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Manzamine A as a Novel Inhibitor of Herpes Simplex Virus Type-1 Replication in Cultured Corneal Cells

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Abstract

This study investigated the putative inhibitory effect of manzamine A on HSV-1 infection. Our results indicated that manzamine A effectively inhibited viral replication and infection in the cell line SIRC, a corneal cell line, at 1 μ M. The existing anti-HSV-1 drug acyclovir was analyzed and showed a comparable activity at 50 μ M. Plaque assays demonstrated that manzamine A reduced the release of infectious virus by 10^{11} -fold. RTPCR assays indicated that HSV-1 virion host shutoff (vhs) activity and ICP0 transcription were decreased by manzamine A treatment. These results bode well for the development of manzamines as potential leads to reduce viral infection in corneal cells and to prevent HSV-1-induced eye infections such as keratitis.

Keywords

manzamine A; herpes simplex virus; corneal cells; acyclovir

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 from recurrent infections [1,2]. Of all the carriers, about one percent suffers monthly outbreaks of the latent herpes infection. These infections last for 4 to 10 days and can extend up to 30 days in immunocompromised patients where lesions may develop extensive necrosis [1]. HSV-1 is a leading cause of corneal disease and blindness in humans, largely because of its recurrent nature [3]. Herpetic keratitis (HK) manifests predominantly as either infectious epithelial keratitis (IEK) or herpetic stromal keratitis (HSK). The drugs of choice for treatment of HK are nucleoside analogues, such as acyclovir (ACV), gancyclovir (GCV), and less frequently the viral DNA-synthesis inhibitors Foscarnet (FOS) and Cidofovir [4]. Acyclovir is a synthetic analogue of the nucleoside guanosine with

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marked inhibitory activity against HSV types 1 and 2 and VZV [5]. The most frequent cause of resistance to ACV are mutations in the gene of the viral thymidine kinase (TK) which result in reduced or abrogated production of this enzyme and in consequent decrease or lack of subsequent phosphorylation [6, 7]. The widespread use of ACV has led to the emergence of HSV strains resistant to ACV. Resistant strains were soon reported and the first cases of clinical resistant strains were published [8]. Hence there is an urgent need for the development of new drugs which can target the HSV infections with different mechanisms of action. Manzamines are marine-derived β -carboline alkaloids first reported in 1986 from the Okinawan sponge genus *Haliclona* [9]. Manzamines possess a fused and bridged tetra- or pentacyclic ring system, which is attached to a β -carboline moiety (Fig. 1). Since the first report of manzamine A, several manzamine-type alkaloids have been reported from sixteen sponge species belonging to the Chalinidae, Niphatidae, Petrosidae, Thorectidae, and Irciniidae families [10,11]. Manzamines exhibit a diverse range of bioactivities including cytotoxicity [9], insecticidal [12], antibacterial [13], and antileishmaniasis effects [14]. Manzamines also show potent activity against HIV-1 [15], 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 [10, 11]. Anti-inflammatory and GSK-3 β inhibitory activities of manzamines were also reported [16, 17]. Of all these compounds, manzamine A, 8-hydroxymanzamine, and 8-methoxymanzamine A showed moderate activity against HSV-2 with MIC of 0.05–0.1 μ g/mL [18]. The isolation of the manzamine A-producing putative symbiotic actinobacterium, *Micromonospora* sp., would guarantee a sustainable cost-effective source of this complex molecule for future development as a drug entity [31].

In this study, the putative inhibitory effect of manzamine A on the HSV-1 was investigated using a recombinant virus expressing EGFP. Results showed that manzamine A effectively inhibited viral replication and infection in several cell lines. Further studies indicated that manzamine A produced potent repressive effects on viral replication and release of infectious viruses in Serum Institut Rabbit Cornea (SIRC) cells at a concentration of 1.0 μ M. This was quite effective when compared to the existing drugs such as ACV (50 μ M). These results suggested that manzamine A may become an alternative therapeutic agent to prevent HSV-1 induced eye infections such as HSV-1 keratitis.

Materials and Methods

Cell lines and virus

The rabbit cell line SIRC (ATCC Cat#: CCL-60) was derived from normal rabbit cornea cells and was maintained in MEM medium containing 10% fetal bovine serum. African green monkey kidney (CV-1) cells (ATCC Cat#: CCL-70) were maintained in DMEM with 10% fetal bovine serum. EGFP HSV-1 virus, a gift of Dr. Timothy Foster (LSUHSC at New Orleans), was used for infection. It is a recombinant virus expressing engineered enhanced green fluorescent protein (EGFP) under the control of the human cytomegalovirus immediate-early gene promoter (HCMV-IEP) inserted in place of the gK gene [19].

Compounds

Manzamine A was isolated from an Indo-Pacific sponge species of the genus *Acanthostrongylophora* using column chromatography on silica gel and neutral alumina as previously described [10, 11, 20]. The identity was established by ^1H and ^{13}C NMR data and TLC co-chromatography with an authentic manzamine sample [10]. A > 99% purity was established based on spectrofluorimetric analysis [20]. The phenylmethyle hydantoin GSK-3 β inhibitors, (*Z*)-5-(4-(methylthio)-benzylidene)-hydantoin, (*Z*)-5-(4-(ethylthio)benzylidene)-hydantoin, and (*Z*)-5-(4-(diethyl-amino)benzylidene)-hydantoin (MAK13, ZR, and ZN, respectively), were chemically synthesized by condensation of hydantoin with corresponding substituted aldehyde as previously reported [21]. Their structure is shown in Fig. 5a. Acyclovir (acycloguanosine) 99% (HPLC) was purchased from Sigma-Aldrich.

Cytotoxicity assay

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

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 hour post-infection (hpi) medium was removed and washed by 1 mL PBS three times followed by the addition of fresh medium.

Manzamine A removal assays

SIRC cells were subjected to infection in the absence or presence of manzamine A. At 24 hpi, the cells were washed three times with PBS followed by addition of media containing manzamine A or DMSO as manzamine A washout control. The infected cells were subjected to fluorescent microscopy for analysis.

Plaque assays

CV-1 cells were plated in a 24-well plate with 80% confluence a day before the 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 2 days for plaque formation. At 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.

Fluorescent microscopy

The expression of enhanced green fluorescent protein (EGFP) from the virus infected cells was accessed by an Olympus fluorescence microscope (IX71) coupled with an Olympus digital camera photo apparatus (DP71). Imaging analysis was performed by using Olympus

DP controller software. Exposure time between treatments was exactly the same and determined accordingly.

RT-PCR

RT-PCR was performed using Superscript One-Step RT-PCR (Invitrogen) with 0.5 µg of total RNA and two primer sets per reaction tube: one set for the actin as a control and another for the experimental sample. The primers were designed by MacVector 7.2.2 (MacVector, Inc.) and synthesized by Invitrogen. Their sequences are as follows: Actin: 5'-ATT CCT ATG TGG GCG ACG AG-3' and 5'-TGG ATA GCA ACG TAC ATG GC-3', product length 270 bp, (Gen-Bank Accession NM_001100); ICP0: 5'-CCC CCA CAA CAA ACA CAC AAG-3' and 5'-TCA ATC AGC ACC CAC GAG TTC C-3', product length 302 bp (GenBank Accession FJ593289); EGFP: 5'-GCA GAA GAA CGG CAT CAA GGT G-3' and 5'-TGG GTG CTC AGG TAG TGG TTG TC-3', product length 143 bp. The reverse transcription/PCR reaction was carried out at 45°C for 20 min followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 30 s, and finally at 68°C for 10 min. The RT-PCR products were analyzed by 2% agarose gel electrophoresis.

Statistical analysis

Statistical calculations were carried out with Microsoft Excel 2003 from Windows software package (Microsoft Office). Results are expressed as the mean ± SEM of 3 replicates.

Results and Discussion

The toxicity of manzamine A was examined on SIRC cell line. The SIRC cells were treated with manzamine A at different concentrations (0.1, 0.5, 1, 2, 3, 5, and 10 µM) and were assayed for cell viability after 72 hours. It showed that manzamine A at 1 µM or lower concentration exhibited no toxicity on SIRC, and the IC₅₀ was found to be 5.6 µM (Fig. 2).

HSV-1 EGFP virus, which upon infection expresses GFP in the infected cells, was used for infection, followed by observation under fluorescent microscope. The reduction in the number of GFP expressing cells correlates with a reduction in infection. Rabbit corneal cells SIRC were either mock-infected, infected with the virus treated with DMSO, or infected with the virus in the presence of 1 µM manzamine A. The results demonstrated that the cells treated with manzamine A exhibited a significantly reduced number of green cells, suggesting that the HSV-1 infection was inhibited by manzamine A in SIRC cells (Fig. 3). To quantitate the reduction in the number of green cells, plaque assays were done.

Plaque assay was used to assess the viral release from the infected cells. SIRC cells were infected with virus (moi = 5) in a 35-mm dish, either in the absence or presence of 1 µM manzamine A, followed by plaque assay with serial dilution using CV-1 cells. The results revealed that the media from the infected cells treated with manzamine A produced much less plaques compared to nontreatment groups (Fig. 4a). The plaque counting indicated that 1 µM manzamine A treatment reduced the plaque formation by 10¹¹-fold (Fig. 4b). These data suggested that manzamine A treatment extensively reduced the viral release from the SIRC cells.

The potency of manzamine A was compared to ACV, a commonly used drug for the HSV treatment. Fluorescent microscopy showed that 1 μ M of manzamine A is more effective than 50 μ M acyclovir to reduce viral replication as the number of green cells was 4-fold less in the manzamine A treated group at 24 hpi (Fig. 5a, compare panel I, II, and III). When cells were treated 1 h after viral infection, manzamine A was effective as well (Fig. 5a, compare panel I, II, and IV).

To test whether manzamine A-mediated inhibition was reversible, viral infection was performed in the presence of manzamine A with or without washout at 24 hpi. Fluorescent microscopy was performed at 48 hpi and the results revealed that viral replication became active after manzamine A removal (Fig. 5b). Additional experiments showed that ACV washout did not restore the viral replication as the number of green cells remained the same (data not shown). These observations indicated that manzamine A did not block the entry of virus into cells, and the inhibition was reversible since the virus resumed replication after manzamine A removal.

Manzamine A was suggested to exert its action by inhibiting GSK-3 β [13]. Other synthetic phenylmethylene hydantoins that exhibit GSK-3 β specific inhibition were used to examine whether the antiviral activity is exerted through GSK-3 β signaling [21]. Structures of phenylmethylene hydantoins MAK13, ZR, ZN are shown in Fig. 6a, and these compounds were tested for anti-HSV-1 activity on SIRC cells. The results did not exhibit any inhibitory effect on HSV-1 replication as the number of green cells remained the same as in the control group (Fig. 6b). This observation suggested that manzamine A utilized a different mechanism other than GSK-3 β inhibition to inhibit HSV-1 infection.

To investigate the targets of manzamine A-mediated antiviral activity during infection, RTPCR assays were performed to analyze manzamine A-initiated regulatory effects on cellular and viral transcripts. RTPCR assays using EGFP primers as a control demonstrated that the same amount of viruses entered into cells and initiated EGFP transcription under CMV promoter at 8 hpi (Fig. 7, top panel). Further studies showed that HSV-1 immediate-early gene ICP0 transcription was reduced by manzamine A (Fig. 7, compare lane 2 and 3 in middle panel), suggesting that manzamine A played a role in downregulating at least one critical viral immediate-early gene. Cellular gene actin, nonetheless, was degraded by HSV-1 infection due to vhs activity during viral infection but was reinstated by manzamine A treatment (Fig. 7, compare lane 2 and 3 in bottom panel). Taken together, these results suggest that manzamine A used multiple pathways to control viral infection in SIRC cells.

Herpes simplex virus infection is usually controlled by the ACV treatment [22]. However, widespread use of ACV has produced HSV-1 that are resistant to treatment [23] so there is a need for the development of new drugs which will act through different mechanisms. EGFP HSV-1 provides a unique feature for this purpose since this virus exhibits full capacity for infection and produces a green fluorescence protein that can be visualized under fluorescent microscopy for high throughput screening [19]. Our results strongly indicated that manzamine A is a potential hit that inhibits HSV-1 replication in corneal cells.

The mechanism of manzamine A-induced antiviral activity is unknown. It was originally hypothesized that manzamine A blocked the viral entry into cells. However, our manzamine A washout assays demonstrated that viruses were able to enter cells in one hour during infection in the presence of manzamine A and the removal of manzamine A initiated the replication (Fig. 5b). In addition, the efficient restoration of viral replication after the removal of manzamine A further demonstrated that manzamine A-mediated inhibition, unlike the one of ACV which was converted into acyclo-GTP to inhibit viral DNA polymerase, was not long-acting but quickly reversible (Fig. 5b). This rapid turnover rate may be beneficial as an alternative therapeutic agent for HSV-1 eye infection since ACV could be mutagenic due to the fact that it can incorporate into cellular DNA. The HSV-1 tegument protein vhs assists viral mRNAs to utilize cellular translational machinery by reducing competition from cellular mRNAs [24]. ICP0 is produced at the earliest stage of infection after entering the host cells [25]. The newly synthesized ICP0 protein is transported to the nucleus, promotes viral transcription and disrupts nuclear structures to alter the host gene expression [26]. Our results showed that manzamine A does not affect CMV-driven transcription of EGFP but reduced HSV-1 vhs activity and repressed ICP0 transcription. However, it is not known whether manzamine A directly influenced the vhs activity and ICP0 expression, or these effects are just a net result of the decreased copy number of the viral genome. Experiments were designed to assess the issue using individual infected cells by flow cytometry. Manzamine A is a known GSK-3 β enzyme inhibitor [16]. Glycogen synthase kinase-3 β (GSK-3 β) is an ubiquitously expressed constitutively active serine/threonine kinase that phosphorylates cellular substrates and thereby regulates a wide variety of cellular functions, including development, metabolism, gene transcription, protein translation, cytoskeletal organization, cell cycle regulation, and apoptosis [27]. It has also been implicated in the control of cellular response to damaged DNA. To check whether GSK-3 β inhibition is involved in reducing the viral infection, we used the potent inhibitors of GSK-3 β , phenylmethylene hydantoins MAK13, ZR, and ZN [21]. However, they did not show any reduction in the HSV-1 infection at a concentration that was sufficient to inhibit the GSK-3 β enzyme. Therefore, it appears that manzamine A utilized other mechanisms than inhibiting the GSK3 β enzyme. HSV-1 has been suggested as an etiological agent in the pathogenesis of Alzheimer's disease (AD) manifested by abnormally phosphorylated forms of a microtubule-associated protein tau [28–30]. Recent studies demonstrated that HSV-1 induces the expression of GSK3 β and protein kinase A, the enzymes that cause tau phosphorylation [28]. It is thus likely that manzamine A may inhibit HSV-1 replication as well as virus-induced GSK3 β activity to prevent AD. Additional experiments are being performed to assess this hypothesis.

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Abbreviations

ACV	acyclovir
HSV-1	herpes simplex virus type 1

ICP0	infectious cellular protein 0
vhs	virion host shutoff
TK	thymidine kinase
hpi	hours post-infection
GSK3β	glycogen synthase kinase 3 beta

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Manzamine A

Fig. 1.

Structure of manzamine A. Manzamines possess a fused and bridged tetra- or pentacyclic ring system attached to a β -carboline moiety.

Fig. 2.

Manzamine A had no toxicity at 1 μM concentration on SIRC cornea cells. Cells (4000/well) were plated in 96-well plates. After 24 hr growth, cells were shifted to 5% FBS medium containing increasing concentrations of manzamine A and grown for an additional 72 hr. Cell viability was measured using the CellTiter-Glo luminescent cell viability assay. The IC_{50} of manzamine A on SIRC cornea cells was found to be 5.6 μM .

Fig. 3. Manzamine A repressed HSV-1 infection in SIRC cornea cells at 24 hpi. SIRC cells infected with EGFP HSV-1 at an moi of 5 observed under microscope in bright field (BF) or fluorescence (FL). Cells treated with 1 μ M manzamine A exhibited a decreased number of green cells, indicating that viral replication was blocked.

Fig. 4.

a Manzanine A decreased the release of infectious virus. CV-1 cells were plated in 24-well plates and infected with the media collected from SIRC cells at 24 hpi. The manzanine A treated groups showed much less plaque compared to no treatment control infection.

b Manzanine A decreased the release of infectious virus. Plaques were counted as 1 mL of media from SIRC-infected cells applied on CV-1 monolayer to determine the virus titer. Manzanine A produced a 10¹¹-fold reduction in contrast to no treatment infection groups. The mean number of virus released calculated by triplicates in the control group was

$1.1650E + 17 \pm 2.8868E + 16$, while in the manzamine A treated group the mean number of virus released was $1.5E + 7 \pm 5E + 6$. The p value was 0.0022, which shows a significant difference between the two groups.

Fig. 5.

a Manzamine A is more effective than ACV (24 hpi). Antiviral activity of manzamine A and ACV was assessed by infection of SIRC followed by fluorescent microscopy. (I) Control infection with DMSO; (II) infection treated with acyclovir 50 μ M; (III) infection treated with manzamine A; and (IV) infection followed by manzamine A addition at 1hpi.

b Manzamine A is more effective than ACV (24 hpi). Removal of manzamine A reversed the inhibition. (I) Infection with DMSO; (II) infection with manzamine A treatment for 48 h;

(III) infection with manzamine A for 24 h followed by manzamine A washout. Pictures were taken 48 hpi.

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Name:	R group:
MAK13:	SCH ₃
ZR:	SCH ₂ CH ₃
ZN:	N(CH ₂ CH ₃) ₂

a Phenylmethylene hydantoin

Fig. 6.

a GSK3 β inhibitors had no effect after 24 hpi. Structures of GSK3 β inhibitors. The R of the following compound is listed below. i. (*Z*)-5-(4-(methylthio) benzylidene)-hydantoin (MAK13): SCH₃; ii. (*Z*)-5-(4-(ethylthio) benzylidene) -hydantoin (ZR): SCH₂CH₃; iii. (*Z*)-5-(4-(diethylamino)benzylidene)-hydantoin (ZN): N(CH₂CH₃)₂.

b GSK3 β inhibitors had no effect after 24 hpi. SIRC cells were infected with different GSK3 β inhibitors at 5 μ M. (I) Control infection with DMSO; (II) infection with MAK13; (III) with ZR; and (IV) with ZN.

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Fig. 7.

Manzamine abolishes vhs activity and ICP0 expression at 8 hpi. Effects of manzamine A on virus-mediated cellular response and viral transcription were analyzed by RTPCR assays.

Lane 1: mock-infection; lane 2: control infection; lane 3: infection with manzamine A. The product length of actin is 270 bp, ICP0 302, and EGFP 143 bp.