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Inhibition of herpes simplex virus type 1 entry by chloride channel inhibitors tamoxifen and NPPB

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ABSTRACT

Herpes simplex virus type 1 (HSV-1) infection is very common worldwide and can cause significant health problems from periodic skin and corneal lesions to encephalitis. Appearance of drug-resistant viruses in clinical therapy has made exploring novel antiviral agents emergent. Here we show that chloride channel inhibitors, including tamoxifen and 5-nitro-2-(3-phenyl-propylamino) benzoic acid (NPPB), exhibited extensive antiviral activities toward HSV-1 and ACV-resistant HSV viruses. HSV-1 infection induced chloride ion influx while treatment with inhibitors reduced the increase of intracellular chloride ion concentration. Pretreatment or treatment of inhibitors at different time points during HSV-1 infection all suppressed viral RNA synthesis, protein expression and virus production. More detailed studies demonstrated that tamoxifen and NPPB acted as potent inhibitors of HSV-1 early entry step by preventing viral binding, penetration and nuclear translocation. Specifically the compounds appeared to affect viral fusion process by inhibiting virus binding to lipid rafts and interrupting calcium homeostasis. Taken together, the observation that tamoxifen and NPPB can block viral entry suggests a stronger potential for these compounds as well as other ion channel inhibitors in antiviral therapy against HSV-1, especially the compound tamoxifen is an immediately actionable drug that can be reused for treatment of HSV-1 infections.

1. Introduction

Herpes simplex virus type 1 (HSV-1), a member of the *Herpesviridae*, is ubiquitous and contagious that cause a variety of clinically significant manifestations in adults and neonates [1]. Besides, HSV-1 can establish its latent infection in neurons and periodically reactivates to lead to significant psychosocial distress for infected patients [2,3]. Conventional treatments against the herpes are acyclovir (ACV) and related nucleoside analogs, which mainly inhibit HSV-encoded DNA polymerase through competition with deoxyguanosine triphosphate as a substrate for the enzyme [4,5]. However, ACV-resistant HSV occurs frequently in immunocompromised patients when using nucleoside analogs since ACV had been reported to have strong anti-HSV activity in the 1970s [6]. Thus exploring novel anti-HSV drugs with different mechanisms of actions is emergent.

Generally, viral proteins or host cell proteins that are essential to any steps of viral life cycle, from viral binding to release, have the potential to be valuable drug targets. Cell entry of viruses is an attractive target to therapeutic intervention, with opportunities to protect 'naive' cells [7]. HSV-1 can enter cells either by direct fusion of the viral envelope with host cell membrane or by endocytic pathways depending on the cell lines [8–10], and several compounds have been reported to interrupt such entry process and thereby effectively inhibited HSV-1 infection. For example, the cobalt chelate complex CTC-96 inhibited HSV-1 entry by interrupting the membrane fusion process which is independent on the presence of viral cellular receptors [11]. Nanoparticles coupled with chemical compounds also showed the antiviral activity by

Abbreviations: ACV, acyclovir; CtxB, choleratoxin beta subunit; ER, estrogen receptor; FDA, food and drug administration; HCV, hepatitis C virus; HSV, herpes simplex virus; MQAE, N-(Ethoxycarbonylmethyl)-6-methoxyquinolinium bromide; NPPB, 5-nitro-2-(3-pheny l-propylamino) benzoic acid.

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mimicking cell-surface-receptor sulfate [12]. In addition, vaccines [13] and natural products [14] probably are the alternative therapeutic strategies.

Ion channels play a key role in the regulation of all aspects of cell physiology including cell proliferation and apoptosis [15]. However, only limited information about the involvement of ion channels in virus infection has been obtained. In this report we demonstrated the antiviral activities of chloride channel inhibitors. Chloride channels represent a relatively under-explored target class for drug discovery and dysfunction of chloride channels correlates well with several diseases [16]. We used tamoxifen [17,18], a food and drug administration (FDA)-approved selective estrogen receptor modulators for the treatment of breast cancer [19], and 5-nitro-2-(3-pheny l-propylamino) benzoic acid (NPPB) [16] to further characterize the mechanism by which these drugs affected HSV-1 infection. These finding demonstrate that targeting the cell host chloride channel, as well as identifying approved drugs and probes with previously undocumented antiviral activity, is promising approaches in the development of antiviral therapies to HSV infection.

2. Materials and methods

2.1. Cells, virus, antibodies, and reagents

Vero cells (purchased from ATCC) were cultured at 37 °C in a humid atmosphere with 5% CO_2. HSV-1 strain F (ATCC VR733) was obtained from Hong Kong University. ACV-resistant clinical isolate of HSV-1 strains were obtained from the Guangzhou Institutes of Biomedicine and Health [20]. For a full list of antibodies, inhibitors, primers and reagents used, please refer to supplemental tables (Tables S1–S3). All inhibitors were used in a non-cytotoxic concentration. The cytotoxicity of chemical inhibitors were determined with a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay.

2.2. In vitro antiviral activity assay

Confluent cell monolayers were treated with increasing non-cytotoxic concentrations of the inhibitors. Four wells were used for each concentration. Afterwards, the cells were infected with HSV at 37 °C and observed daily for cytopathic effect (CPE) using a light microscope. ACV (20 μg/ml) served as positive control. The EC_{50} value was calculated by MTT method.

2.3. Intracellular chloride ion detection

After HSV-1 infection, cells were washed with Krebs-HEPES buffers (20 mM HEPES, 128 mM NaCl, 2.5 mM KCl, 2.7 mM $CaCl_2$, 1 mM $MgCl_2$, 16 mM glucose, pH 7.4) three times and then loaded with 5 mM MQAE in Krebs-HEPES for 60 min at 37 °C. Then cells were washed for five times to remove non-specific dye staining and images were acquired by confocal microscopy. MQAE is a kind of fluorescent probes specific for chloride ions, and its fluorescence intensity quenches when binds to chloride ions. Thus increased intracellular chloride concentration leads to a lower MQAE fluorescence. Images were pseudocolored in order to better visualize Cl^- mobilization with blue = low Cl^- and red = high Cl^-. The fluorescence intensities of the images were processed and quantified using Image J software.

2.4. Measurement of HSV-1 binding or penetration

Viral binding or penetration was detected as described previously [21,22]. Briefly, for penetration assay, Vero cells pretreated with inhibitors at 37 °C for 1 h were incubated with HSV-1 (MOI = 20) for 1 h at 4 °C in the absence of inhibitors. Then the cells were washed and incubated at 37 °C with the indicated compounds for 1 h. The cells were then washed three times with cold-PBS (pH 3.0) to remove bound but not penetrated virus. The cells were then harvested and the internalized viral DNA was isolated. Viral UL47/UL46 genes were assessed using quantitative real-time PCR (qRT-PCR) and were expressed relative to control infections without the addition of inhibitors. The PCR amplification product of UL46/UL47 was purified, diluted serially, and used as a standard for quantitative analysis. For HSV-1 binding assay, cells were incubated with virus in the presence of inhibitors at 4 °C for 1 h, and the cells were then washed to remove unbounded virus. Total viral DNA was extracted and measured by qRT-PCR.

2.5. RNA extraction and quantitative real-time PCR

For the mRNA expression level assay, total RNA was extracted with TRIzol reagent (Invitrogen) according to manufacturer's protocol and 1 μg of RNA was then reverse transcribed with a Prime Script RT reagent kit (TaKaRa) as described previously [21,22]. A quantitative real-time PCR assay was performed using a Bio-Rad CFX96 real-time PCR system. Messenger RNA transcription levels were standardized against housekeeping gene GAPDH.

2.6. Western blotting

Cells were lysed in RIPA buffer (Beyotime, China). The lysates were normalized to equal amounts of protein, and the proteins were separated by 6–15% gradient SDS–PAGE, transferred to nitrocellulose and probed with the indicated primary antibodies. Detection was conducted by incubation with species-specific HRP-conjugated secondary antibodies. Immunoreactive bands were visualized by enhanced chemiluminescence (Millipore).

2.7. Immunofluorescence staining and analysis

Vero cells were challenged with virus at 4 °C for 1 h and then incubated at 37 °C for indicated times. Then samples were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100. The samples were blocked in 5% BSA and incubated with anti-ICP5 antibody (1:3000) and fluorochrome-conjugated secondary antibodies (1:1000). Additionally, 1 mg/ml DAPI-PBS and 5 μM TRITC-phalloidin were added to label nuclei (15 min) and F-actin (40 min), respectively. Images were captured with a Zeiss LSM510 Meta confocal system (Carl Zeiss).

For lipid rafts staining, cells incubated on ice with Alexa Fluor 488-conjugated Choleratoxin beta subunit (CtxB) (1 μg/ml) for 1 h were transferred to 37 °C for the indicated time to visualize raft-resident ganglioside M1 (GM1). Proteins of interest were visualized using a common immunofluorescence staining protocol, and images were taken by confocal microscopy.

2.8. Intracellular calcium detection

Vero cells loaded with 5 μM Furo-3 AM (Beyotime, China) were pretreated with NPPB (100 μM) and tamoxifen (10 μM) at 37 °C for 1 h, then cells were incubated with HSV-1 (MOI 10) at 4 °C for 1 h and the cells were then transferred to 37 °C for 1 h in the presence of inhibitors. Then images were captured by confocal microscopy. Increased intracellular calcium concentration leads to a higher furo-3 AM fluorescence intensity.

2.9. Statistical analysis

Student's two-tailed *t*-test was used for all statistical analysis, with the level of significance set at $^{***}p < 0.0005$; $^{**}p < 0.005$; $^{*}p < 0.05$.

3. Results

3.1. Assessment of anti-HSV activities of tamoxifen and NPPB

First the effects of chloride channel inhibitors on HSV replication and the cytotoxicity of inhibitors were examined in Vero cells, where the active drug concentrations could be refined and an accurate inhibitory concentration of 50% (IC_{50}) determined [23] (Fig. 1A). Tamoxifen and NPPB inhibited HSV-1 infection with an IC_{50} of 4.89 and 35.24 μM respectively, while the IC_{50} for ACV toward HSV-1 was 3.5 μM. Three clinical isolated ACV-resistant viruses (IC_{50} value of ACV >200, namely HSV-1 106, HSV-1 153 and HSV-1 blue) were also used to test the antiviral activities of tamoxifen and NPPB (Fig. 1B). Apparently, tamoxifen and NPPB were both represented significant inhibition activities. The antiviral activity of tamoxifen was more potent against ACV-resistant HSV-1 strain than that against HSV-1 F strain. In addition, inhibitory effects of tamoxifen and NPPB on HSV RNA synthesis and protein expression were assayed by quantitative Real-time PCR and western blotting respectively [21,22]. At indicated times post infection, mRNA expression levels of HSV-1 immediate early (IE) gene (UL54), early (E) gene (UL52) and late (L) gene (UL27) were significantly reduced by inhibitors (Fig. 1C). Besides, tamoxifen and NPPB showed inhibitory effects on viral proteins ICP5 (L gene-encoded) and ICP27 (IE gene-encoded) expression (Fig. 1D).

3.2. Intracellular chloride concentration is increased by HSV-1 infection and reduced by tamoxifen and NPPB treatment

Because tamoxifen and NPPB are chloride channel inhibitors, we determined whether HSV-1 infection affected homeostasis of

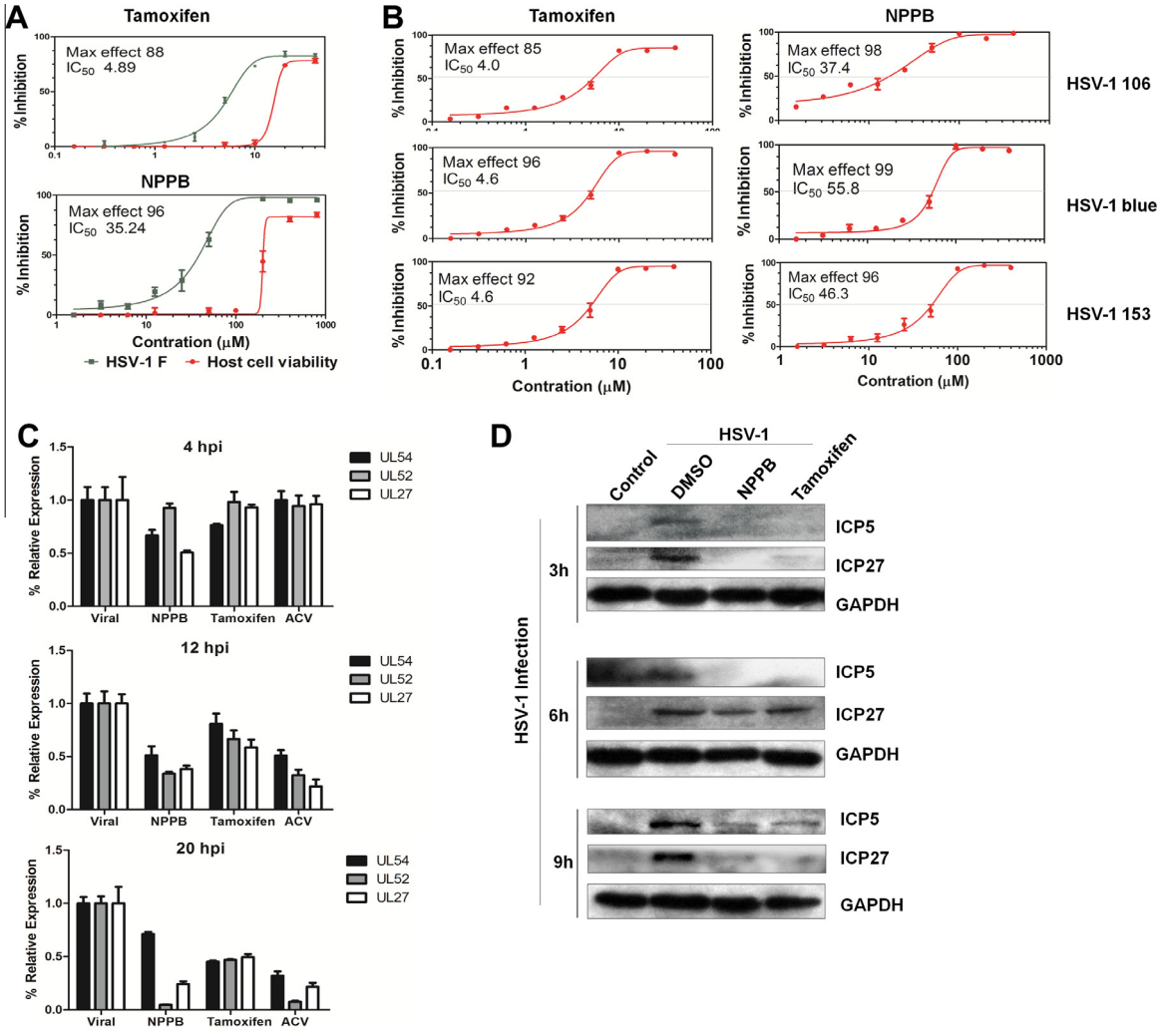

Fig. 1. Assessment of anti-HSV activities of tamoxifen and NPPB. (A, B) In vitro dose–response curves for tamoxifen and NPPB toward standard HSV-1 (HSV-1 F) and three clinical isolated ACV-resistant viruses. (A) The percent inhibition of the compounds is shown in green and the cytotoxic effect of the compounds is shown in red. The maximum percent inhibition observed (Max effect) and IC_{50} are indicated. (B) Shown is the Max effect (% inhibition) along with the IC_{50} values (in μM). Results indicate that both inhibitors are effective across all clinical isolated virus strains. Error bars indicate SEM. Results are from three replicates. (C) Vero cells were infected with HSV-1 (MOI = 1) for 4, 12 and 20 h in the presence of tamoxifen (10 μM), NPPB (100 μM) or ACV (20 μg/ml) and mRNA expression levels of viral IE (UL54), E (UL52) and L (UL27) genes were assayed by qRT-PCR. Each value represents the mean ± SD of 3 separate experiments. (D) Cells infected with HSV-1 for indicated times were lysated and probed with anti-ICP5 and anti-ICP27 antibodies. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

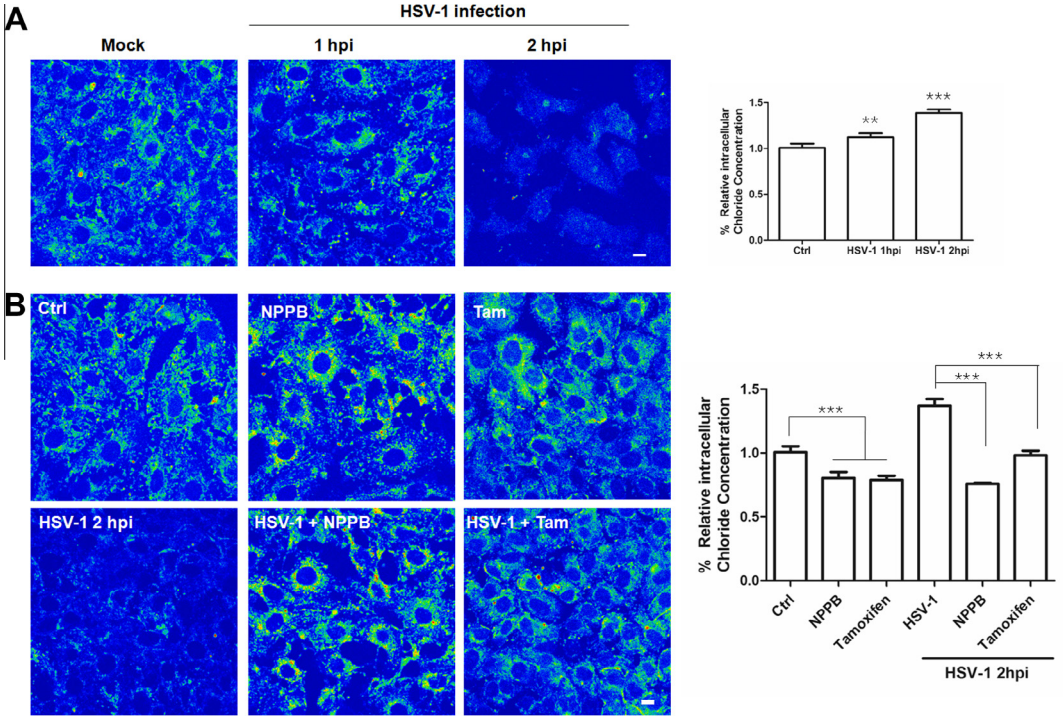

Fig. 2. Chloride concentration increased by HSV-1 infection and reduced by tamoxifen and NPPB. (A) HSV-1 infection increased intracellular chloride concentration. Vero cells infected with HSV-1 (MOI = 10) at different time points were loaded with 5 mM MQAE and fluorescent images were acquired by confocal microscopy. (B) Still images captured at the indicated times in vero monolayers pretreated with tamoxifen (10 μM) and NPPB (100 μM) for 1 h, infected with HSV-1 for 2 h in the presence of inhibitors. Scale bar = 10 μm.

intracellular chloride ion. As shown in Fig. 2A, HSV-1 infection increased intracellular chloride concentration at 1 or 2 h post-infection (hpi). In the presence of tamoxifen and NPPB, chloride concentration was reduced to normal or even lower level comparing to control group (Fig. 2B). These results suggest an important role for chloride influx in HSV-1 early infection.

3.3. Tamoxifen and NPPB inhibit HSV-1 entry and nuclear translocation

To determine the stage when tamoxifen and NPPB exerts their antiviral activity, a time of addition assay was performed (Supplemental Figs. 1 and 2). Results suggest that tamoxifen and NPPB inhibit mainly viral entry. Next to further characterize the inhibitory effects during the early stage of HSV-1 infection, we evaluated whether tamoxifen or NPPB affected HSV-1 binding and penetration (Fig. 3A). After incubation for HSV-1 binding (at 4 °C) or penetration (at 37 °C), viral DNA was extracted and assayed by quantitative-PCR [22]. We found that both binding and penetration of HSV-1 were reduced in a concentration-dependent manner. Western blotting assay also showed a reduction of viral binding (Fig. 3B). The effect of tamoxifen or NPPB on HSV-1 entry was also evaluated by confocal microscopy by immunostaining with a viral late gene-encoded protein ICP5. As shown in Fig. 3C, HSV-1 virions were localized efficiently in the cytoplasm at 1 and 2 hpi in control group. However, only few viral particles could be detected and the ICP5 protein signal declined noticeably in the presence of 100 μM NPPB and 10 μM tamoxifen. Both inhibitors reduced the percentage of infected cells and the average number of viral particles per cell. Furthermore, inhibitors prevented intracellular migration of viral particles (Fig. 3D). ICP5 is a late gene encoded protein, thus at a relatively early time post-infection (4 h), de novo production of IC5 has not been started and the signal for ICP5 represents the entry of virus. Cells were treated with vehicle, tamoxifen, NPPB, or Nocodazole, the latter as a positive control for inhibition viral trafficking by disrupting microtubule [24]. At 4 hpi, we observed strong inhibition of viral nuclear transportation with tamoxifen, NPPB, as well as nocodazole. The percentage of positive nuclei (nuclei docked with ICP5-postivie dot) was determined to evaluate the viral trafficking efficiency and all the inhibitors reduced the percentages as 72% (tamoxfien), 66% (NPPB), and 63% (Noc), respectively. Overall, these results indicate that tamoxifen and NPPB inhibit HSV-1 replication mainly through inhibiting viral entry.

3.4. Tamoxifen and NPPB reduce viral membrane localization

Next we sought to determine the mechanism of entry inhibition. Lipid rafts, acting as concentrating the receptors required for binding and penetration or inducing signal transduction upon binding of the viral protein, has been reported to participate in HSV-1 fusion and entry [22,25]. Thus we evaluated whether lipid rafts was involved in HSV-1 entry. We examined raft organization by labeling the lipid raft marker ganglioside GM1 using Alexa Fluor-conjugated CtxB (Fig. 4A). During HSV-1 entry, most viral particles colocalized with lipid rafts (arrows indicated). However, in the presence of tamoxifen and NPPB, few or none of viral particles colocalization was observed while both inhibitors did not affect the components or the numbers of lipid rafts. Thus the compounds are likely affect the triggering of fusion process by reducing viral membrane localization or binding.

3.5. Tamxifen and NPPB reduce intracellular calcium release

Calcium is involved in many signaling cascades and HSV-1 has been reported to utilize Ca^{2+}-signaling pathway for virus-cell fusion and entry [26,27]. Treatment with calcium channel blockers diminishes virus-cell fusion and entry into cells. Thus we asked

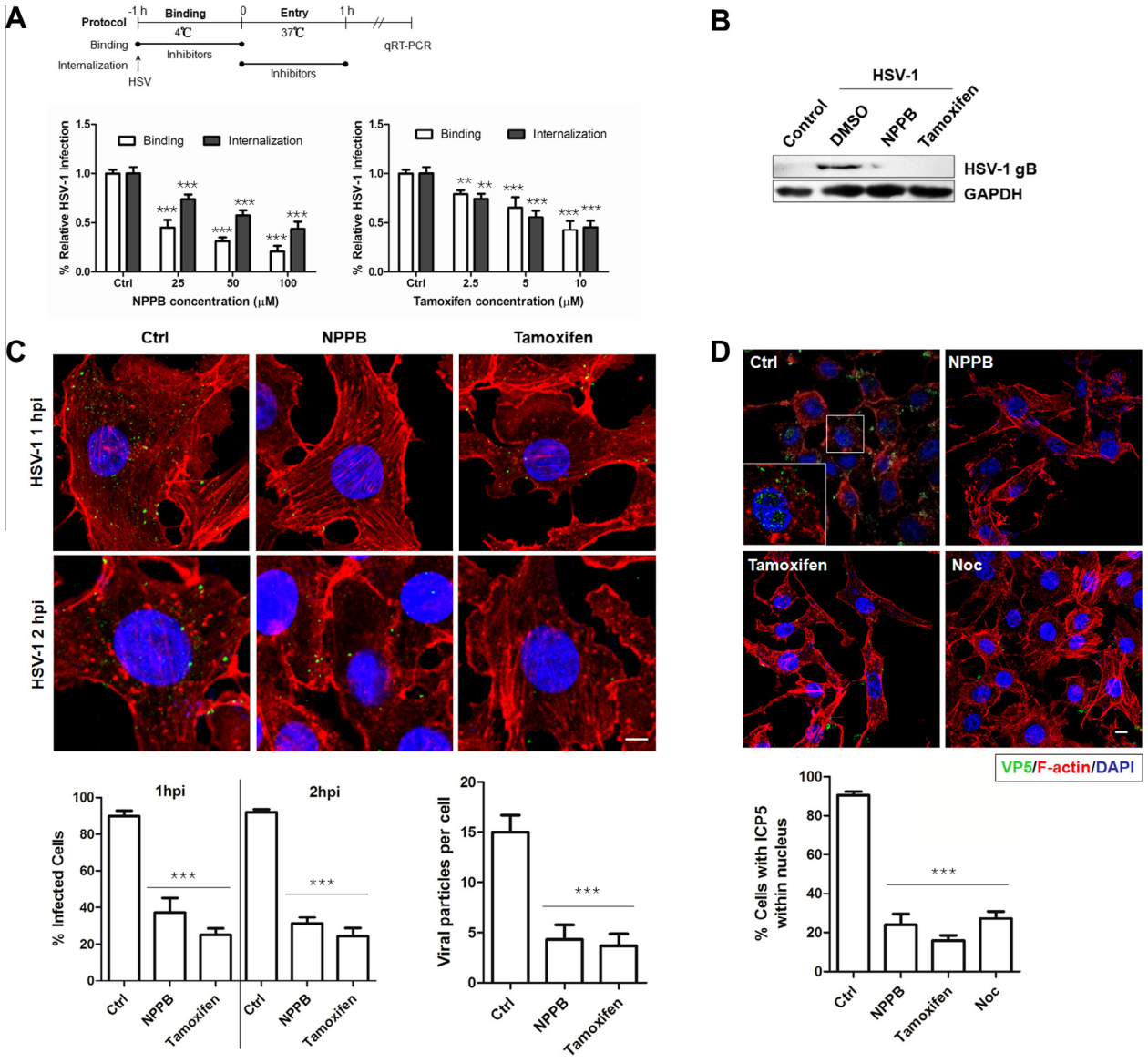

Fig. 3. Tamoxifen and NPPB inhibit viral binding, penetration and nuclear translocation. (A) Quantitative PCR assay shows the inhibitory effects of tamoxifen and NPPB on HSV-1 binding and penetration. Experimental design was shown. Vero cells pretreated with different concentrations of inhibitors were incubated with HSV-1 (MOI = 10) at 4 °C for 1 h in the presence or absence of inhibitors as the protocol shows. The cells were then washed, transferred to 37 °C for 1 h and viral DNA was extracted and analyzed by quantitative PCR, see also materials. (B) Western blotting shows the inhibitory effect of tamoxifen and NPPB on viral binding. Vero cells incubated with HSV-1 at 4 °C for 1 h in the presence or absence of tamoxifen (10 μM) and NPPB (100 μM) were lysed and probed with an anti-gB antibody. (C) Inhibition of HSV-1 entry. The cells were infected with HSV-1 for the indicated time and then fixed, immunostained with anti-HSV ICP5 antibody (green) and TRITC-phalloidin (red). The percentage of infected cells and the average number of viral particles per cell were quantified. At least 100 cells with from 5 representative fields were counted in each independent experiment. (D) Inhibitors impair viral nuclear transportation. At 4 h post infection in the presence of inhibitors, the cells were fixed and stained. 5 μM nocodazole was used as a positive control. The percentage of positive nuclei (nuclei docked with at least 1 ICP5-positive dot) was determined to evaluate the viral trafficking efficiencies. Each value represents the mean and SD of 3 separate experiments, and at least 100 cells from 5 representative fields were counted in each experiment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

whether tamoxifen or NPPB affected calcium homeostasis and thereby reduced HSV-1 entry. As shown in Fig. 4B, HSV-1 entry apparently increased intracellular calcium concentration while the calcium response to virus was significantly reduced if the cells were pretreated with tamoxifen and NPPB. In summary, the above observed entry inhibition indicates that these compounds are likely affecting HSV-1 fusion process possibly by affecting calcium homeostasis and the viral colocalization with lipid rafts.

4. Discussion

Interfering with the virus entry steps has become an attractive therapeutic strategy except for targeting viral enzymes that are indispensable for replication [28]. The proof of concept for entry targets has mostly been obtained from the safe and efficacious HIV fusion inhibitor enfuviritide [7]. In this work, for the first time, we demonstrate the antiviral activities of chloride channel inhibitors tamoxifen, an FDA-approved drug, and NPPB through blocking HSV-1 entry.

Chloride channels are transmembrane proteins that facilitate chloride ion transport through a membrane [16]. Unlike other ion channels, identification and analysis of chloride channel has lagged behind those of other ions partly due to technical challenges in screening for specific chloride channel modulators. Researches seldom pay close attention to elucidating the role for chloride channels in virus infection. Recently, Sasvari et al. have shown that

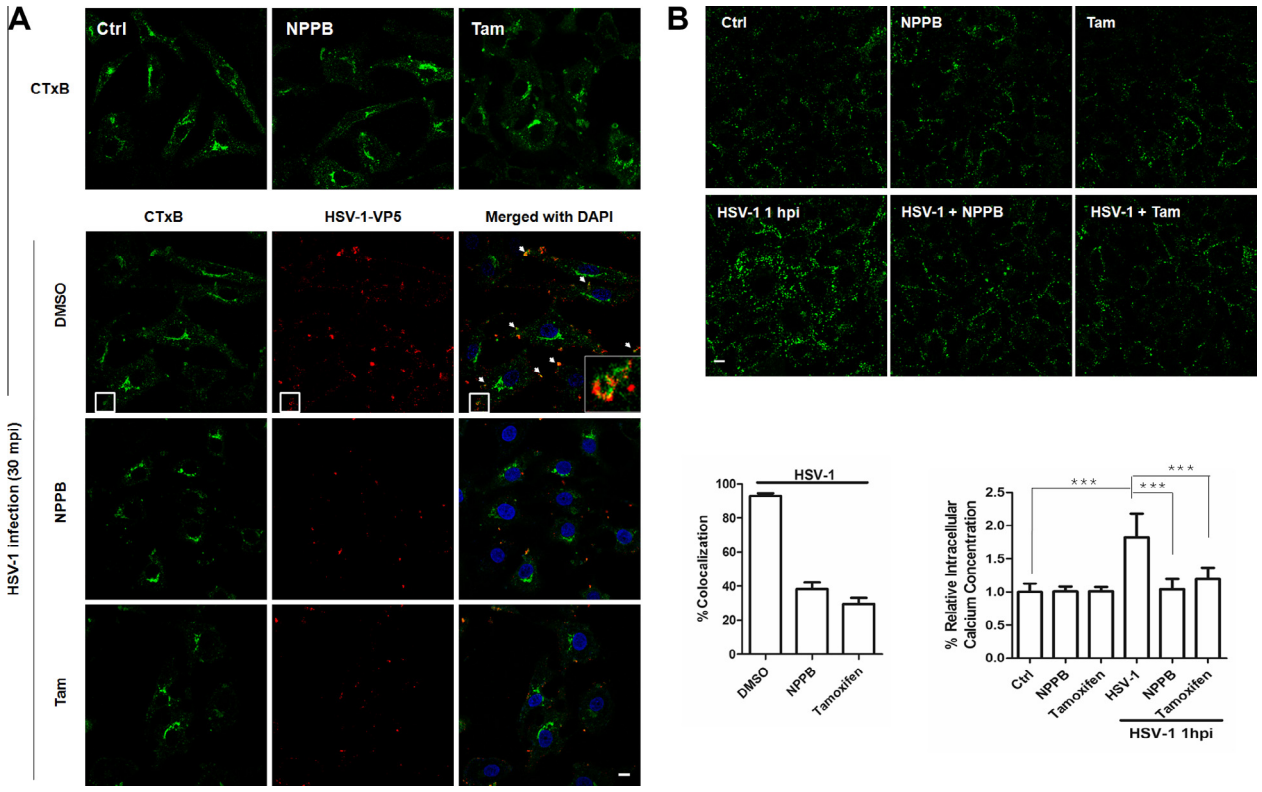

Fig. 4. Evaluation of tamoxifen and NPPB on calcium concentration and the colocalization between virions and lipid rafts. (A) Vero cells were pretreated with tamoxifen (10 μM) and NPPB (100 μM) for 1 h at 37 °C, and the cells then were incubated on ice with Alexa Fluor 488-conjugated choleratoxin beta subunit (CtxB) (1 μg/ml) and HSV-1 for 1 h. The cells were then shifted to 37 °C for additional 30 min in the presence of inhibitors and fixed, immunostained with anti-ICP5 antibody. Arrows indicated the colocalization. (B) Tamoxifen and NPPB reduce intracellular calcium concentration. Vero cells preloaded with furo-3 AM in the presence of absence of tamoxifen (10 μM) and NPPB (100 μM) were infected with HSV-1 for 1 h and the cells were then fixed, photographed with a confocal microscope. Fluorescent intensity was calculated using Image J software. Scale bar = 10 μm.

by blocking of chloride channels, the accumulation of Tomato bushy stunt virus genomic RNA has been reduced [29]. Herein, our results indicate that chloride channels may play a critical role in HSV-1 entry because intracellular chloride ion concentration was increased by HSV-1 infection (Fig. 2A) and chloride channel inhibitors, as tamoxifen and NPPB, exhibited extensive anti-HSV-1 activities.

Tamoxifen, an antagonist of estrogen receptor (ER-α), has been widely used clinically for the treatment α of breast cancer and there were few reports of undocumented activity of tamoxifen. Previously, tamoxifen was reported to have an antiviral activity toward HCV [30], and the antiviral activity was independent of the activity of estrogen receptor [31]. Similarly in our experiments, tamoxifen affected HSV-1 early infection through other mechanisms and the antiviral activity is also independent of ER function because the cells we used (Vero cells) did not express ER-α [23]. Reduced viral colocalization with lipid rafts and intracellular calcium concentration indicate that fusion process of HSV-1 was impaired by tamoxifen. It is not known whether tamoxifen and NPPB interact directly with viral protein or mediate their effects indirectly through inhibition of chloride channels function. Further experiments are required to investigate the detailed mechanism of chloride channels in viral entry and virus-induced Ca^{2+}-signaling. In addition, tamoxifen has been shown to induce the expression of interferon and interferon-stimulated genes, which would have dramatic effects on virus replication [32].

In conclusion, tamoxifen and NPPB were shown to be effective against both wild-type and ACV-resistant HSV strains. They exhibited their inhibitory activities at the early phase by preventing viral fusion process. Because it takes a great deal of time and money to develop a new drug from a novel chemical compound, it may be easier to use previously developed drugs that can be used for new applications. Tamoxifen is such a drug that has been in use for an extended period of time. Therefore, tamoxifen, as well as NPPB were confirmed to be a promising anti-HSV drug candidate. Furthermore, insights emerging from this basic research indicate broad opportunities and challenges for chloride-channel-based antiviral therapies.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.050.

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