iPSC Neuronal Assay Identifies Amaryllidaceae Pharmacophore with Multiple Effects against Herpesvirus Infections

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S Supporting Information

ABSTRACT: The Amaryllidaceae alkaloid *trans*-dihydrolycoricidine **7** and three analogues **8–10** were produced via asymmetric chemical synthesis. Alkaloid **7** proved superior to acyclovir, the current standard for herpes simplex virus, type 1 (HSV-1) infection. Compound **7** potently inhibited lytic HSV-1 infection, significantly reduced HSV-1 reactivation, and more potently inhibited varicella zoster virus (VZV) lytic infection. A configurationally defined (3*R*)-secondary alcohol at C3 proved crucial for efficacious inhibition of lytic HSV-1 infection.

KEYWORDS: *Herpesvirus, HSV-1, varicella zoster, stem cells, Amaryllidaceae, alkaloid*

Herpes simplex virus, type 1 (HSV-1) is a ubiquitous DNA virus from the Herpesviridae family that causes substantial human morbidity and mortality.¹ With seropositivity rates exceeding 70% in elders, it causes recurrent cold sores, corneal infection, blindness, and, rarely, encephalitis.¹ It establishes latent infection in sensory neurons that is refractory to clearance, producing an intractable lifelong reservoir that cycles with lytic infections. Though acute HSV-1 infection is readily treatable with nontoxic nucleoside analogues such as acyclovir (ACV), no drug successfully eliminates the latent infection. In addition, growing ACV drug resistance has further fueled the search for novel antivirals.²

Plants of the Amaryllidaceae family produce numerous structurally diverse, biologically active alkaloids.^{3,4} In particular, alkaloids of the lycorane subclass including lycorine **1**, pseudolycorine **2**, pancratistatin **3**, its 7-deoxy analogue **4**, and the unsaturated derivatives narciclasine **5** and deoxy analogue **6** have demonstrated a variety of antiviral activities (Figure 1).^{5–13} The restricted availability of other natural lycorane-type alkaloids and the absence of a rapid synthetic route toward structural analogues has impeded further development of the antiviral pharmacophore.

The development of synthetic strategies that allow rapid access to functionally dense, stereochemically defined cores not

Figure 1. Structure of antiviral Amaryllidaceae alkaloids. Alkaloids **1–7** are natural products. Compounds **7–10** were accessed via total synthesis. Lycorine **1**, pseudolycorine **2**, pancratistatin **3**, 7-deoxypancratistatin **4**, narciclasine **5**, 7-deoxynarciclasine **6**, *trans*-dihydrolycoricidine **7**, 3-*epi-trans*-dihydrolycoricidine **8**, and 3-deoxy-*trans*-dihydrolycoricidine **9**.

only enables the asymmetric synthesis of biologically active natural products, but opens synthetic entry to non-natural analogues. These analogues expand the structural diversity

Received: August 6, 2015

Accepted: November 19, 2015

Published: November 19, 2015

available for biological assessment facilitating the discovery of previously unknown effects, improving our understanding of the structural requirements for such biological activity.

We recently reported the total asymmetric synthesis of *trans*-dihydrolycoricidine **7** employing a convergent, stepwise organocatalytic [3 + 3]-Michael-aldol annulation of substituted acetone derivative **13a** onto functionalized cinnamaldehyde **12**,¹³ catalyzed by diarylprolinol ether **11** (TMS = trimethylsilyl). This crucial step allows for the asymmetric entry to functionalized, nitrogen-substituted cyclohexanes, in one step, and could be conducted at gram scale. In this case,¹³ compound **14a** was converted to alkaloid **7** in a short sequence of subsequent transformations involving epoxidation and 2,3-diaxial diol installation, *Scheme 1*.

Scheme 1^a

^aAsymmetric organocatalytic Michael-aldol [3 + 3]-cycloaddition reaction of cinnamaldehyde derivative **12** and azido-acetone derivatives **13a** allows a one-step access to stereochemically defined cyclohexanol **14a** permitting rapid asymmetric entry to natural alkaloid **7**, while reaction of **13b** leads to **14b**, permitting access to C3-epimeric and deoxy analogues **8**, **9**, and **10**.

In order to extend the [3 + 3]-Michael-aldol methodology to obtain more oxygenated cyclohexane derivatives directly, we have now investigated the reaction of **12** with the nonsymmetrical 1,3-disubstituted benzyloxy-azidoacetone derivative **13b** (*Scheme 1*). To our delight, this reaction was found to proceed in a highly regioselective fashion with the initial Michael-addition being fully directed by the azido group, allowing a direct entry to cyclohexanol **14b**. The complex cyclohexane product **14b** was obtained directly as a precipitate that formed during the reaction in 73% isolated yield and did not require chromatographic purification. The reaction also proved to be highly diastereoselective (dr = 96%) with the major diastereomer being essentially homochiral (>99% ee). The relative and absolute stereochemistry of this major cycloadduct was determined as α -benzyloxy at C3 as shown in **14b**. This data was confirmed by single crystal X-ray analysis of the 4-bromobenzoate analogue **15** (see *Scheme 2* and *Supporting Information*).

With rapid access to compounds of the C3-epimeric series now available (in comparison to natural products **3–7**), we converted **14b** to compound **8**, the C3-epimer of natural product **7**, as shown in *Scheme 2*. Catalytic hydrogenation of the azido group and *in situ* protection as the methoxycarbonylamino ketone **16** was successfully achieved from **14b** using dimethoxydicarbonate (DMDC). The ketone **16** was subjected to axial hydride reduction and the desired C4-equatorial alcohol protected alongside the C2-axial hydroxyl group to give the orthogonally protected triol **18**. Cyclization of **18** was achieved using the Banwell modification of the Bischler–Napieralski reaction to give **19** with high regioselectivity (no minor diastereomer detected), which was now deprotected to give the target molecule **8**.

Scheme 2^a

^aReagents and conditions: Yields of isolated products (%) are indicated. (a) **13b** (1.00 equiv), **12** (1.05 equiv), **11** (10 mol %), quinidine (10 mol %), CH₂Cl₂, −10 °C → RT, 73%. (b) DMDC (3.0 equiv), H₂, 10% Pd/C (0.075 equiv), EtOAc, 50 psi, RT, 79%. (c) Me₄NHB(OAc)₃ (4.0 equiv), AcOH (10.0 equiv), MeCN, RT, 90%. (d) AcCl (100 equiv). (e) Tf₂O (5.0 equiv), DMAP (3.0 equiv), CH₂Cl₂, 0 °C → RT, 73%. (f) K₂CO₃ (0.10 equiv), H₂O/MeOH 9:1, RT, 81%. DMDC = dimethyl dicarbonate, Tf = triflic, DMAP = 4-dimethylaminopyridine, Moc = methyloxycarbonyl.

Access to compound **8** was exceedingly efficient given the one-pot asymmetric entry to the azido-cyclitol **14b** and high yields obtained in conversion to **8** as shown in *Scheme 2*. The C3-deoxy analogue **9** and the acyclic intermediate **10** were now prepared via a similar [3 + 3]-Michael-aldol annulation of azidoacetone **13** and cinnamaldehyde derivative **12**, as outlined in *Supplemental Schemes 1 and 3*.

With synthetic access to the natural alkaloid **7**, C-3-epimer **8**, C-3-deoxy analogue **9**, and acyclic compound **10** now on-hand and, in conjunction with natural products **3** and **5**, we proceeded with their antiviral assessment.

Based on an earlier report showing the anti-HSV-1 activity of alkaloids derived from Amaryllidaceae,¹² we initially evaluated the efficacy of compounds **3**, **5**, **7**, **8**, **9**, and **10** against HSV-1 infection. We employed a genetically engineered HSV-1 construct, incorporating enhanced green fluorescent protein (EGFP) and red fluorescent protein (RFP) as reporter genes, whose expression is driven by the viral promoters ICP0 and glycoprotein C (gC), respectively.¹⁴ ICP0 is an HSV-1 immediate early (IE) gene, while gC is expressed only after onset of viral DNA replication. Vero cells initially infected with HSV-1 at multiplicity of infection (MOI) 1 were cultured at 2 h postinfection (hpi) in media containing compounds **3**, **5**, **7**, **8**, **9**, or **10** (10 μ M) or ACV (50 μ M). Flow cytometry analysis (FC) showed that the percentage of EGFP positive (EGFP⁺) cells in infected cultures exposed to **3**, **5**, and **7** was reduced 8.3-, 10.6-, and 19.6-fold, respectively, compared with untreated, HSV-1 infected cultures 24 hpi (*Figure 2a*), while ACV caused 4.3-fold reduction of EGFP⁺ cells. Productive infection, estimated in the culture supernatant by virus yield reduction assay using Vero cells 48 hpi was also suppressed in cell cultures treated with **3**, **5**, and **7** (10 μ M), similar to ACV (50 μ M) (*Figure 2b*). Conversely, compounds **8**, **9**, and **10** (10 μ M) did not inhibit HSV-1 infection (*Figure 2a*); furthermore, these compounds did not suppress productive infection (*Figure 2b*). These results reveal a critical role played by the C3- β -hydroxyl group, found only in natural products **3**, **5**, and **7**, as a requirement for antiviral activity.

Figure 2. Natural and synthetic Amaryllidaceae alkaloids selectively inhibit HSV-1 infection in Vero cells and iPSC-neurons. (a) Flow cytometry (FC) analysis of uninfected and HSV-1 infected Vero cells (MOI 1) 24 hpi. (b) Viral titer in Vero cell culture supernatants estimated with virus yield reduction assay using Vero cells 48 hpi. Plaques were not observed in cultures treated with compounds 3, 5, and 7. A very low titer was observed for ACV (33.3 pfu/mL). (c) FC analysis of uninfected and HSV-1 infected iPSC-neurons (MOI 0.3) 24 h postinfection (hpi). (d) Viral titer in iPSC-neuronal culture supernatants estimated with virus yield reduction assay using Vero cells 48 hpi. Plaques were observed only in cultures infected with supernatants deriving from untreated, infected neurons. Dimethyl sulfoxide (DMSO) is the vehicle. The data represent an average of three independent experiments. Error bars represent standard deviations.

As humans are the natural hosts for HSV-1, and human neurons are the only known reservoir of HSV-1 infection, compounds 3, 5, and 7 were also tested in neurons derived from human induced pluripotent stem cell (iPSCs).^{15,16} iPSC-neuronal cultures were infected with the genetically engineered HSV-1 construct (MOI 0.3) and subsequently treated with 3, 5, 7, or ACV 2 hpi. FC analysis showed ~62-, 69-, and 28-fold reduction in the percentage of EGFP⁺ cells in iPSC-neuronal cultures treated with 3, 5, and 7 (10 μ M) 24 hpi, respectively, while ACV caused more modest inhibition (~8.5-fold at 50 μ M; Figure 2c). Suppression of productive infection in iPSC-neurons treated with compounds 3, 5, and 7 (10 μ M) was also noted (Figure 2d).

Next, using fluorescent *in situ* hybridization (FISH) we investigated whether the test compounds affected HSV-1 DNA replication in iPSC-neurons similar to ACV. FISH analysis of HSV-1 infected iPSC-neurons treated with 3, 5, 7 (10 μ M), or ACV (50 μ M) indicated no hybridization with HSV-1 DNA, whereas infected cells treated with 8, 9, or 10 (10 μ M) showed numerous hybridizing signals, suggesting ongoing viral replication (Supplemental Figure 1a). Thus, compounds 3, 5, and 7 appear to exert an inhibitory effect on HSV-1 DNA replication like ACV. Since compound 7 is the simplest analogue from a functional and stereochemical perspective to exhibit these novel antiviral effects and is readily available through our total synthetic strategy, we selected it for further analyses.¹³

iPSC-neurons were infected with HSV-1 as described above, and DNA was extracted 24 hpi. Quantitative PCR (qPCR) analysis indicated dramatic reduction in viral DNA copy number in cells treated with 7 (over 1800-fold reduction at 10 μ M) and ACV (2300-fold reduction at 50 μ M), compared with infected, untreated cells (Supplemental Figure 1b). The HSV-1 immediate early gene ICP4 is essential for viral replication;¹⁷ therefore, we utilized RT-qPCR to test the expression of ICP4 and viral DNA polymerase 24 hpi in infected iPSC-neurons treated with 7 or ACV.

A substantial reduction in the expression of ICP4 and viral DNA polymerase was observed in iPSC-neurons treated with 7 (10 μ M) and ACV (50 μ M) (Supplemental Figure 1c). The reduction of ICP4 expression level was further confirmed by Western blotting (Supplemental Figure 1d). Taken together, these results indicate that 7 influences one or more stages of HSV-1 lytic cycles at or before DNA replication. Alternatively, the reduced expression of IE genes and viral DNA polymerase with both drugs may result from the inhibition of multiple rounds of viral replication. As the drugs were added 2 hpi, it was unnecessary to test effects on viral entry. The concentration of 7 that caused a decrease in the percentage of EGFP⁺ cells by 50% (EC₅₀) was estimated to be 0.10 μ M, similar to ACV (EC₅₀ = 0.07 μ M) (Supplemental Figure 2a). At concentrations from 100 nM to 50 μ M, 7 or ACV did not significantly affect iPSC-neuronal viability (Supplemental Figure 2b).

Given the remarkable anti-HSV-1 activity of 7, we next investigated its effect on nonlytic HSV-1 infection. By adapting conventional rodent latency protocols, we have previously used an antiviral cocktail of (E)-5-(2-bromovinyl)-2'-deoxyuridine (SBVdU, 30 μ M) + interferon- α (IFN- α , 125 U/mL) for 7 days to induce "quiescent" infection in iPSC-neurons that mimics several aspects of HSV-1 latency.¹⁶ As reported before, up to 5 days after SBVdU + IFN- α were withdrawn, only low levels of productive infection were noted (Figure 3c), but HSV-1 could be reactivated using sodium butyrate (NaB, 5 mM), a histone deacetylase inhibitor.¹⁶ Cells treated with 7 (10 μ M) or 7 + IFN- α showed 2.9- and 2.1-fold greater increase, respectively, in the percentage of EGFP⁺ cells compared with SBVdU + IFN- α treated cells after withdrawal of agents for 5 days (Figure 3a), but infectious viral particles were nevertheless undetectable in the supernatant harvested from any of the cells (Figure 3c). Unexpectedly, the EGFP mean fluorescence intensity (MFI) in cells treated with 7 or 7 + IFN- α was approximately 1.6- and 2.5-fold lower than the SBVdU + IFN- α treated cells, 5 days after the drugs were withdrawn (Figure 3b). Cells treated with 7 or 7 + IFN- α also differed from the SBVdU + IFN- α treated cells following incubation with NaB; whereas the latter showed robust reactivation with respect to percentage of EGFP⁺ cells or EGFP MFI, cells treated with 7 or 7 + IFN- α did not show significant reactivation (Figure 3a,b). Similarly, the proportion of EGFP-RFP⁺ cells and EGFP-RFP MFI were significantly reduced in cells treated with 7 or 7 + IFN- α after treatment with NaB when compared to the cells treated with SBVdU + IFN- α (Figure 3a,b). Taken together, these experiments suggest that the repression induced by 7 or 7 + IFN- α is distinct from BVdU + IFN- α or ACV, as it is not reactivated to the same extent by NaB.

To explore whether the antiviral effects of 7 extend beyond HSV-1, we compared 7 and ACV against varicella zoster virus (VZV), another common herpes virus that causes chicken pox, shingles, and neuralgia.¹⁸ We used a VZV construct expressing

Figure 3. Compound 7 significantly reduces HSV-1 reactivation and inhibits VZV lytic infection. (a–c) Sodium butyrate (NaB) induces HSV-1 reactivation in quiescent neuronal cultures (QCs) but not in infected cultures treated with 7 or 7 + IFN- α . Quiescent neuronal cultures were produced by incubating iPSC-neurons with HSV-1 in the presence of 5-bromodeoxyuridine (5BdU) and interferon- α (IFN- α). Cells were also infected with HSV-1 in the presence of 7 or 7 + IFN- α . After 7 days, drugs were withdrawn (wd) and cells were either cultured in the presence of NaB to induce HSV-1 reactivation or vehicle for an additional 5 days. (a) Percentage of EGFP+ and EGFP-RFP+ cells. (b) Mean fluorescence intensity (MFI) of EGFP+ cells and EGFP-RFP+ cells. (c) Viral titer in culture supernatants estimated with virus yield reduction assay using Vero cells 48 hpi. Indicated p-values for comparisons in panel b involving EGFP and EGFP-RFP are depicted in green and red, respectively. The data represent an average of three independent experiments. Error bars represent standard deviations.

luciferase from the late ORF9 promoter.¹⁹ ARPE-19 retinal pigment epithelial cells were infected with VZV at 1000 PFU/well. Compound 7 or ACV were added 2 hpi and assayed 48 hpi. Compound 7 caused ~220-fold reduction at 10 μ M in luciferase counts per second and showed an EC₅₀ of 0.15 μ M, while ACV (50 μ M) caused only 1.8-fold reduction (Supplemental Figure 2c). No significant changes in cell viability were observed at tested concentrations for 7 and ACV (Supplemental Figure 2d).

Various antiviral activities of Amaryllidaceae alkaloids have been reported over the last three decades.⁸ A comprehensive evaluation on the antiviral activity of several of these alkaloids revealed that pancratistatin 3, narciclasine 5, *trans*-dihydrolycoricidine 7, and its 7-hydroxy analogue were active against Japanese encephalitis, yellow fever, and dengue viruses.⁵ Antiviral activity of lycorine 1 against the avian influenza virus was recently reported.⁶ Lycorine can also inhibit other RNA-viruses, including severe acute respiratory syndrome (SARS)-associated coronavirus,⁹ poliovirus,¹⁰ and West Nile virus. The present work significantly expands knowledge on the antiviral activity of these alkaloids by identifying derivatives that exhibit novel, distinctive, and diverse inhibitory effects against HSV-1 and VZV infection that are superior to ACV. The facile method reported here for the rapid asymmetric synthesis of natural and non-natural lycorine-type alkaloids permitted discovery of a clear structure–activity pattern, demonstrating that a crucial role is played by the (3*R*)-hydroxy substituent in these alkaloids as a requirement for this antiviral activity. Only compounds 3, 5, and 7 containing the (3*R*)-stereochemistry demonstrate potent antiviral activity, the C3-epimeric compound 8 and C3-deoxy derivative 9 are significantly less active.

This allowed identification of alkaloid 7 as a functionally minimum antiviral pharmacophore. Further applications on harnessing the iPSC-neuron based platform with additional pathogens with the current and additional analogues prepared through refinements on the synthetic protocol are under active investigation. Most importantly, as latent HSV-1 infection is lifelong and drug-refractory, the multiple effects of compound 7 on HSV-1 infection, including its ability to significantly reduce HSV-1 reactivation from quiescence, motivate additional studies of viral entry and viral heterochromatinization. Additional studies are needed to evaluate whether compound 7 alone or in combination with currently known antihherpetic drugs can repress HSV-1 irreversibly to confer protection of immunocompromised individuals who may be at risk for ACV-resistance²⁰ or patients with renal failure.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.5b00318.

Full details on the biological and synthetic procedures and characterization data for all compounds reported; crystallographic data for compound 15 are deposited under CCDC 1001464 (PDF)

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Author Contributions

#These authors contributed equally. The manuscript was written through contributions of all authors. J.McN. designed and C.Z.V. and S.L. performed all synthetic organic experiments. H.A.J. performed X-ray analysis. V.L.N., L.D., Y.Z., and L.M. designed/performed the HSV-1 studies. P.R.K., M.B.Y., L.D., and L.M. designed/performed the VZV studies. All authors aided in manuscript preparation and editing. All authors have given approval to the final version of the manuscript.

Funding

This work was supported by NSERC (to J.McN.), the Stanley Medical Research Institute (to J.McN., V.L.N.), and NIMH (MH63480). C.Z.V. thanks CONACyT for a graduate fellowship. P.R.K. acknowledges EY015291, EY08098, Research to Prevent Blindness Inc., and the Eye and Ear Foundation of Pittsburgh.

Notes

The authors declare no competing financial interest.

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