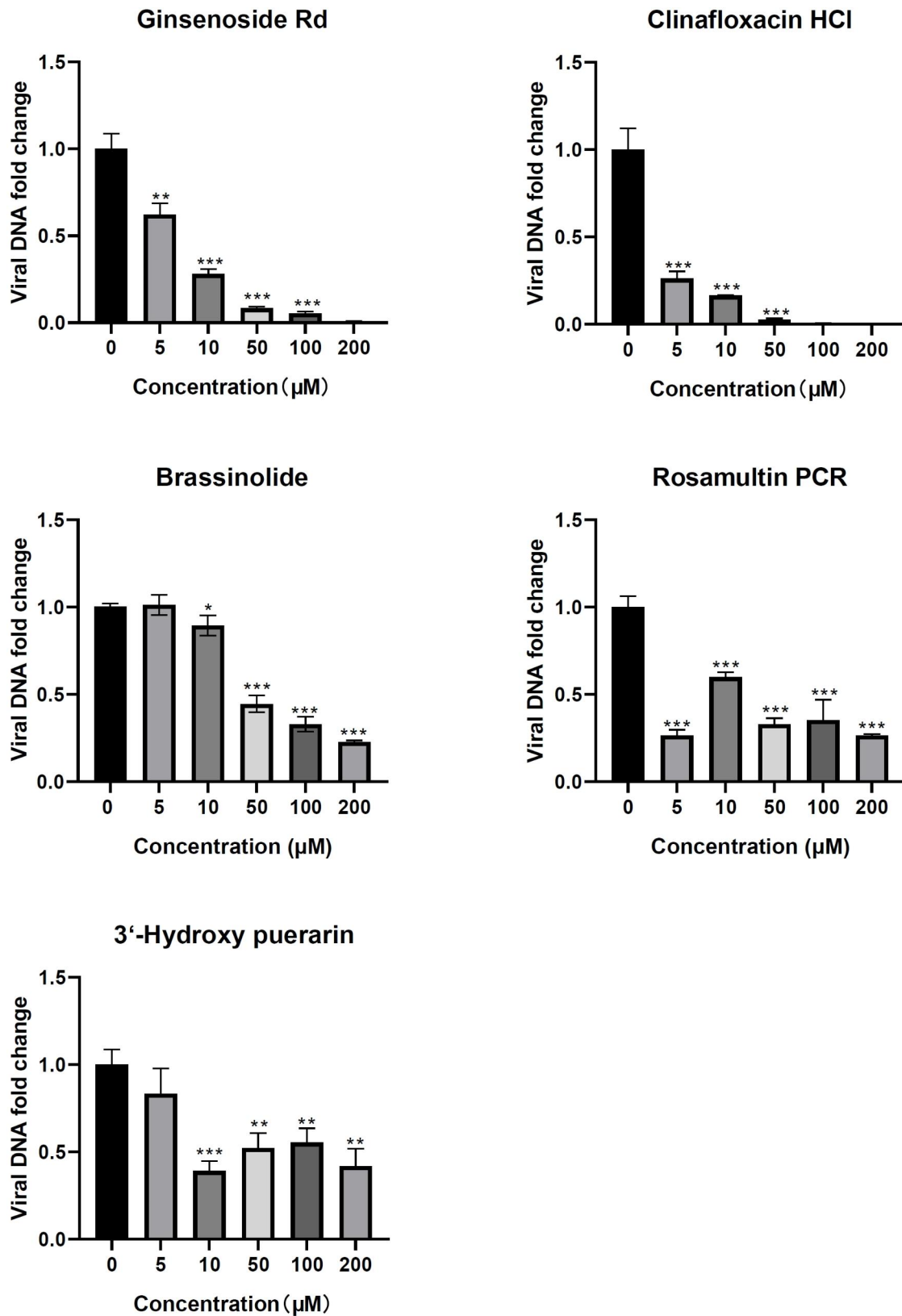
**e**



**f**



**Fig. 3** Percent inhibition of 11 candidate inhibitors against HSV-1 and dose-response effects of five inhibitors(a) Percent inhibition at the concentration of 100 μM. This is based on results of plaque reduction assay. This concentration is also used in first-round screening. (b-g) Dose-response curves of five inhibitors. A log transformation of these concentrations has been conducted



**Fig. 4** Inhibitory effects by relatively quantifying viral DNA in infected SK-N-SH cells. Data are presented as mean + SD. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 vs. '0' group

levofloxacin, and moxifloxacin have been reported to exhibit low antiviral activity against SARS-CoV-2 and MERS-CoV in vitro, no literature has shown that clinafloxacin has antiviral effects [38]. In our study, clinafloxacin HCl, with much higher solubility than clinafloxacin itself, demonstrated significant anti-HSV-1 activity in vitro, although its antiviral mechanism remains unclear.

Brevianamide F, a tryprostatin-type compound isolated from actinomycete, is a potential natural plant growth inhibitor or a broad-spectrum systemic herbicide [39]. On the other hand, brevianamide F has shown good anti-BCG (tuberculosis) activity [40].

6-biopterin, a cofactor of NO synthase, is an oxidized product of (6R)5,6,7,8 tetrahydrobiopterin (6-BH4) and exhibits extreme cytotoxicity to human melanocytes [41]. In our cytotoxicity test, 6-biopterin seems to be able to promote SK-N-SH cell growth in vitro (Fig. 2). On the other hand, it can suppress HSV-1 growth in host cells (Fig. 3).

Taurochenodeoxycholic acid (TCDCa), a bioactive substance of animal bile produced by the liver, acts as an agonist of farnesoid X receptor (FXR) in the digestive system [42, 43]. While it has been proposed as a compound for treating enveloped viral infections such as influenza, parainfluenza, human immunodeficiency, and herpes viruses, no antiviral activity of this compound has been reported against HSV-1 so far [44]. Allopregnanolone, an endogenous reproductive neurosteroid, plays a vital role in controlling inflammatory processes and behavior. Owing to its neuroprotective effect, allopregnanolone also plays an important role in promoting fetal brain development [45]. It can affect the inflammatory reaction and repair alveolar respiratory epithelium damaged by influenza virus infection [46]. Epiandrosterone (EA) and Dehydroepiandrosterone (DHEA) demonstrated in vitro antiviral activity against Junin virus (JUNV) and adenovirus (AdV) replication by inhibiting protein synthesis [47, 48].

In a previous study, a screening method was employed to discover anti-EV71 inhibitors from a small library, and several candidate drugs belonging to flavonoids were identified in the final [16]. This screening strategy does not target any specific part of viruses or host cells, allowing for the inclusion of almost all kinds of antiviral compounds in screened libraries. While this may increase the success rate of discovering effective antiviral drugs, it may also result in a larger workload for efficacy verification and mechanism research, particularly when numerous candidate drugs are identified after the final screening round.

The plaque inhibition test was performed to screen for potential inhibitors of HSV-1 infection. A total of 88 compounds were identified as candidates at a concentration of 100  $\mu$ M, which completely blocked the viral life-cycle and prevented the formation of plaques on Vero

cell monolayers by single infectious HSV-1 particles. This concentration was chosen to simplify the interpretation of results by using a binary criterion (presence or absence of plaques). A dose-dependent reduction of plaque size would be expected in a plaque inhibitory test, but it was not assessed in this screening round.

We have identified 11 potential inhibitors of HSV-1 infection by using an improved plaque inhibition test in a two-round screening process. Five of these inhibitors were confirmed by two independent methods in a short time. These inhibitors may have novel mechanisms of action that are not yet known, or they may have existing antiviral effects that have not been reported before. To further explore the mode of action of these inhibitors, we plan to conduct more experiments to investigate how they affect different stages of the viral replication cycle, such as attachment, entry, uncoating, genome replication, assembly, and release. This will help us understand the molecular basis of their antiviral activity and optimize their therapeutic potential.

#### Abbreviations

HSV-1	Herpes simplex virus type 1
qPCR	Real time polymerase chain reaction
MOI	Multiplicity of infection
CC <sub>50</sub>	The 50% cytotoxic concentration
IC <sub>50</sub>	The half maximal inhibitory concentration

#### Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12879-023-08843-3>.

Supplementary Material 1

Supplementary Material 2

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Not applicable.

#### Authors' contributions

Yingxian Yin and Jiahui Li wrote the main manuscript text. Qingqun Lv and Misi Xiao cultured the cells and finished drug screening. Changbing Wang, Dan Zeng and Yiling Gu tested the titer of virus. Minxia Chen and Fengxia Yang prepared the compound library. Ling Su and Zhiying Ou analysed the results. Shaojuan Feng, Wanming Hu and Fengling Bu did statistical analysis. Bing Zhu and Yi Xu edited the manuscript. All authors reviewed the manuscript.

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#### Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.



## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare no competing interests.

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## References

- Whitley RJ, Roizman B. Herpes simplex virus Infections. *Lancet*. 2001;357(9267):1513–8. [https://doi.org/10.1016/S0140-6736\(00\)04638-9](https://doi.org/10.1016/S0140-6736(00)04638-9).
- Arduino PG, Porter SR. Herpes simplex virus type 1 Infection: overview on relevant clinico-pathological features. *J Oral Pathol Med*. 2008;37(2):107–21. <https://doi.org/10.1111/j.1600-0714.2007.00586.x>.
- Rowe AM, St Leger AJ, Jeon S, Dhaliwal DK et al. 2013. Herpes keratitis. *Prog Retin Eye Res*. 32:88–101. <https://doi.org/10.1016/j.preteyeres.2012.08.002>.
- Pinninti SG, Kimberlin DW. Neonatal herpes simplex virus Infections. *Semin Perinatol*. 2018;42(3):168–75. <https://doi.org/10.1053/j.semperi.2018.02.004>.
- Kennedy PG, Chaudhuri A. Herpes simplex encephalitis. *J Neurol Neurosurg Psychiatry*. 2002;73(3):237–8. <https://doi.org/10.1136/jnnp.73.3.237>.
- Pires de Mello CP, Bloom DC, Paixao IC. Herpes simplex virus type-1: replication, latency, reactivation and its antiviral targets. *Antivir Ther*. 2016;21(4):277–86. <https://doi.org/10.3851/IMP3018>.
- Rechenchoski DZ, Faccin-Galhardi LC, Linhares REC, Nozawa C. Herpesvirus: an underestimated virus. *Folia Microbiol (Praha)*. 2017;62(2):151–6. <https://doi.org/10.1007/s12223-016-0482-7>.
- Yan C, Luo Z, Li W, Li X, et al. Disturbed Yin-Yang balance: stress increases the susceptibility to primary and recurrent Infections of herpes simplex virus type 1. *Acta Pharm Sin B*. 2020;10(3):383–98. <https://doi.org/10.1016/j.apsb.2019.06.005>.
- Klysik K, Pietraszek A, Karewicz A, Nowakowska M. Acyclovir in the Treatment of Herpes Viruses - a review. *Curr Med Chem*. 2020;27(24):4118–37. <https://doi.org/10.2174/0929867325666180309105519>.
- Vere Hodge RA, Field HJ. Antiviral agents for herpes simplex virus. *Adv Pharmacol*. 2013;67:1–38. <https://doi.org/10.1016/B978-0-12-405880-4.00001-9>.
- De Clercq E. Antivirals: past, present and future. *Biochem Pharmacol*. 2013;85(6):727–44. <https://doi.org/10.1016/j.bcp.2012.12.011>.
- Anton-Vazquez V, Mehra V, Mbisa JL, Bradshaw D, et al. Challenges of acyclovir-resistant HSV Infection in allogeneic bone marrow transplant recipients. *J Clin Virol*. 2020;128:104421. <https://doi.org/10.1016/j.jcv.2020.104421>.
- Smith RL, Morroni J, Wilcox CL. Lack of effect of treatment with penciclovir or acyclovir on the establishment of latent HSV-1 in primary sensory neurons in culture. *Antiviral Res*. 2001;52(1):19–24. [https://doi.org/10.1016/S0166-3542\(01\)00155-3](https://doi.org/10.1016/S0166-3542(01)00155-3).
- Viegas DJ, Edwards TG, Bloom DC, Abreu PA. Virtual screening identified compounds that bind to cyclin dependent kinase 2 and prevent herpes simplex virus type 1 replication and reactivation in neurons. *Antiviral Res*. 2019;172:104621. <https://doi.org/10.1016/j.antiviral.2019.104621>.
- Deschamps T, Waisner H, Dogrammatzis C, Roy A, et al. Discovery of small-molecule inhibitors targeting the E3 ubiquitin ligase activity of the Herpes Simplex Virus 1 ICPO protein using an in Vitro High-Throughput Screening Assay. *J Virol*. 2019;93(13). <https://doi.org/10.1128/JVI.00619-19>.
- Yin Y, Xu Y, Ou Z, Yang X, et al. An antiviral drug screening system for enterovirus 71 based on an improved plaque assay: a potential high-throughput method. *J Med Virol*. 2019;91(8):1440–7. <https://doi.org/10.1002/jmv.25463>.
- Luo Z, Kuang XP, Zhou QQ, Yan CY, et al. Inhibitory effects of baicalin against herpes simplex virus type 1. *Acta Pharm Sin B*. 2020;10(12):2323–38. <https://doi.org/10.1016/j.apsb.2020.06.008>.
- Lakeman FD, Whitley RJ. Diagnosis of herpes simplex encephalitis: application of polymerase chain reaction to cerebrospinal fluid from brain-biopsied patients and correlation with Disease. National Institute of Allergy and Infectious Diseases Collaborative Antiviral Study Group. *J Infect Dis*. 1995;171(4):857–63. <https://doi.org/10.1093/infdis/171.4.857>.
- Gege C, Bravo FJ, Uhlig N, Hagmaier T et al. 2021. A helicase-primase drug candidate with sufficient target tissue exposure affects latent neural herpes simplex virus Infections. *Sci Transl Med*, 13 (598). <https://doi.org/10.1126/scitranslmed.abf8668>.
- Uhlig N, Donner AK, Gege C, Lange F, et al. Helicase primase inhibitors (HPIs) are efficacious for therapy of human herpes simplex virus (HSV) Disease in an Infection mouse model. *Antiviral Res*. 2021;195:105190. <https://doi.org/10.1016/j.antiviral.2021.105190>.
- Saini S, Sharma I, Pati PK. Versatile roles of brassinosteroid in plants in the context of its homeostasis, signaling and crosstalks. *Front Plant Sci*. 2015;6:950. <https://doi.org/10.3389/fpls.2015.00950>.
- Sadava D, Kane SE. The effect of brassinolide, a plant steroid hormone, on drug resistant small-cell lung carcinoma cells. *Biochem Biophys Res Commun*. 2017;493(1):783–7. <https://doi.org/10.1016/j.bbrc.2017.08.094>.
- Maria Crisler ED, J.Phillip KENNEDY. In: SHAMAN NATURALS,LLC,Rock, Springs, editors. Composition for preventing and treating viral Infections. WY(US); 2021. United States.
- Jana Oklestkova LH, Steigerova J, Kohout L, Kolar Z. Miroslav Strnad.2010. Natural brassinosteroids for use for treating hyperproliferation, treating proliferative diseases and reducing adverse effects of steroid dysfunction in mammals, pharmaceutical composition and its use. In., United States.
- Kang S, Im K, Kim G, Min H. Antiviral activity of 20(R)-ginsenoside Rh2 against murine gammaherpesvirus. *J Ginseng Res*. 2017;41(4):496–502. <https://doi.org/10.1016/j.jgr.2016.08.010>.
- Wright S, Altman E. Inhibition of Herpes Simplex Viruses, types 1 and 2, by Ginsenoside 20(S)-Rg3. *J Microbiol Biotechnol*. 2020;30(1):101–8. <https://doi.org/10.4014/jmb.1908.08047>.
- Wang H, Jiang N, Lv J, Huang H, et al. Ginsenoside Rd reverses cognitive deficits by modulating BDNF-dependent CREB pathway in chronic restraint stress mice. *Life Sci*. 2020;258:118107. <https://doi.org/10.1016/j.lfs.2020.118107>.
- Zhang N, An X, Lang P, Wang F, et al. Ginsenoside Rd contributes the attenuation of cardiac hypertrophy in vivo and in vitro. *Biomed Pharmacother*. 2019;109:1016–23. <https://doi.org/10.1016/j.biopha.2018.10.081>.
- Ren K, Li S, Ding J, Zhao S, et al. Ginsenoside Rd attenuates mouse experimental autoimmune neuritis by modulating monocyte subsets conversion. *Biomed Pharmacother*. 2021;138:111489. <https://doi.org/10.1016/j.biopha.2021.111489>.
- Liu GM, Lu TC, Sun ML, Jia WY et al. 2020. Ginsenoside Rd inhibits Glioblastoma Cell Proliferation by Up-Regulating the expression of miR-144-5p. *Biol Pharm Bull*, 43 (10):1534–41. <https://doi.org/10.1248/bpb.b20-00338>.
- Song JH, Choi HJ, Song HH, Hong EH, et al. Antiviral activity of ginsenosides against coxsackievirus B3, enterovirus 71, and human rhinovirus 3. *J Ginseng Res*. 2014;38(3):173–9. <https://doi.org/10.1016/j.jgr.2014.04.003>.
- Cheol Park J, Kim C, Moon Hur S, Choi J, S.H., et al. Anti-hepatotoxic effects of Rosa rugosa root and its compound, rosamultin, in rats intoxicated with bromobenzene. *J Med Food*. 2004;7(4):436–41. <https://doi.org/10.1089/jmf.2004.7.436>.
- Zhang L, Liu Y, Li JY, Li LZ et al. 2018. Protective Effect of Rosamultin against H2O2-Induced Oxidative Stress and Apoptosis in H9c2 Cardiomyocytes. *Oxid Med Cell Longev*, 2018:8415610. <https://doi.org/10.1155/2018/8415610>.
- Park JC, Kim SC, Choi MR, Song SH, et al. Anti-HIV protease activity from rosa family plant extracts and rosamultin from Rosa rugosa. *J Med Food*. 2005;8(1):107–9. <https://doi.org/10.1089/jmf.2005.8.107>.
- Li S, Li S, Liu C, Liu C, et al. Extraction and isolation of potential anti-stroke compounds from flowers of Pueraria lobata guided by in vitro PC12 cell model. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2017;1048:111–20. <https://doi.org/10.1016/j.jchromb.2017.02.009>.
- Huang Y, De Bruyne T, Apers S, Ma Y, et al. Complement-inhibiting cucurbitacin glycosides from Picria fel-terrae. *J Nat Prod*. 1998;61(6):757–61. <https://doi.org/10.1021/np9705773>.
- Jorgensen JH, Weigel LM, Swenson JM, Whitney CG, et al. Activities of clinafloxacin, gemifloxacin, and trovafloxacin against recent clinical isolates of levofloxacin-resistant Streptococcus pneumoniae. *Antimicrob Agents Chemother*. 2000;44(11):2962–8. <https://doi.org/10.1128/AAC.44.11.2962-2968.2000>.
- Scroggs SLP, Offerdahl DK, Flather DP, Morris CN et al. 2020. Fluoroquinolone antibiotics exhibit low antiviral activity against SARS-CoV-2 and MERS-CoV. *Viruses*, 13 (1). <https://doi.org/10.3390/v13010008>.
- Zhang Q, Wang SQ, Tang HY, Li XJ, et al. Potential allelopathic indole diketopiperazines produced by the plant endophytic aspergillus fumigatus using the one strain-many compounds method. *J Agric Food Chem*. 2013;61(47):11447–52. <https://doi.org/10.1021/jf403200g>.

40. Huang P, Xie F, Ren B, Wang Q, et al. Anti-MRSA and anti-TB metabolites from marine-derived *Verrucosipora* sp. MS100047. *Appl Microbiol Biotechnol*. 2016;100(17):7437–47. <https://doi.org/10.1007/s00253-016-7406-y>.
41. Schallreuter KU, Buttner G, Pittelkow MR, Wood JM et al. ,1994. Cytotoxicity of 6-biopterin to human melanocytes. *Biochem Biophys Res Commun*, 204(1):43–8. <https://doi.org/10.1006/bbrc.1994.2423>.
42. Li L, Liu C, Mao W, Tumen B et al. ,2019. Taurochenodeoxycholic Acid inhibited AP-1 activation via stimulating glucocorticoid receptor. *Molecules*, 24(24). <https://doi.org/10.3390/molecules24244513>.
43. Waise TMZ, Lim YM, Danaei Z, Zhang SY, et al. Small intestinal taurochenodeoxycholic acid-FXR axis alters local nutrient-sensing gluco regulatory pathways in rats. *Mol Metab*. 2021;44:101132. <https://doi.org/10.1016/j.molmet.2020.101132>.
44. Sharpe C. James 1989. Bile acids for treatment of viral infections. In Office, E. P, editor, *WO1990003172A2*. United Kingdom.
45. Brunton PJ, Russell JA, Hirst JJ. Allopregnanolone in the brain: protecting pregnancy and birth outcomes. *Prog Neurobiol*. 2014;113:106–36. <https://doi.org/10.1016/j.pneurobio.2013.08.005>.
46. Pinna G. Sex and COVID-19: a protective role for Reproductive steroids. *Trends Endocrinol Metab*. 2021;32(1):3–6. <https://doi.org/10.1016/j.tem.2020.11.004>.
47. Acosta EG, Bruttomesso AC, Bisceglia JA, Wachsman MB, et al. Dehydroepiandrosterone, epiandrosterone and synthetic derivatives inhibit Junin virus replication in vitro. *Virus Res*. 2008;135(2):203–12. <https://doi.org/10.1016/j.virusres.2008.03.014>.
48. Romanutti C, Bruttomesso AC, Castilla V, Galagovsky LR, et al. Anti-adenovirus activity of epiandrosterone and dehydroepiandrosterone derivatives. *Chemotherapy*. 2010;56(2):158–65. <https://doi.org/10.1159/000313530>.

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