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A dihydro-pyrido-indole potently inhibits HSV-1 infection by interfering the viral immediate early transcriptional events

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ABSTRACT

In our continued quest for identifying novel molecules from ethnomedicinal source we have isolated an alkaloid 7-methoxy-1-methyl-4,9-dihydro-3*H*-pyrido[3,4-*b*]indole, also known as Harmaline (HM), from an ethnomedicinal herb *Ophiorrhiza nicobarica*. The compound exhibited a potent anti-HSV-1 activity against both wild type and clinical isolates of HSV-1. Further we demonstrated that HM did not interfere in viral entry but the recruitment of lysine-specific demethylase-1 (LSD1) and the binding of immediate-early (IE) complex on ICP0 promoter. This leads to the suppression of viral IE gene synthesis and thereby the reduced expression of ICP4 and ICP27. Moreover, HM at its virucidal concentration is nontoxic and reduced virus yields in cutaneously infected Balb/C mice. Thus, the interference in the binding of IE complex, a decisive factor for HSV lytic cycle or latency by HM reveals an interesting target for developing non-nucleotide antiherpetic agent with different mode of action than Acyclovir.

1. Introduction

Herpes simplex virus (HSV) is one of the most serious public health concerns, responsible for variety of diseases including recurrent cold sores, keratoconjunctivitis, genital herpes and encephalitis. Following entry into the host cells, the virus undergoes replication to establish primary infection and is then transported to the sensory ganglia for life-long latency (Du et al., 2013). During infection, HSV replicates within the nucleus in three phases: immediate-early (IE), early (E) and late (L) (Lehman and Boehmer, 1999). In the IE phase the viral transcription is initiated by the release of a tegument protein VP16, that in association with host cell factors Oct1 and HCF1, forms IE complex (VP16-HCF1-Oct1) to bind at 5′-TAATGARAT of ICP0 promoter for transcriptional regulation and reactivation (Kim et al., 2012). Oct1, a versatile factor of POU family of homeo-domain proteins, modulates host transcription whereas HCF1 helps in the recruitment of LSD1 that demethylates histone H3K4 for transcriptional activation of IE promoter. Thus, the depletion of HCF1 results in increased levels of repressive histone H3K9 methylation, suggesting a crucial role of HCF1 in HSV gene expression (Liang et al., 2009). Moreover, the transcriptional activation of E and L genes requires two IE gene products ICP4 and ICP27 to regulate DNA synthesis and completion of the viral life cycle (Zhou and Knipe, 2002).

The clinical management of herpesvirus diseases by nucleoside analogs Acyclovir (ACV) and related drugs target viral DNA polymerase with limited efficacy (Piret and Boivin, 2011) due to their inability to eliminate the virus; while their extensive clinical use leads to the emergence of drug-resistant viruses, particularly in neonates and immunocompromised patients (Prichard et al., 2011). Till date there is no effective HSV vaccine (Coleman and Shukla, 2013) and the deduced animal efficacy has not been translated in man (Johnston et al., 2011). However, a recent study demonstrated that anti-HSV therapy significantly reduces HIV-1 RNA load in HSV-2 co-infected patients (Modjarrad and Vermund, 2010). Therefore, alternative treatments to minimize

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the development of resistance, less side-effects with better efficacy are urgently needed. Earlier studies reported that small molecules can selectively inhibit herpesvirus early gene expression (Kutluay et al., 2008; Xiang et al., 2011; Weber et al., 2002) and epigenetically block HSV replication (Liang et al., 2013). We found that the ethnomedicinal herb *Ophiorrhiza nicobarica*, used for skin ailments by the Shompen tribes of Great Nicobar Islands, India (Dagar and Dagar, 1991) has antimicrobial, antiviral and anti-inflammatory activities (Chattopadhyay et al., 2006, 2007). Herein, we report the isolation of a potent anti-HSV alkaloid Harmaline from *O. nicobarica* that interferes with the IE transcriptional events necessary for HSV replication and reactivation, with reduced virus yield in infected mice.

2. Materials and methods

2.1. Plant materials

O. nicobarica Balkr. (Rubiaceae, International index: 758538-1), a wild herb, was collected from the Galathia River Village, Great Nicobar Islands, India. The isolation and identification of the compound has been described in the Supplementary information.

2.2. Cells and viruses

Vero cells (ATCC, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum (FBS; Invitrogen, USA), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in 5% CO_2. The viral strains used were HSV-1F (ATCC733), clinical isolates 1-4 and TK^- strain gifted by Professor M. Sengupta, Calcutta Medical College and Hospital, India and passaged in Vero cells.

2.3. Cytotoxicity and antiviral assay

The Vero cells were exposed to various concentrations of HM and incubated at 37 °C in 5% CO_2, using ACV and DMSO (0.1%) as controls. After 72 h, MTT assay was carried out following manufacturer's protocol (MTT; Sigma) and OD was read at 570 nm. The 50% cytotoxic concentration (CC_{50}) was calculated by linear regression of the dose–response curves. For antiviral activity the plaque reduction assay (PRA) was used. Briefly, Vero cells infected with laboratory and clinical isolates of HSV-1 (100 PFU) were exposed to serial dilutions of test drugs and then overlaid with 1% methylcellulose. The plaques developed after 72 h were counted and virus titers were calculated by scoring the plaque-forming units. The effective concentration of test drugs that reduced plaques number by 50% (EC_{50}) was interpolated from the dose-response curves (Bertol et al., 2011).

2.4. Time-of-addition and removal assay

The effect of drug addition over time was performed to determine the possible step(s) of viral life cycle targeted by HM. Following three different approaches Vero cells were exposed to HM (5.0 μg/ml) before, after and during infection with HSV-1F (100 PFU). For pre-infection, cells were treated with HM for 1 or 3 h, washed with PBS and then infected with HSV. For co-infection, the cells were treated simultaneously with HSV and HM. After 1 h the virus-drug mixture was removed and the treated cells were subjected to PRA. While for post-infection (p.i) the cells were first infected with HSV for 1 h, washed with PBS, and then treated with HM at intervals of 2, 3, 4, 5, 6, 8, 12 and 24 h and finally harvested after 24 h for PRA. For time-of-removal assays the cells were infected with HSV and after 1 h treated with HM. The drug mixture was removed at intervals of 2, 3, 4, 5, 6, 8, 12 and 24 h p.i., then the cells were washed with PBS and subjected to PRA (Bertol et al., 2011).

2.5. Immunoblotting

Equal amounts of protein (40 μg/sample) from HSV-1 infected, untreated or HM treated cells for 2–8 h p.i. in buffer (200 μl/well) containing 20 mM Tris (pH 7 ± 0.5), 50 mM NaCl, 5% NP-40, 0.05% DOC was subjected to SDS–PAGE and blotted to pre-equilibrated PVDF membrane (Thermo Scientific, USA). The membrane was blocked in 5% NFDM in 1X TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Tween 20), rinsed and incubated with monoclonal anti-ICP4, anti-ICP27 and polyclonal anti-β-actin antibodies (Santa Cruz Biotech Inc., USA) and visualized using ECL Western blotting detection kit (Millipore, USA) (Rao et al., 2011).

2.6. Quantitative real-time PCR

HSV-1 (5 moi) infected Vero cells were treated with HM (1.1 and 5.0 μg/ml) for 2 and 4 h p.i, and the RNA was isolated using RNeasy Mini kit (Qiagen, Germany). The RNA (1–2 μg) in RNase-free water containing 20 μl of RT mix was subjected to cDNA synthesis using GeneAmp PCR System 9700 (Applied Biosystems, USA). The real-time PCR (95 °C for 10 min followed by 40 cycles of 15 s at 95 °C, 60 s at 60 °C) was performed using SYBR Green Master Mix in ABI Prism 7500 Sequence Detection System (Applied Biosystems, USA) (Frazia et al., 2006). Primer sequences are available on request.

2.7. EMSA

Oligonucleotide sequence 5′-GCATGCTAATGATATTCTTTG-3′ of the ICP0 promoter was biotinylated using Biotin 3′-end DNA labeling Kit (Thermo Scientific, USA). Reaction mixtures (20 μl) containing 3 μg nuclear extracts (presented in Supplementary file) prepared from HSV-1 infected untreated or HM treated cells, 20 fmol Biotin 3′ end-labelled probe, 50 ng/μl poly (dI-dC), 2.5% glycerol, 0.05% NP-40 (1%), 5 mM $MgCl_2$ and 1X binding buffer were incubated for 20 min at room temperature. The mixtures were subjected to 4% polyacrylamide gel, transferred to Nylon membrane and developed according to the manufacturer's instructions (Thermo Scientific, USA). For super shift assay, the nuclear extracts were pre-incubated with specific antibodies for 30 min on ice (Huang et al., 2012).

2.8. Co-immunoprecipitation

The HSV-1 infected untreated or HM treated (5.0 μg/ml) cells for 4 h p.i. were washed with ice-cold PBS and mixed with a solution containing 10 mM Tris (pH 8.0), 170 mM NaCl, 0.5% NP40 and protease inhibitors, and kept on ice for 30 min with subsequent three freeze/thaw cycles at −80 °C to lyse nuclei. After centrifugation the supernatants were precleared with protein A-coupled Sepharose beads for 2 h. The lysates were then immunoprecipitated with HCF1 or LSD1 antibodies along with isotype-matched control antibodies plus protein A-Sepharose for 4 h or overnight. Beads were washed four times with wash buffer (200 mM Tris at pH 8.0, 100 mM NaCl and 0.5% NP-40), once with ice-cold PBS and boiled in 2X loading buffer. Finally the proteins were resolved by SDS–PAGE before probing with indicated antibodies (Halder et al., 2011).

2.9. Tryptophan fluorescence study

Binding of HM to LSD1 or HCF1 was examined by monitoring changes in the emission of intrinsic Tryptophan fluorescence (Yang

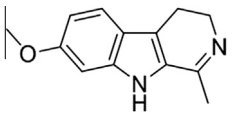

Fig. 1. The chemical structure of isolated compound Harmaline (HM) from *O. nicobarica*.

et al., 2005). Briefly, the purified protein (0.5 μM; Supplementary file) was mixed with HM, incubated for 1 h at room temperature to measure the excitation/emission at 285/340 nm (Jasco FP-6300 spectrofluorometer, Thermo Spectronic, USA). The percent fluorescence reduction was calculated: 100×[1-intensity (drug)/intensity (buffer only)].

2.10. Animals

Female BALB/c mice (18–20 g) housed in polypropylene cages in Animal House facility were used in accordance with the OECD guidelines approved by the Institutional Animal Care and Use Committee, Jadavpur University, Kolkata (No: 367/01/C/CPCSEA).

2.11. Efficacy of oral and topical treatment of HM in infected mice

Seven weeks old acclimatized mice were divided into six groups (n = 10). Group-I served as control, while groups II–VI were cutaneously infected with 5 μl of HSV-1F (1×10^6 pfu), after scarification of the shaved right midflank with a 27-gauge needle. The groups III–V were orally administered with HM (0.1, 0.25, 0.5 mg/kg) and VI with ACV (5 mg/kg), once at 8 h before and then daily for 7 successive days after viral inoculation, while group-II served as infection control. The development of skin lesions and mortality were observed three times daily for next eight days. The severity of the lesion was scored in a composite scale (Kurokawa et al., 1995) as: no lesion (0); vesicle in local area (2); mild zosteriform or small clustered lesion (4); moderate zosteriform lesion with redness and swelling (6); severe zosteriform lesions with erosion/ulceration (8); and death (10).

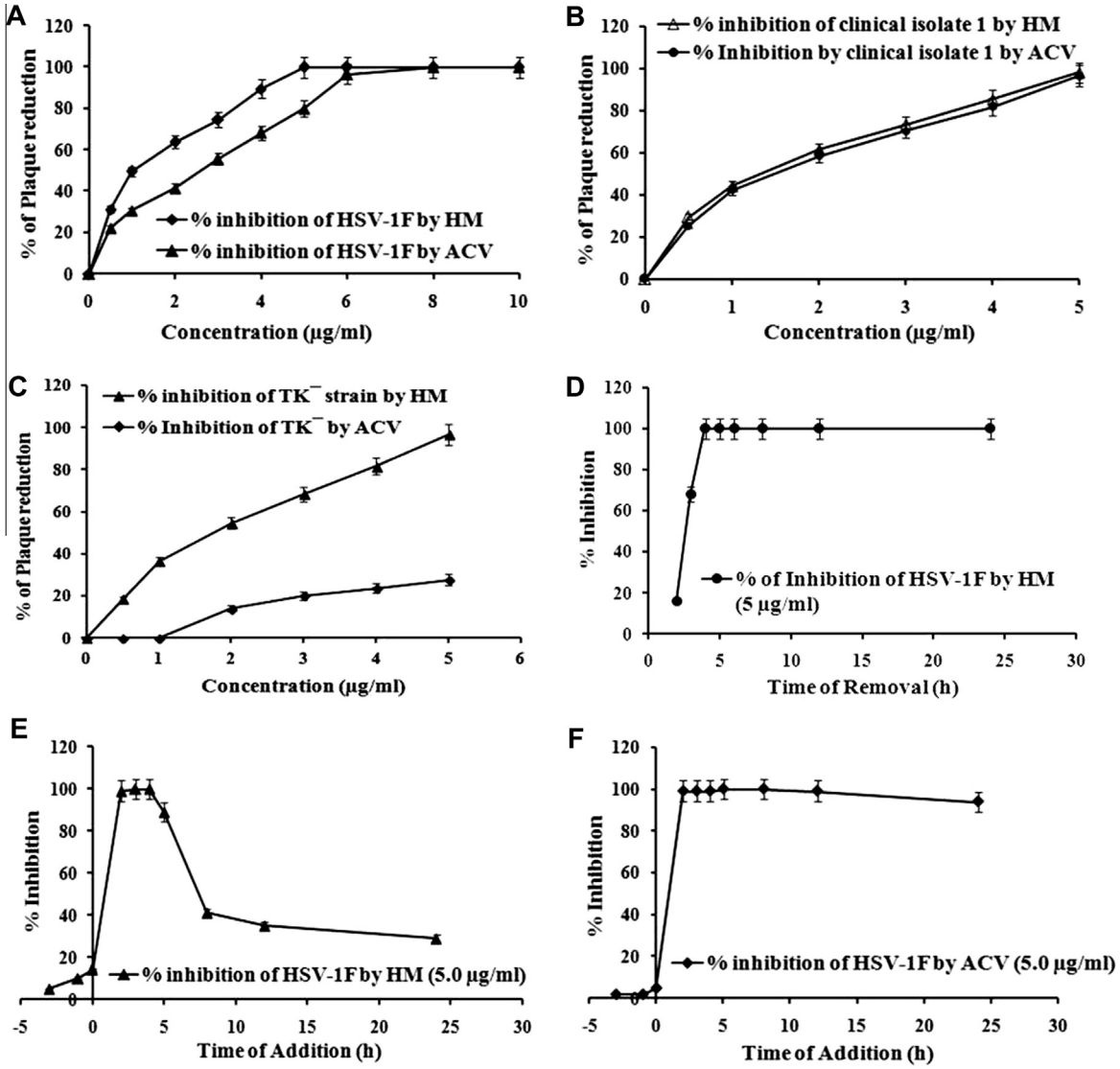

Fig. 2. Anti-HSV activities of the isolated HM: Vero cells were infected with HSV-1F (A), clinical isolate 1 (B) and TK⁻ deficient strain (C), untreated or treated with HM or ACV at 0.5–10 μg/ml and were subjected to the plaque reduction assay. The % of plaque number reduction was calculated and the effective concentration of drug that inhibited the number of viral plaques was interpolated from the dose-response curve. (D) Inhibitory effect of HM using time of removal assay. Inhibitory effect of HM (E) or ACV (F) at various time points: pre-infection (3 h, 1 h), co-infection (0 h) and post-infection (1–24 h) with HSV-1 (100 PFU/well), determined by the plaque reduction assay. Each bar represents the mean ± S.E.M of three independent experiments.

To test the efficacy of HM ointments, the animals were divided into two batches and infected as above. The first batch (six groups, $n = 10$) includes two test groups treated with HM ointment (0.25 and 0.5%, presented in Supplementary file), one with ACV ointment (5%), vehicle (ointment base), no treatment (virus control) and uninfected control. The ointment was applied topically with sterile cotton swabs on the scratched area (5 mg/cm^2/dose) 1 h post-infection and twice daily for 7 days. The animals were observed for at least 10 days post-infection to score the lesions and mortality as described above. The second batch consisting of five groups ($n = 10$) including two experimental and three controls, received ointment on the scratched area 1 h post-infection, three or five times daily for 7 days (Chuanasa et al., 2008).

3. Results and discussion

3.1. Isolation and identification of antiviral compound

Powdered herb was extracted in MeOH and evaporated to a residue. A part of the residue (32 g) was extracted with *n*-butanol and purified in Silica-gel CC into three fractions (A–C). All fractions were further purified. The fractions A and B yielded ursolic acid and β-sitosterol by spectral analysis. Whereas the fraction C yielded two sub-fractions, of which sub-fraction 2 (Retention-factor 0.33) with significant anti-HSV activity was purified. The ^{1}H and ^{13}C NMR spectra's, and ESI-TOF mass revealed that the compound was 7-methoxy-1-methyl-4,9-dihydro-3*H*-pyrido[3,4-*b*]indole, called as harmaline (HM, Fig. 1). The isolated HM (95% purity) was a light yellow powder, soluble in DMSO with chemical formula $C_{13}H_{14}N_2O$, melting point (229 °C), Molecular Mass 214.3 g/mol, and pKa 9.8 (Supplementary information).

3.2. Effect of test drugs on isolates of HSV

We have evaluated the *in vitro* antiviral activity of HM isolated from *O. nicobarica*, using wild-type and TK$^-$ HSV-1 strains (Fig. 2A–C). The plaque reduction assay revealed that HM has potent anti-HSV activities against all the isolates, less than their CC_{50} (Table 1). Moreover, the susceptibility of TK$^-$ strain indicated that HM has significant anti-HSV activity with different mode of action than ACV.

To find out the possible stage of viral life cycle interfered by HM we performed the time-of-addition and removal assay. The results show that HM exerts its inhibitory effect during 0–4 h post-infection; whereas ACV was effective at 6–12 h post-infection. Concordantly, the antiviral activity was significantly reduced up to 3 h p.i. when HM was removed. However, no effect was observed when HSV was pre-exposed or co-infected with HM (Fig. 2D–F). Further, the attachment and penetration assays (presented in Supplementary information) reveal that HM fails to block the virus entry, which was confirmed by immunoblotting of glycoprotein B from

Table 1
Assessment of cytotoxicity and antiviral activity of fraction C and HM against HSV-1 isolates.

HSV strains	Fraction C (μg/ml)			HM (μg/ml)			Acyclovir (μg/ml)
	CC_{50}[a]	EC_{50}[b]	SI[c]	CC_{50}[a]	EC_{50}[b]	SI[c]	EC_{50}[b]
HSV-1F (ATCC)	215 ± 15.5	13.2 ± 0.21	16.29	30 ± 0.32	1.1 ± 0.1	27.27	2.1 ± 0.21
Clinical isolate 1	13.8 ± 0.32	15.58	1.4 ± 0.3	21.43	2.3 ± 0.32		
Clinical isolate 2	12.9 ± 0.09	16.7	0.9 ± 0.09	33.33	2.5 ± 0.71		
Clinical isolate 3	14.7 ± 0.4	14.62	1.7 ± 0.4	17.64	2.7 ± 0.52		
Clinical isolate 4	14.4 ± 0.95	14.93	1.4 ± 0.23	21.43	3.1 ± 0.31		
TK$^-$ strain	17.4 ± 1.1	12.35	2.6 ± 0.7	11.54	>30		

[a] The CC_{50} (50% cytotoxic concentration for Vero cells in μg/ml); mean ± S.E., determined by MTT reduction assay.
[b] The EC_{50} (Concentration of compound producing 50% inhibition of virus-induced cytopathic effect), at moi: 0.5, was expressed as the mean (μg/ml) ± S.E. of six independent experiments.
[c] SI = Selectivity index, determined by the ratio of CC_{50} to EC_{50} (CC_{50}/EC_{50}), EC_{50} or 50% inhibitory concentration, mean ± S.E., determined by three independent experiments.

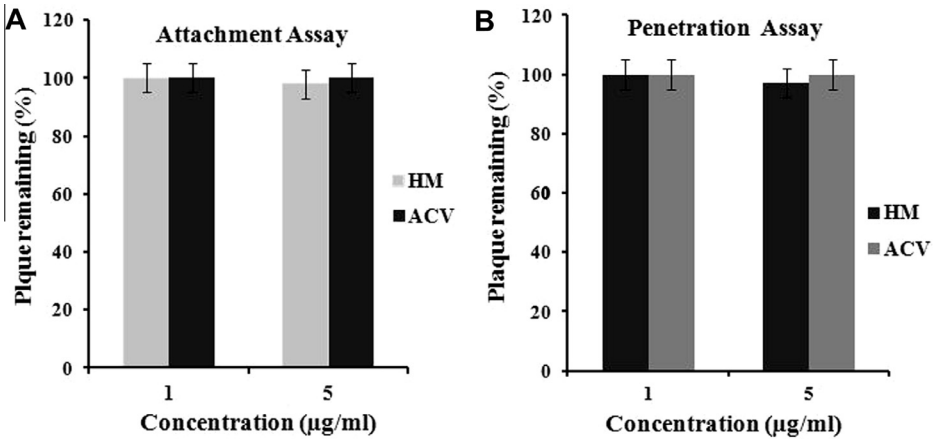

Fig. 3. Effect of HM on HSV-1 entry: (A) In attachment assay, cells were prechilled at 4 °C for 1 h, then challenged with HSV-1 (500 PFU/well) in absence or presence of HM (5.0 μg/ml) and kept for 3 h at 4 °C and then the plaque assay was carried out. (B) In penetration assay, prechilled infected cells were incubated for 3 h at 4 °C and then untreated or treated with HM, re-incubated for 20 min at 37 °C to facilitate viral penetration. The extracellular virus was inactivated by citrate buffer (pH 3.0) for 1 min and then the plaque assay was carried out. The graphs represent data from three independent experiments. Error bars are standard errors of the means.

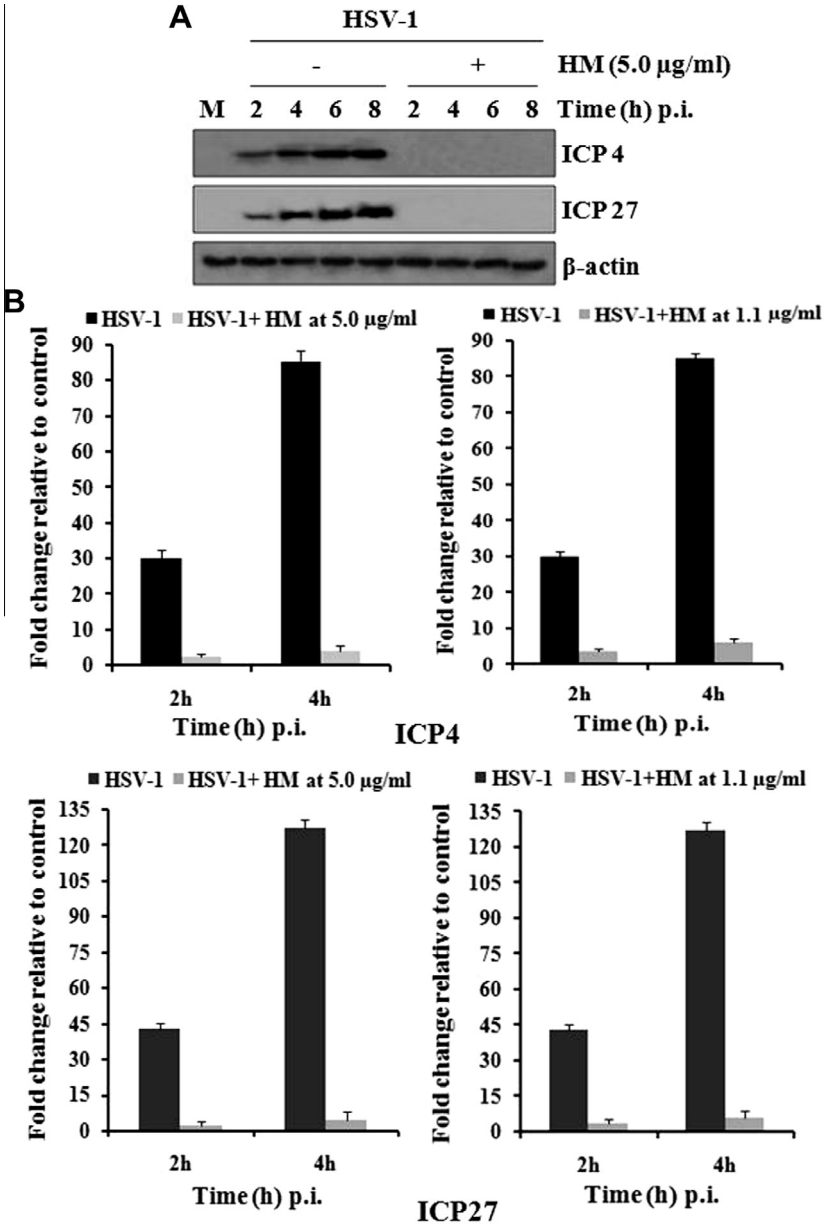

Fig. 4. Effect of HM on IE gene expression: (A) HSV-1 infected Vero cells were treated with HM (5 µg/ml) for 2–8 h post-infection and then the cells were lysed. The protein sample (40 µg) from whole cell extracts were separated by SDS–PAGE, blotted to PVDF and incubated with monoclonal anti-ICP4, anti-ICP27 or polyclonal anti-β-actin antibodies followed by peroxidase-labeled anti-rabbit polyclonal antibodies and visualized by using ECL Western blotting detection kit. (B) HSV-1 (5 moi) infected cells were treated with HM (1.1 and 5.0 µg/ml) for 2 and 4 h post-infection. Then RNA was isolated and subjected to cDNA synthesis, followed by the quantitative real-time PCR of ICP4 and ICP27 using SYBR Green. The graphs represent data from three independent experiments and Student's *t*-test was used to evaluate the significance of differences between groups.

HM treated HSV-infected cells (Fig. 3), suggesting that HM perhaps interferes with the viral early replication process.

To further analyze the effect of HM on HSV replication cycle, we determined the expressions of two IE gene products. The immunoblotting data reveals that ICP4 and ICP27 fails to express in virus-infected HM treated cells, but is well expressed in untreated controls (Fig. 4A). This observation was further strengthened by the qRT-PCR study where expression of both transcripts was significantly decreased in a time-dependent manner (Fig. 4B).

3.3. HM down regulates IE transcriptional events

Next we investigated the effect of HM on viral IE transcriptional events by assessing the binding of IE complex on ICP0 promoter. The EMSA analysis of nuclear extract (NE) from HSV-infected Vero cells for 2 and 4 h post-infection showed slower migrating band with increased binding of IE complex on ICP0 promoter (Fig. 5A, lanes 3–4), while in HM-treated virus-infected cells relatively faster migration of IE complex was evident (Fig. 5A, lanes 5–6). Further, when the binding of IE complex was analyzed with anti-HCF1, anti-VP16, anti-Oct1 and anti-LSD1 antibodies, the results also demonstrated faster migrating band in HM-treated NE (Fig. 5B–E, Lane 4) than the untreated one (Fig. 5B–E, lane 3), indicating that the slower migrating complex could contain additional proteins, recruited in response to the HSV-1 infection, than the infected HM-treated cells.

It is well known that HCF1-dependent recruitment of LSD1 plays an important role in the initiation of HSV infection, and the depletion of LSD1 activity with monoamine oxidase (MAO) inhibitors can block the HSV gene expression (Liang et al., 2009).

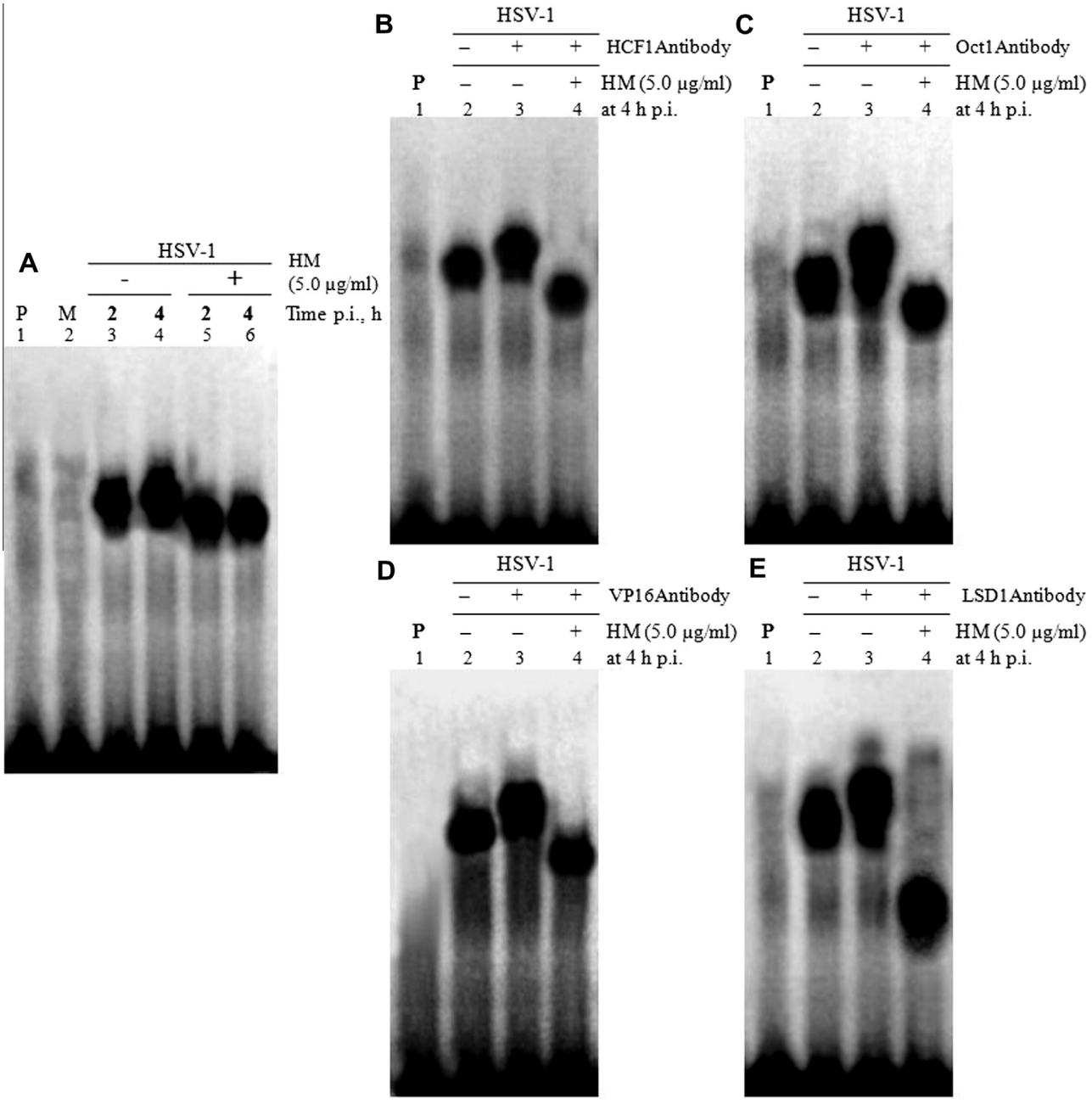

Fig. 5. Effect of HM on the binding of IE complex on ICP0 promoter: (A) Reaction mixtures (20 µl) containing 3 µg nuclear extracts, prepared from HSV-1 infected untreated or HM-treated Vero cells for 2 and 4 h post-infection, 20 fmol Biotin 3′ end-labelled probe, 50 ng/µl poly (dI-dC), 2.5% glycerol, 0.05% NP-40 (1%), 5 mM $MgCl_2$ and 1X binding buffer was incubated for 20 min at room temperature. The protein–DNA complexes were then separated by native gel electrophoresis on a 4% polyacrylamide gel in 0.5X TBE buffer transferred to Nylon membranes and developed according to the manufacturer's instructions (Thermo Scientific, USA). Lane 1: P, free probe; Lane 2: M, mock infected nuclear extract; Lane 3 to 4: HSV-1 infected nuclear extract for 2 and 4 h post-infection; Lane 5 to 6: HSV-1 infected nuclear extract treated with HM for 2 and 4 h post-infection. (B–E) For the gel shift assay, the binding reactions were performed as described above in panel A, following 30 min pre-incubation with specific antibodies against HCF1, VP16, Oct-1 and LSD1. Lane 1: P, free probe; Lane 2: Infected nuclear extract; Lane 3: HSV-1 infected nuclear extract in presence of respective antibodies; Lane 4: HSV-1 infected HM treated nuclear extract for 2–4 h in presence of respective antibodies.

Therefore, we have investigated the effect of HM on the association of HCF1 with LSD1 in infected cells by co-immunoprecipitation. A significant reduction of the association between HCF1 and LSD1 was observed in HM-treated cells, compared to untreated control (Fig. 6A), confirming that HM indeed interferes with the recruitment of LSD1 by HCF1, a vital component of IE transcription. As it is known, the reduced expression of ICP4 and ICP27 in inducible LSD1-RNAi cell line (Fig. 6B and C) showed the critical role of LSD1 in IE gene expression of HSV during infection. Further, the dose-dependent reduction in the intrinsic tryptophan fluorescence emission was observed when LSD1 was incubated with HM but not with HCF1 or VP16 (Fig. 7), suggesting that HM specifically interacts with LSD1. Thus, our results demonstrate that HM interferes with the binding of IE complex on ICP0 promoter and down-regulates the association of HCF1-LSD1 for its antiviral activity.

3.4. HM exhibit anti-HSV activity in infected mice

Acute toxicity studies revealed that HM is safe below 50 mg/kg without any obvious toxicity in treated mice. The sub-acute toxic-

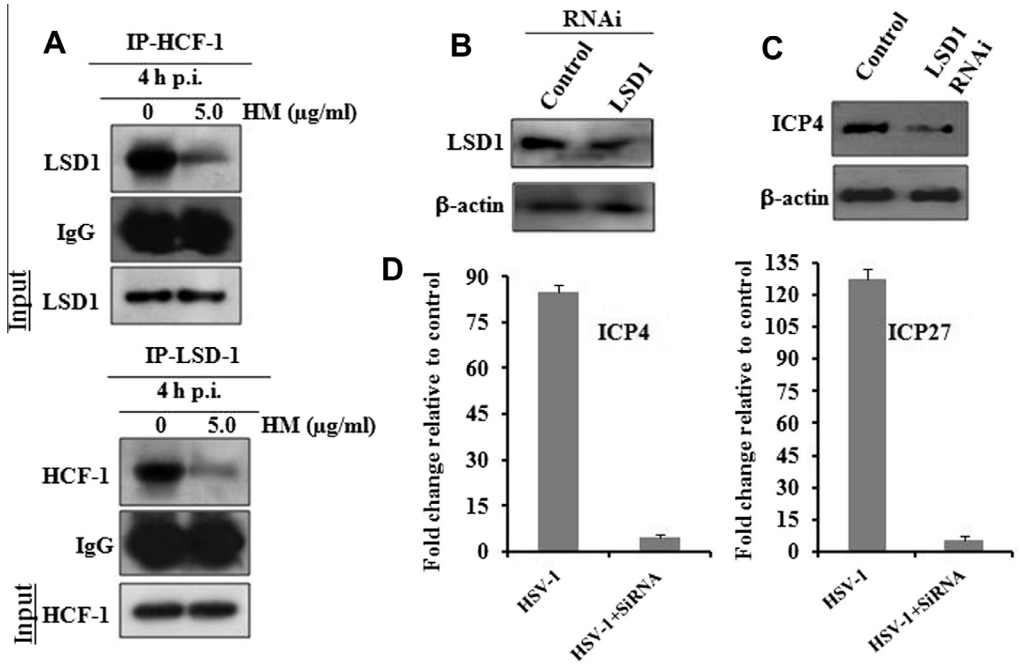

Fig. 6. Effect of HM on IE transcriptional events: (A) Western blot of HCF-1, LSD1 and control IgG immunoprecipitates. (B) Western blot of LSD1 and β-actin showing depletion of LSD1 relative to cells transfected with control scrambled RNAi. (C) Western blot of ICP4 and β-actin showing depletion of ICP4 in LSD1 SiRNA transfected cells relative to control (HSV-1 infected cells). (D) Quantitative real-time PCR showing the depletion of ICP4 and ICP27 in LSD1 transfected cells relative to HSV infected cells.

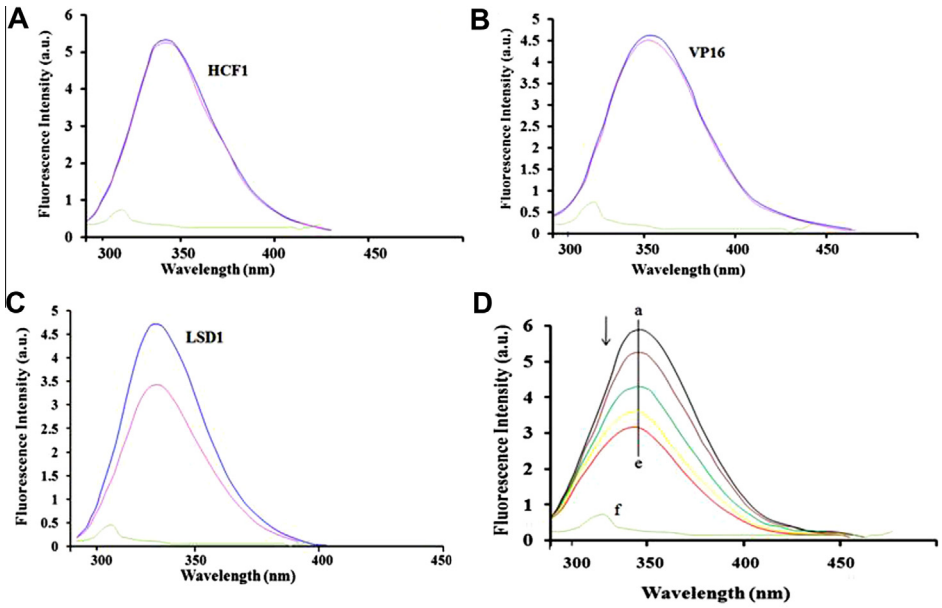

Fig. 7. Tryptophan fluorescence spectra analysis in presence of HM: (A–C) Tryptophan fluorescence spectra of HCF1, VP16, LSD1 in presence of HM (3.0 μg/ml) and the respective controls. (D) Tryptophan fluorescence spectra of LSD1 (30 μM) in presence of various concentration of HM (a, 0; b, 1.5; c, 3; d, 4; e, 5.0 μg/ml) at excitation wavelength of 285 nm.

ity study corroborated the safety profile of HM at antiviral concentration with normal haematological and biochemical profile, along with histopathology of major organs of the treated mice (Supplementary information).

In cutaneously-infected mice, the oral treatment of HM at its antiviral concentrations [0.1 (data not shown), 0.25, 0.5 mg/kg] delayed the development ($p < 0.05$) and progression of skin lesion in a dose-dependent manner at 4–8 days post-infection, compared to vehicle control (Fig. 8). However, ACV treatment showed significant reduction ($p < 0.001$) in survival time and skin lesion development. The topical treatment of HM-ointment showed that the lesion scores of untreated or Vaseline-treated groups were not significantly different ($P > 0.05$), while 0.25% and 0.5% ointment group had significant antiviral activity ($P < 0.001$) compared to control groups ($P < 0.001$) (Fig. 8). Interestingly, the infected mice treated with 0.5% HM, 1 h before and

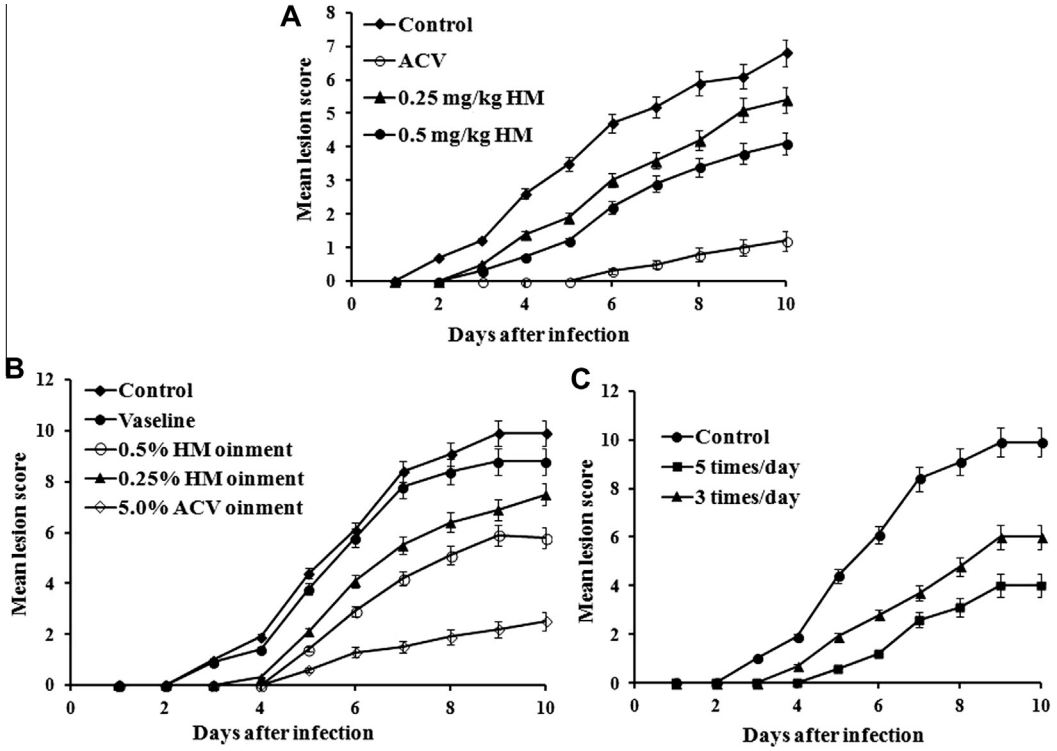

Fig. 8. Effect of HM administration on mice infected with HSV-1: (A) Cutaneously infected BALB/c mice were orally administered with HM (0.25 or 0.5 mg/kg) or ACV (5 mg/kg) and scored the skin lesions. (B) HM ointment (0.25% and 0.5%) was topically applied 1 h after HSV-1 infection and twice daily for 7 days, with no treatment or treated with Vaseline base as control, and scored the skin lesion(s). (C) HM ointment (0.5%) was topically applied 1 h after HSV-1 infection three or five times daily for 7 days, with no treatment group as control. Five and three times daily treatments were significantly effective compared with control. The graphs represent the mean, SEM of three independent experiments, and ANOVA tests were carried out as appropriate.

five-times a day provided better therapeutic effect than three-times, compared to no treatment control ($P < 0.001$). Thus, HM reduced HSV-1 load in a dose and time-dependent manner in infected animals.

The contemporary literature reveals that harmaline (Wang et al., 2008) is a CNS stimulant that stimulates striatal dopamine release with vasorelaxant activity (Berrougui et al., 2006), along with acetyl-cholinesterase and histamine N-methyltransferase inhibition. Harmaline is a reversible MAO-A inhibitor (Zhang et al., 2007) and may increase forelimb tremor (Stanford and Fowler, 1998) with weak neurotoxic effect in rats at a high dose (O'Hearn and Molliver, 1993). However, our study showed that isolated harmaline has potent anti-HSV-1 activity in mice at 0.25 and 0.5 mg/kg without any visible toxic effect, probably due to less purity or its less psychoactive nature than the related compounds (Jahaniani et al., 2005).

4. Conclusion

We have isolated a known alkaloid harmaline from *O. nicobarica* having potent anti-HSV-1 activity. The compound interferes with the viral IE-transcriptional events, and significantly reduces virus yield in mice at well-tolerated dose. As IE complex is a critical component of herpes virus reactivation mechanism, so harmaline may help to prevent both the multiplication and reactivation of HSV, and provide an interesting molecular target for the development of better therapy for efficient HSV management.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.02.007.

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