

## Anti-varicella-zoster virus activity of cephalotaxine esters *in vitro*

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**Harringtonine (HT) and homoharringtonine (HHT), alkaloid esters isolated from the genus *Cephalotaxus*, exhibit anti-tumor activity. A semisynthetic HHT has been approved for treatment of chronic myelogenous leukemia. In addition to antileukemic activity, HT and HHT are reported to possess potent antiviral activity. In this study, we investigated the effects of HT and HHT on replication of varicella-zoster virus (VZV) *in vitro*. HT and HHT, but not their biologically inactive parental alkaloid cephalotaxine (CET), significantly inhibited replication of recombinant VZV-pOka luciferase. Furthermore, HT and HHT, but not CET, strongly induced down-regulation of VZV lytic genes and exerted potent antiviral effects against a VZV clinical isolate. The collective data support the utility of HT and HHT as effective antiviral candidates for treatment of VZV-associated diseases.**

**Keywords:** varicella zoster virus, antiviral, harringtonine, homoharringtonine

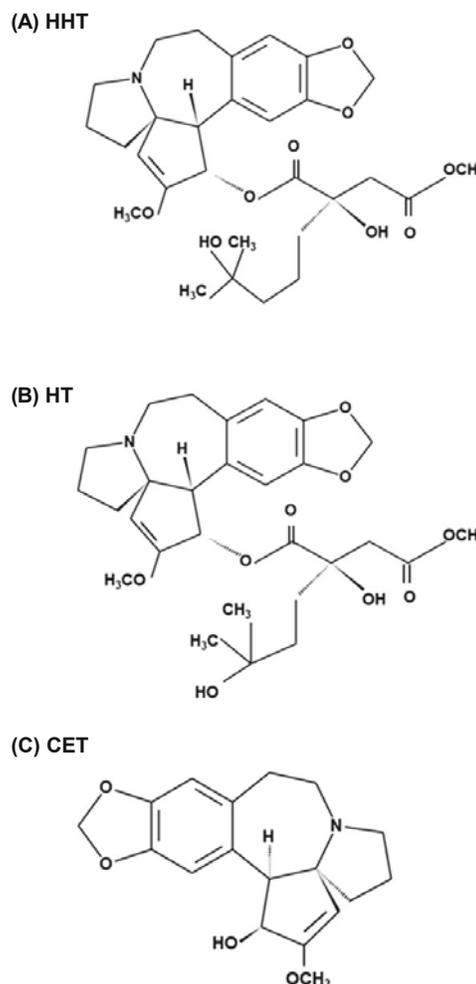
### Introduction

Varicella-zoster virus (VZV), a member of the herpesvirus family, is a causative agent of varicella (chickenpox) and zoster (shingles) (Arvin, 1996; Arvin and Gilden, 2013). VZV is highly species-specific with its natural host range restricted to humans. Primary VZV infection in childhood causes a diffuse vesicular rash of varicella, followed by resolution of illness by the host immune system and establishment of latent VZV infection in the dorsal root ganglia. Reactivation of latent VZV infection results in herpes zoster, which provokes severe pain associated with postherpetic neuralgia (Arvin, 1996; Arvin and Gilden, 2013).

Inhibitors of VZV DNA polymerase are currently employed in the clinic to treat VZV-associated diseases (Cohen *et al.*, 1999). Acyclovir and famciclovir/penciclovir are nucleoside analogs which lacks the 3'-OH for the phosphodiester bond between nucleotides. Upon activation by viral thymidine kinase (TK), these drugs are incorporated into the viral DNA synthesis to prevent DNA elongation (Cohen *et al.*, 1999). Foscarnet, a pyrophosphate analog, binds the pyro-

phosphate binding site to inhibit viral DNA polymerase (Cohen *et al.*, 1999; Gnann Jr, 2007). Although these anti-VZV drugs are highly effective, more concerted efforts to develop novel therapeutic strategies for VZV-associated diseases are necessary due to the emergence of drug resistance and side-effects of available antiviral agents (Ida *et al.*, 1999; Arvin, 2002).

Members of the genus *Cephalotaxus* produce various alkaloids, such as cephalotaxine (CET), harringtonine (HT), homoharringtonine (HHT), isoharringtonine, and deoxyharringtonine (Lu and Wang, 2014). CET, a benzazepine-bearing pentacyclic alkaloid, is a parent structure of C3-ester compounds, including HT and HHT (Fig. 1). HT is a C3  $\alpha$ -hydroxyl succinate ester of CET while HHT contains a methylene group in the side-chain (Quintas-Cardama *et al.*,



**Fig. 1.** Structures of *Cephalotaxus* alkaloids used in this study. (A) HHT, (B) HT, (C) CET.

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2009). Although CET is biologically inactive, HT and HHT exert potent antileukemic effects (Powell *et al.*, 1972). Indeed, omacetaxine mepesuccinate, a semisynthetic HHT produced by direct esterification of CET, has been approved by the Food and Drug Administration (FDA) for treatment of chronic myelogenous leukemia (CML) patients resistant or intolerant to tyrosine kinase inhibitors (Tujebajeva *et al.*, 1992; Chen *et al.*, 2011).

In addition to antileukemic effects, HT and HHT are reported to exhibit antiviral activity. HT inhibits chikungunya virus (CHIKV) replication through down-regulation of viral protein expression while HHT displays activity against hepatitis B virus (HBV) and coronavirus (Romero *et al.*, 2007; Kaur *et al.*, 2013; Cao *et al.*, 2015). In the current study, we investigated whether HT and HHT exert antiviral effects against VZV, in accordance with previous reports.

## Materials and Methods

### Cells, viruses, chemicals, and biological assays

Primary human foreskin fibroblast (HFF) cells were cultured in Dulbecco's modified eagle's medium (GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (Capricorn Scientific), penicillin (100 U/ml) and streptomycin (100 µg/ml). Recombinant VZV-pOka expressing luciferase reporter (VZV-pOka-luciferase) and the clinical YC01 strain of VZV (VZV-YC01) have been described previously (Zhang *et al.*, 2007; Jeon *et al.*, 2016). HT and HHT were purchased from Santa Cruz Biotechnology and Sigma-Aldrich, respectively. CET was obtained from Glentham Life Sciences and acyclovir (ACV) from Calbiochem. The luciferase activities of VZV-pOka-luciferase were measured using the luciferase assay system from Promega. The CellTiter-Glo luminescent cell viability assay was performed according to the manufacturer's instructions (Promega). Plaque reduction assay was performed as described previously (Bae *et al.*, 2017).

### Quantification of VZV DNA and transcripts

For quantitative analysis of VZV DNA, quantitative polymerase chain reaction (qPCR) was employed as described previously (Bae *et al.*, 2017). Briefly, total DNA was isolated using an AccuPrep Genomic DNA Extraction kit (Bioneer), and VZV DNA was amplified and quantified on a StepOne-Plus Real-Time PCR system (Applied Biosystems) using HOT FIREPol® EvaGreen qPCR master mix Plus (Solis BioDyne) and specific primers for amplification of VZV or human GAPDH DNA. VZV transcripts were analyzed via quantitative reverse transcription PCR (qRT-PCR) according to a previous report (Bae and Song, 2017). The primer sequences used for amplification were as follows: VZV ORF63 (IE), forward 5'-ATTGAGGCGCCGAATGTTC-3' and reverse 5'-CTTCACCACCATCAGATACG-3'; VZV ORF28 (E), forward 5'-CGAACACGTTCCCCATCAA-3' and reverse 5'-CCCGGCTTTGTTAGTTTGG-3'; VZV gB, forward 5'-GATGGTGCATACAGAGAACATTCC-3' and reverse 5'-CCGTTAAATGAGGCGTGAATAA-3'; GAPDH, forward 5'-CATGAGAAAGTATGACAACAGCCT-3' and reverse 5'-AGTCCTTCCACGATACCAAAGT-3'.

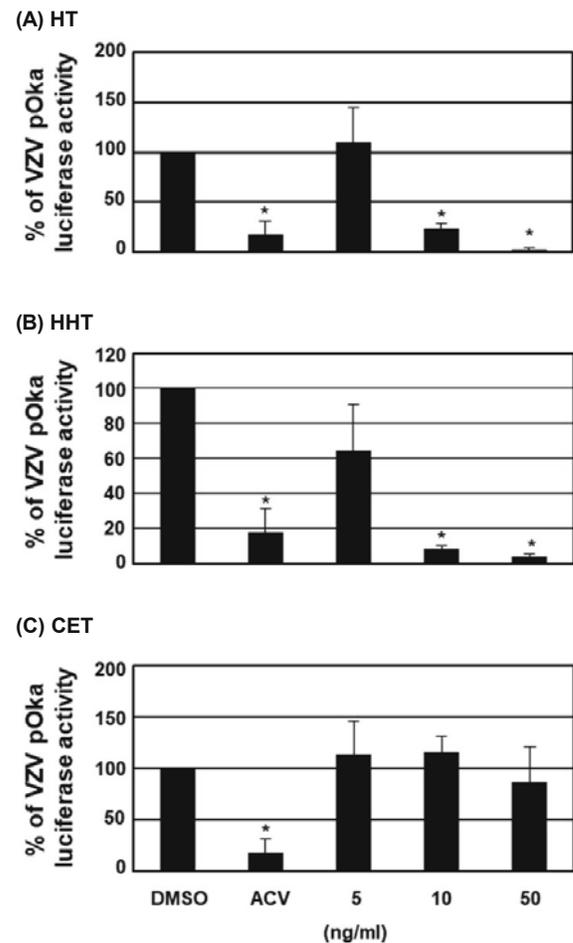
### Western blot analysis

Cells were harvested, fractionated and transferred onto nitrocellulose membrane, as described previously (Kim *et al.*, 2014). Antibodies against VZV ORF62 and tubulin were purchased from Abcam and Sigma-Aldrich, respectively. Secondary peroxidase-labeled anti-mouse immunoglobulin G antibodies (Amersham Biosciences) and enhanced chemiluminescence detection reagent for visualization of proteins (Pierce) were used according to the manufacturer's specifications.

## Results

### Anti-VZV activities of ester alkaloids from *Cephalotaxus*

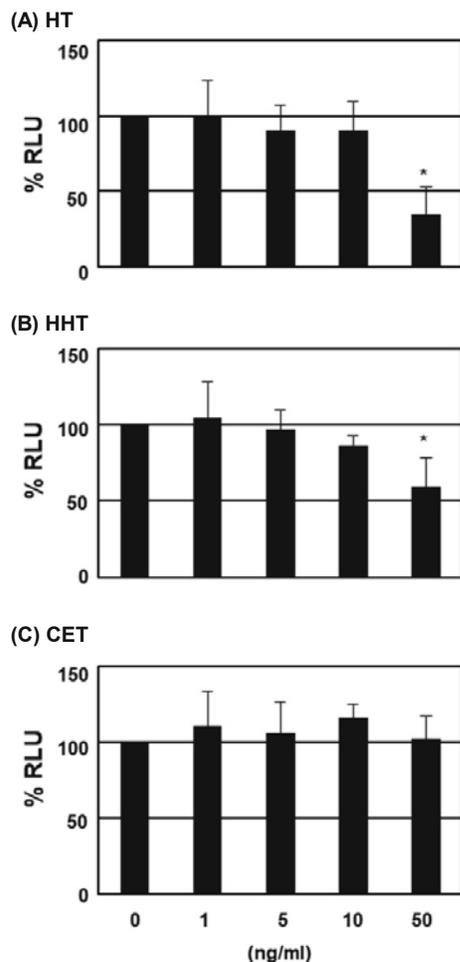
To investigate the potential anti-VZV activities of alkaloids from *Cephalotaxus*, uninfected HFF cells were inoculated



**Fig. 2.** Inhibition of VZV-pOka-luciferase activity by HT and HHT. HFF cells were inoculated with VZV-pOka-luciferase-infected HFF cells at an MOI of 0.1 and treated with (A) HT, (B) HHT, or (C) CET at concentrations of 5, 10, and 50 ng/ml. Cells treated with ACV (5 µM) served as a positive control. Luciferase activities were measured at 72 h after inoculation. VZV pOka-luciferase activity in DMSO-treated cells was set as 100%. Data are represented as means  $\pm$  SD of three independent experiments. The asterisk (\*) denotes a significant difference between samples determined using the two-sample *t*-test ( $P < 0.05$ ).

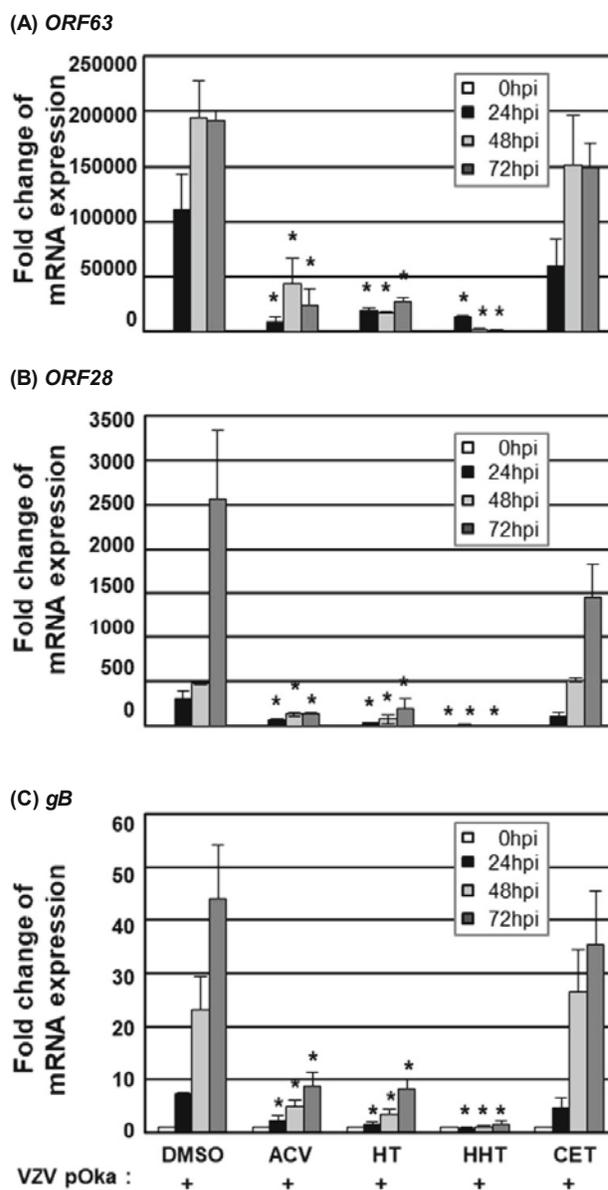
with VZV-pOka-luciferase-infected HFF cells at an MOI of 0.1, followed by treatment with DMSO, acyclovir, HT, HHT or CET. At 72 h after inoculation, VZV replication was determined based on luciferase activity (Fig. 2). Both HT and HHT reduced VZV-pOka-luciferase activity in a dose-dependent manner (Fig. 2A and B), while CET had no effect (Fig. 2C). Acyclovir treatment at a concentration of 5  $\mu$ M (~1.13  $\mu$ g/ml) reduced VZV-pOka-luciferase activity by 82%. Interestingly, both HT and HHT were more potent than acyclovir reducing VZV-pOka-luciferase activity by 76.1% and 91.3%, respectively, at a concentration of 10 ng/ml. Overall, HHT was more effective than HT in inhibiting replication of VZV-pOka-luciferase, with 50% effective concentration ( $EC_{50}$ ) values (at which VZV replication was reduced by 50%) of  $4.654 \pm 1.134$  ng/ml and  $9.574 \pm 3.474$  ng/ml for HHT and HT, respectively.

To determine whether the anti-VZV activities of *Cephalotaxus* ester alkaloids are mediated via toxic effects on cells,

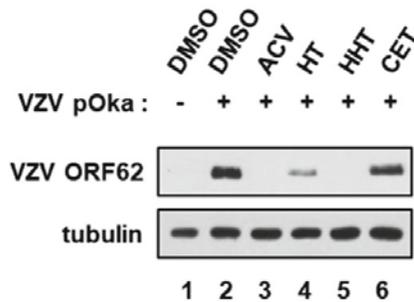


**Fig. 3.** Cytotoxic effects of HT, HHT and CET against HFF cells. HFF cells were treated with (A) HT, (B) HHT, or (C) CET at concentrations of 1, 5, 10, and 50 ng/ml. At 72 h after treatment, cell viability was determined using the CellTiter-Glo luminescent cell viability assay. Luciferase activity in DMSO-treated cells (0 ng/ml) was set as 100%. Data are represented as means  $\pm$  SD of three independent experiments. The asterisk (\*) denotes a significant difference between samples determined using the two-sample *t*-test ( $P < 0.05$ ).

HFF cells were treated with 0, 1, 5, 10, or 50 ng/ml HT, HHT and CET, and cell viability assessed by determining cellular ATP levels using the CellTiter-Glo assay after 72 h (Fig. 3). HT, HHT, and CET exhibited no significant cytotoxicity up to 10 ng/ml against HFF cells (Fig. 3). At a dose of 50 ng/ml, HT and HHT suppressed HFF cell viability by 65.3% and 40.4%, respectively (Fig. 3A and B). The 50% cytotoxicity concentration ( $CC_{50}$ ) values of HT and HHT for HFF cells



**Fig. 4.** Down-regulation of VZV lytic gene transcripts by HT and HHT. HFF cells were inoculated with VZV-pOka-luciferase-infected HFF cells at an MOI of 0.1 and treated with DMSO, HT, HHT or CET (10 ng/ml). HFF cells treated with ACV (5  $\mu$ M) served as a positive control. At 24, 48, or 72 h post inoculation (hpi), total RNA was harvested and reverse-transcribed into cDNA. Relative amounts of VZV [ORF63 (IE), ORF28 (E), or gB (L)] transcripts were measured via qRT-PCR, as described in 'Materials and Methods'. The transcript levels at 0 hpi were set as 1. Data are represented as means  $\pm$  SD of three independent experiments. The asterisk (\*) denotes a significant difference between samples determined using the two-sample *t*-test ( $P < 0.05$ ).



**Fig. 5.** Down-regulation of VZV ORF62 protein by HT and HHT. HFF cells were inoculated with VZV-pOka-infected HFF cells at an MOI of 0.1 and treated with DMSO, HT, HHT, or CET (10 ng/ml). HFF cells were also treated with ACV (5  $\mu$ M) served as a positive control. At 72 h after infection, equal amounts of cell extracts were subjected to western blot with antibodies against VZV ORF62 or tubulin.

(at which cell viability was reduced by 50%) were determined as  $45.82 \pm 12.72$  ng/ml and  $74.71 \pm 15.76$  ng/ml, respectively. In view of the finding that the anti-VZV activities of HT and HHT at 10 ng/ml do not appear to be mediated through cytotoxic effects, this dose was used for subsequent studies.

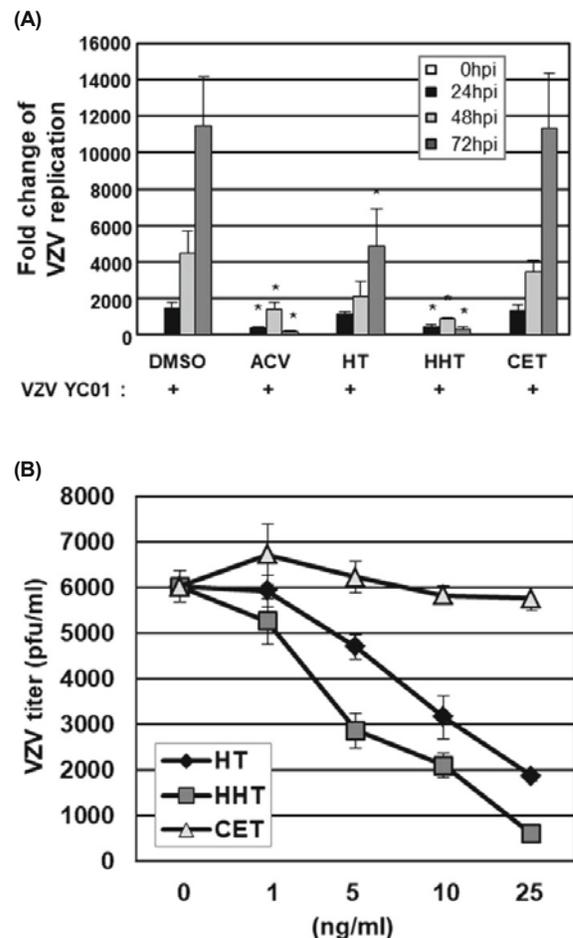
#### Effects of ester alkaloids from *Cephalotaxus* on VZV lytic gene expression

During the lytic cycle of infection, VZV lytic genes are expressed in a sequential cascade composed of immediate early (IE), early (E), and late (L) genes (Arvin and Gilden, 2013). To determine the effects of HT and HHT on VZV lytic gene expression patterns, uninfected HFF cells were inoculated with VZV-pOka-luciferase-infected HFF cells at an MOI of 0.1, followed by treatment with DMSO, acyclovir, HT, HHT, or CET. At 24, 48, and 72 h after inoculation, the relative transcript levels of ORF63 encoding IE63 (IE), ORF28 encoding a subunit of viral DNA polymerase (E) and glycoprotein B (gB) (L) were analyzed via qRT-PCR (Fig. 4). HT treatment reduced ORF63, ORF28, and gB transcript levels by 85.5%, 92.6%, and 81.2% while HHT suppressed the levels by 99.2, 99.8, and 96.4% at 72 h after inoculation (Fig. 4). On the other hand, CET exerted no effects on VZV lytic gene expression (Fig. 4). Furthermore, HT and HHT, but not CET, induced significant down-regulation of IE62 protein, which is essential for VZV lytic replication (Fig. 5).

#### Effects of ester alkaloids from *Cephalotaxus* on replication of VZV clinical isolates

To ascertain the effects of *Cephalotaxus* ester alkaloids on VZV-pOka-luciferase activity, qPCR was employed to assess activity against the clinical isolate of VZV (Fig. 6A). To this end, uninfected HFF cells were inoculated with VZV-YC01-infected HFF cells at an MOI of 0.1, followed by treatment with DMSO, acyclovir, HT, HHT, or CET. Compared to 0 h after inoculation, the VZV DNA level was increased by 11,485-fold in DMSO-treated cells at 72 h after inoculation. Similar to data obtained with VZV-pOka-luciferase, HT and HHT, but not CET, induced significant reduction of VZV-YC01 DNA by 57.8% and 97.1%, respectively (Fig. 6A). The effects of HT and HHT on replication of VZV-YC01 were further

determined using the plaque reduction assay (Fig. 6B). HFF cells were inoculated with serially diluted VZV-YC01-infected HFF cells, incubated for 6 h and treated with HT, HHT or CET at concentrations of 0, 1, 5, 10, or 25 ng/ml. Cells were treated again with HT, HHT, or CET at 72 h after the initial treatment, and the number of plaques was counted to determine the titer expressed as plaque forming units (pfu/ml) at 6 days after infection via the plaque assay. Both HT and HHT treatment significantly reduced the number of plaques with an estimated  $EC_{50}$  of  $16.15 \pm 1.94$  and  $9.96 \pm 0.49$  ng/ml, respectively (Fig. 6B). The anti-VZV activities of HT and HHT were less effective against VZV-YC01 than VZV-pOka-luciferase. Nevertheless, both HT and HHT cle-



**Fig. 6.** Inhibition of VZV clinical isolate replication by HT and HHT. (A) HFF cells were inoculated with VZV-YC01-infected HFF cells at an MOI of 0.1 and treated with DMSO, HT, HHT, or CET (10 ng/ml). HFF cells treated with ACV (5  $\mu$ M) served as a positive control. At 72 h after inoculation, total DNA was harvested and the relative amounts of viral DNA measured using qPCR with primers specific for ORF62, as described in 'Materials and Methods'. The transcript levels at 0 hpi was set as 1. (B) HFF cells were inoculated with serially diluted VZV-YC01-infected HFF cells, incubated for 6 h and treated with HT, HHT, or CET at concentrations of 0, 1, 5, 10, or 25 ng/ml. Cells were treated again with HT, HHT or CET at 72 h after the initial treatment, and the number of plaques was counted to determine the titer expressed as pfu/ml at 6 days after infection via the plaque assay. Data are represented as means  $\pm$  SD of three independent experiments. The asterisk (\*) denotes a significant difference between samples determined using the two-sample *t*-test ( $P < 0.05$ ).

arly exerted strong inhibitory effects against both recombinant VZV-pOka-luciferase and the clinical isolate VZV-YC01.

## Discussion

In the present study, we showed that the cephalotaxine esters, HT and HHT, inhibit replication of both the laboratory strain and clinical isolate of VZV *in vitro*. The finding that CET, the parental alkaloid for HT and HHT, exerted no anti-VZV effects suggests that the ester side-chains at C-3 are critical for inhibition of VZV replication. ACV is widely used to treat VZV-associated diseases, with a reported mean EC<sub>50</sub> of 13 ± 3 μM for VZV-pOka (Morfin *et al.*, 1999). Remarkably, HT and HHT were more potent than ACV in inhibiting VZV-pOka, with mean EC<sub>50</sub> values of 18 ± 6.5 and 8.5 ± 2 nM, respectively. Moreover, HHT was more potent than HT, indicating that, structurally, a methylene group in its side-chain may be responsible for enhanced anti-VZV activity.

Cephalotaxine esters are reported to possess antiviral as well as antileukemic activities (Romero *et al.*, 2007; Kaur *et al.*, 2013; Cao *et al.*, 2015). Following screening of a natural product compound library, Kaur *et al.* (2013) demonstrated that HT inhibits replication of Chikungunya virus, a positive-strand RNA virus belonging to the *Alphavirus* genus of the *Togaviridae* family (Kaur *et al.*, 2013). HHT has additionally been shown to exert antiviral effects against coronavirus, a positive-strand RNA virus, and HBV, a double-strand DNA virus (Romero *et al.*, 2007; Cao *et al.*, 2015). Another recent study demonstrated that HT inhibits replication of Sindbis virus, a member of the *Togaviridae* family (Jia *et al.*, 2018).

Given that HT and HHT inhibit replication of both RNA and DNA viruses, antiviral activities are most likely to be attributable to effects on cellular rather than viral factor(s), which are critical for viral replication. Cephalotaxine esters have been shown to interfere with the elongation step of translation by blocking aminoacyl-tRNA binding to the acceptor site on the large ribosome subunit and peptide bond formation (Fresno *et al.*, 1977). Kaur and co-workers suggested that HT suppresses Chikungunya virus replication by inhibiting the large ribosome subunit, in turn, down-regulating viral protein translation (Kaur *et al.*, 2013).

Interestingly, HT and HHT significantly suppressed the mRNA levels of VZV lytic genes (Fig. 4). Thus, in addition to blocking ribosome function, these compounds may employ other mechanism(s) to inhibit VZV lytic gene expression and replication, which will be a focus of further studies. HT and HHT may interfere with signal transduction pathways and transcription factors involved in VZV lytic gene expression. Additionally, HT and HHT are reported to block cell cycle progression at G<sub>1</sub>/S or G<sub>2</sub>/M transition (Huang, 1975; Zhou *et al.*, 1995). Since cell cycle regulation affects herpesvirus lytic gene expression and replication, one potential mechanism underlying inhibition of VZV replication by HT and HHT may be through effects on the cell cycle in infected cells.

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