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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.

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