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Discovery and preliminary structure-activity relationship of the marine natural product manzamines as herpes simplex virus type-1 inhibitors

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Abstract: Herpes simplex virus type-1 (HSV-1) is a member of alpha-herpesviridae family and is known to cause contagious human infections. The marine habitat is a rich source of structurally unique bioactive secondary metabolites. A small library of marine natural product classes **1–10** has been screened to discover a new hit entity active against HSV-1. Manzamine A showed potent activity against HSV-1 via targeting the viral gene ICPO. Manzamine A is a β -carboline alkaloid isolated from the Indo-Pacific sponge *Acanthostrongylophora* species. Currently, acyclovir is the drug of choice for HSV-1 infections. Compared with 50 μ M acyclovir, manzamine A at 1 μ M concentration produced potent repressive effects on viral replication and release of infectious viruses in SIRC cells in recent studies. The potent anti-HSV-1 activity of manzamine A prompted a preliminary structure-activity relationship study by testing targeted manzamines. These included 8-hydroxymanzamine A (**11**), to test the effect of the C-8 hydroxy substitution at the β -carboline moiety; manzamine E (**12**), to assess the importance of substitution at the azacyclooctane ring; and ircinal A (**13**), to determine whether the β -carboline ring is required for the activity. Manzamine A was chemically transformed to its

salt forms, manzamine A monohydrochloride (**14**) and manzamine A monotartrate (**15**), to test whether improving water solubility and hydrophilicity will positively affect the activity. Compounds were tested for activity against HSV-1 using fluorescent microscopy and plaque assay. The results showed the reduced anti-HSV-1 activity of **11**, suggesting that C-8 hydroxy substitution might adversely affect the activity. Similarly, manzamines **12** and **13** showed no activity against HSV-1, indicating the preference of the unsubstituted azacylooctane and β -carboline rings to the activity. Anti-HSV-1 activity was significantly improved for the manzamine A salts **14** and **15**, suggesting that improving the overall water solubility as salt forms can significantly enhance the activity. Manzamines have significant potential for future development as anti-HSV-1 entity.

Keywords: herpes simplex virus type 1 (HSV-1); infected cell protein 0 (ICPO); manzamine A; manzamine A salts; structure-activity relationship.

1 Introduction

Herpes simplex virus type-1 (HSV-1) is a common and ubiquitous infection of the skin which causes mucocutaneous lesions herpes labialis or fever blisters. It is estimated that approximately 80% of the population worldwide are carriers of the virus, and approximately 40% suffer recurrent infections [1, 2]. Nearly 1% of all carriers suffer monthly outbreaks of the latent herpes infection. These infections last for 4–10 days and can extend up to 30 days in immunocompromised patients where lesions may develop extensive necrosis [1]. HSV-1 is the leading cause of corneal disease and blindness in humans, largely because of its recurrent nature [3]. Herpetic keratitis manifests predominantly as either infectious epithelial keratitis, or herpetic stromal keratitis.

ICPO is one of the five immediate-early proteins encoded by HSV-1, which regulates whether the virus

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progresses to lytic or latent infection [4, 5]. ICP0 is a member of the family of E3 ubiquitin ligases with RING finger zinc-binding domain, which confers on ICP0's ability to induce the proteasome-dependent degradation of several cellular proteins. The E3 ubiquitin ligase activity of ICP0 correlates with its role in the stimulation of lytic virus infection and induction of reactivation of latent or quiescent viral genomes [4, 5]. Therefore, targeting ICP0 activities will directly regulate HSV-1 infection.

Oceans cover over 70% of the earth and nearly possess 80% of the earth's animal life [6, 7]. Marine natural products display an extraordinary chemical and pharmacological scope. Cytarabine (Cytosar-U, Ara-C, Depocyt) and vidarabine (Ara-A, Vira-A) were the first sponge-derived drugs approved by the FDA for cancer and viral infections, respectively [6]. Later, ziconotide as anticancer (ω -conotoxin, Prialt®), omega-3 acid ethyl esters as anti-triglyceridemia (Lovaza®), eribulin mesylate as anticancer (Halaven®), brentuximab vedotin as anticancer (Adcetris®), and *iota*-carrageenan as antiviral (Carragelose®) were approved in the United States by the FDA, while trabectedin, ET-743, as anticancer (Yondelis®) was approved in the European Union [7]. Ten other marine natural products are in phase I, phase II, or phase III clinical trials [7]. Marine environment is an excellent resource for novel entities and drug discovery [8, 9]. Over the past two decades, only one FDA-approved drug (sunitinib for renal carcinoma in 2005) has been discovered based on high-throughput screening of combinatorial chemistry libraries [10]. Natural products-based drugs (parent compounds, analogs, and mimics) are still the major entity sources among the FDA-approved drugs (more than 50%) [10].

Manzamines are marine-derived β -carboline alkaloids first reported by Higa and co-workers in 1986 from the Okinawan sponge genus *Haliclona* [11]. Manzamines possess a fused and bridged tetra- or pentacyclic ring system, which is attached to a β -carboline moiety.

To date, over 80 manzamine-related alkaloids have been reported from more than 16 marine sponge species belonging to five families [12, 13]. Manzamines exhibit a diverse range of bioactivities including cytotoxicity [11], insecticidal [14], antibacterial [15], and antileishmania effects [16]. Manzamines also show potent activity against HIV-1 [13], several AIDS opportunistic infection pathogens, e.g. *Cryptosporidium parvum*, *Toxoplasma gondii*, and *Mycobacterium tuberculosis*, as well as the potential curative activity against malaria in animal models [12, 17]. The anti-inflammatory and GSK-3 β inhibitory activities of

manzamines were also reported [18, 19]. Manzamine A, 8-hydroxymanzamine A, and 8-methoxymanzamine A also showed moderate activity against HSV-2 with an MIC of 0.05–0.1 $\mu\text{g/mL}$ [20, 21].

To discover new natural product classes that can inhibit the growth and control HSV-1 infections, an in-house library of 10 marine compounds representing diverse chemical classes was screened (Figure 1). These compounds were isolated from diverse marine sponges. Selection of tested compounds was based on chemical, pharmacophoric, and mechanistic diversity.

This group has showed earlier that manzamine A has a strong inhibitory activity on HSV-1 via targeting viral gene ICP0, which is essential for the HSV-1 replication cycle [22]. Manzamine A at 1 μM concentration has been shown to have a strong inhibitory (10^{11} -fold reduction in viral replication) activity on HSV-1 [22]. Manzamine A was identified to be more effective than the existing standard antiviral drug acyclovir. To study the structure-activity relationship of manzamine A, three available natural manzamines **11–13** were selected for testing their activity against HSV-1 using HSV-1 enhanced green fluorescent protein (EGFP) virus. Manzamine A is a highly lipophilic and poorly water soluble compound; therefore, its highly water soluble salts **14** and **15** were prepared and tested for anti-HSV-1 activity.

2 Materials and methods

2.1 Biological and chemical material

Manzamines **1** and **11–13** were isolated as natural products from an Indo-Pacific sponge *Acanthostrongylophora* species using column chromatography on silica gel and neutral alumina as previously described [12, 15, 17]. Compounds **2–9** were also isolated from marine sponges as reported earlier [23]. The identity was established by ^1H and ^{13}C NMR data and TLC co-chromatography with authentic samples. A >95% purity was established based on spectrofluorimetric analysis [24]. To prepare manzamine A salts, manzamine A and HCl at equimolar concentrations were added into a round bottom flask and freeze dried to obtain manzamine A monohydrochloride (**14**). Manzamine A monotartrate (**15**) was also synthesized by adding manzamine A and tartaric acid at equimolar concentrations. The dry salts **14** and **15** identity was further confirmed by ^1H NMR analysis in D_2O . The structure of salts **14** and **15** are presented in Figure 1.

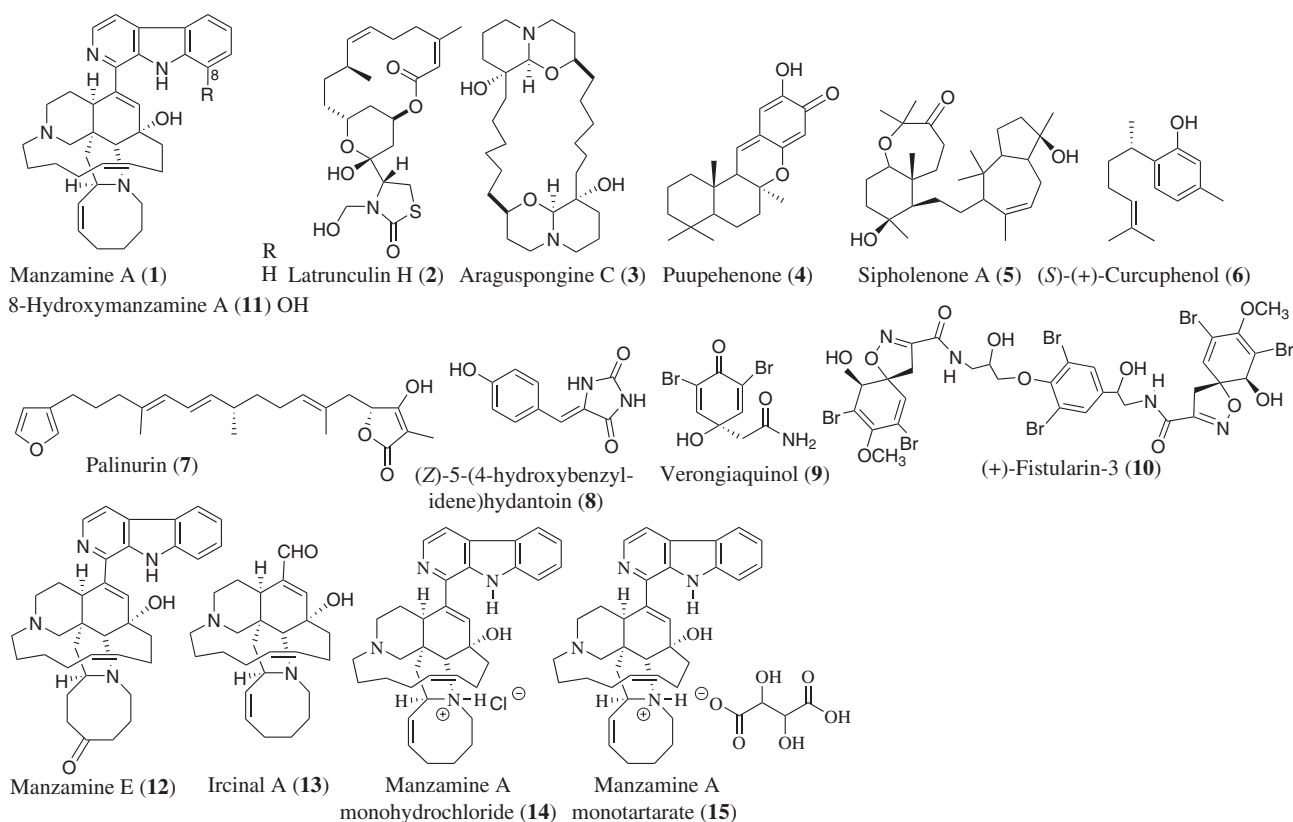


Figure 1: Chemical structures of the marine natural products and manzamine A salts.

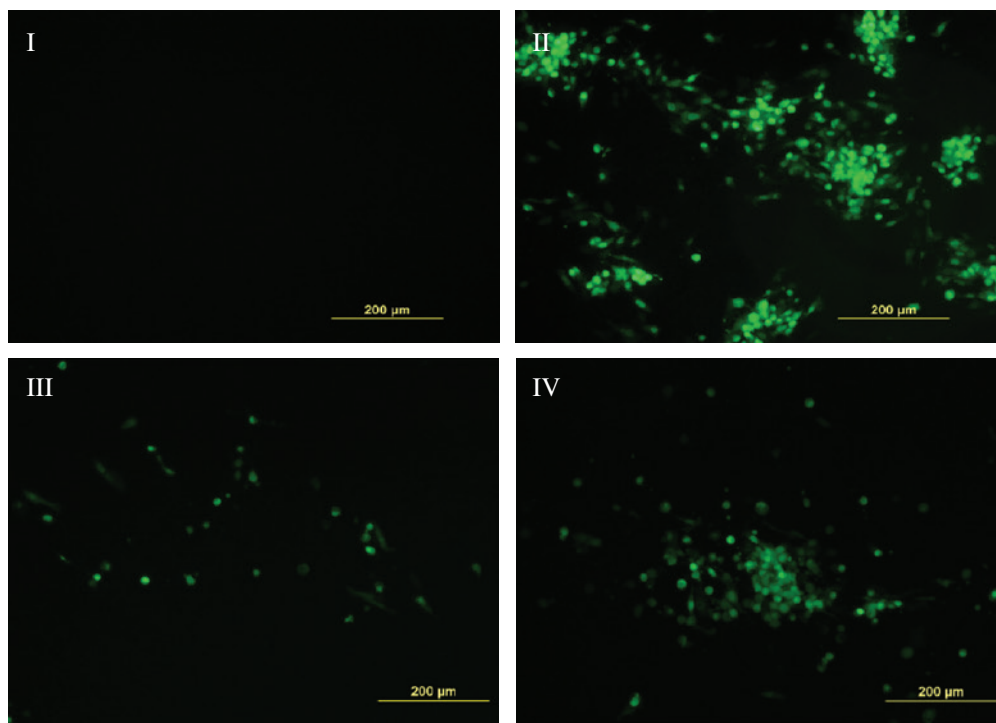


Figure 2: Antiviral activity of compounds 1 and 11 was assessed by infection of SIRC followed by fluorescent microscopy: (I) mock; (II) control infection with DMSO; (III) infection treatment with compound 1; (IV) infection treatment with compound 11.

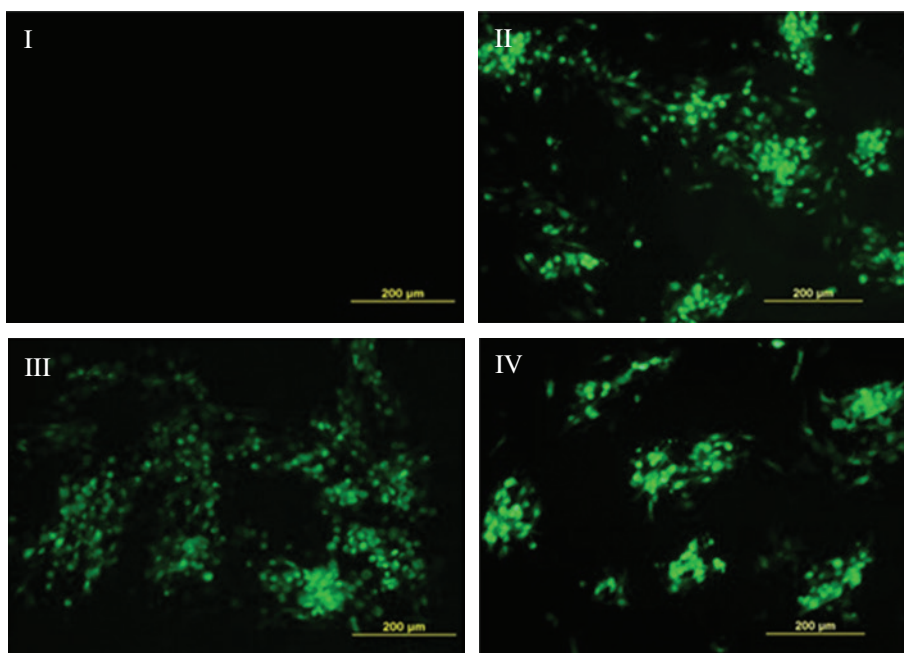


Figure 3: Antiviral activity of compounds **12** and **13** was assessed by infection of SIRC followed by fluorescent microscopy: (I) mock; (II) control infection with DMSO; (III) infection treatment with compound **12**; (IV) infection treatment with compound **13**.

2.2 Biological assays

2.2.1 Infection

The SIRC cells were plated in a 35-mm dish a day before infection. On the day of infection the medium was removed, and the cells were infected with medium containing virus (moi=5). At 1 h post-infection (hpi) medium was removed and washed with 1 mL PBS three times followed by the addition of fresh medium.

2.2.2 Cytotoxicity assay

Cell viability was analyzed by quantitation of ATP, an indicator of active cells, using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI, USA). Briefly, SIRC cells (4000 cells/well) were grown in 96-well plates with MEM medium containing 10% FBS for 24 h. The cells were then incubated with increasing concentrations of tested manzamine in 5% FBS medium for an additional 72 h. Cell viability was determined by the measurement of luminescent ATP in a Synergy HT microplate reader, following incubation with CellTiter-Glo reagent.

2.2.3 Fluorescent microscopy

SIRC cells were plated in a six-well plate. The cells were infected with HSV-1 EGFP virus. The wells were treated

with tested manzamines **1** and **11–15** at different concentrations ranging from 100 nM to 5 µM. The expression of EGFP from the virus-infected cells was assessed by an Olympus fluorescence microscope (IX71) coupled with Olympus digital camera photo apparatus (DP71). Imaging analysis was performed by using the Olympus DP controller software. Exposure times between treatments were exactly the same and determined accordingly.

2.2.4 Plaque assay

CV-1 cells were plated in a 24-well plate with 80% confluence a day before experiment. The medium collected from the infected SIRC cells was prepared for serial dilutions. The CV-1 cells were infected in triplicate. After 1 hpi the medium was removed and washed. Fresh medium was added and incubated for 2 days for plaque formation. At

Table 1: Anti-HSV-1 activity of manzamine derivatives **11–15** in SIRC, rabbit corneal cells.

Compounds	CD50 on SIRC cells	MIC on HSV-1
Manzamine A	5.6 µM	1 µM
11	5.8 µM	3.7 µM
12	3.6 µM	Not effective
13	4.9 µM	Not effective
14	7.8 µM	100 nM
15	8.9 µM	100 nM

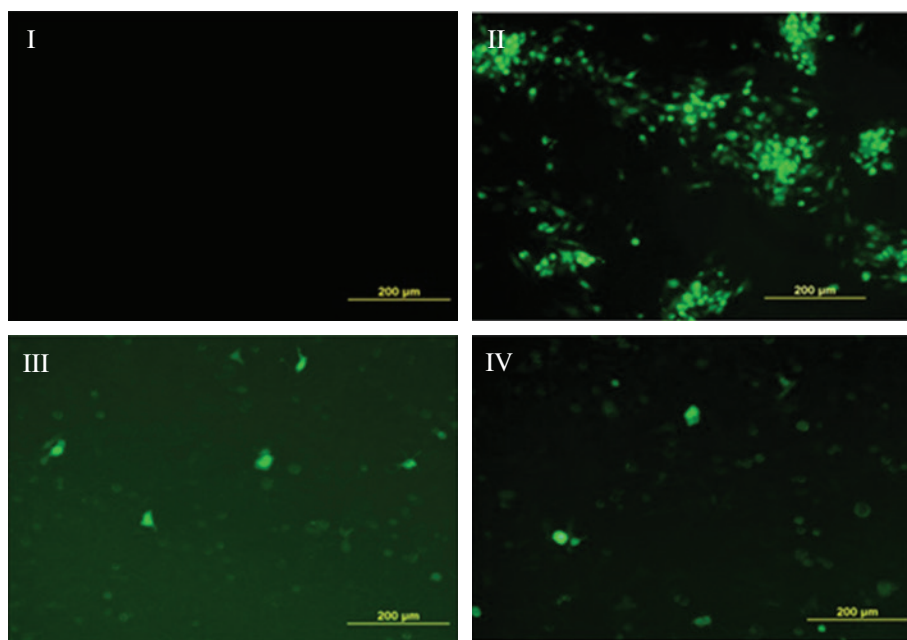


Figure 4: Antiviral activity of compounds **14** and **15** was assessed by infection of SIRC followed by fluorescent microscopy: (I) mock; (II) control infection with DMSO; (III) infection treatment with compound **14**; (IV) infection treatment with compound **15**.

the end of incubation, the cells were fixed with 1% formaldehyde for 40 min followed by the addition of crystal violet. The cells were then washed and the number of plaques was measured.

3 Results and discussion

Screening diverse marine natural products **1–10** revealed manzamine A (**1**) as the only antiviral active hit [23]. Therefore, the available library of natural manzamines **11–13** was tested for their anti-HSV-1 activity using rabbit corneal cells (SIRC) infected with HSV-1 EGFP virus. 8-Hydroxymanzamine A (**11**) showed significantly less activity against HSV-1 infection as suggested by the number of green fluorescent cells observed under a microscope (Figure 2). This result indicated that C-8 hydroxylation diminishes the activity. Manzamine E (**12**) and ircinal A (**13**) did not show any activity against HSV-1 infection, as shown in Figure 3. Furthermore, plaque assay results indicated no activity against HSV-1 by **12** and **13** (Table 1). Manzamine E (**12**) has identical structure such as manzamine A but with a C-21 ketone and saturated $\Delta^{22,23}$ system at the azacyclooctane ring. Ircinal A (**13**) is the biosynthetic precursor of manzamine A, which lacks the β -carboline ring system. These results suggest that any

modification in manzamine A C-8, C21–C23, or elimination of the β -carboline group significantly decreases the activity against HSV-1 infection. Compounds **14** and **15**, which are the manzamine A mono HCl and tartaric acid salts, were highly water soluble and have a significantly higher activity against HSV-1 as observed under fluorescent microscopy and with plaque assay (Figure 4). With the increase in solubility, the toxicity of manzamine A salts was significantly reduced against SIRC cells. The effective concentration of manzamine A was reduced from 1 μ M to 100 nM by the manzamine A salts, imparting 10-fold enhancement in the activity (Table 1).

In conclusion, manzamines **11–13** either had weak or lack activity against HSV-1 infection in SIRC cells. Their toxicity levels on SIRC were also high. Manzamine A had optimal structure features for anti-HSV-1 activity. The mono HCl and tartrate salts of manzamine A had greater water solubility and better activity which reduced their effective concentration against HSV-1 from 1 μ M for the free base to 100 nM in SIRC cells. Manzamine alkaloids are a novel class of HSV-1 inhibitors appropriate for further future optimizations.

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