NSC23766 and Ehop016 Suppress Herpes Simplex Virus-1 Replication by Inhibiting Rac1 Activity

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Herpes simplex virus-1 (HSV-1) infection of the eyes leads to herpes simplex virus keratitis (HSK), the major cause of infectious blindness in the world. As the current therapeutics for HSV-1 infection are rather limited and prolongued use of acyclovir (ACV)/ganciclovir (GCV) and in immunocompromised patients lead to the risk of drug resistant mutants, it underlines the urgent need for new antiviral agents with distinct mechanisms. Our study attempted to establish ras-related C3 botulinum toxin substrate 1 (Rac1) as a new therapeutic target for HSV-1 infection by using Rac1-specific inhibitors to evaluate the in vitro inhibition of virus growth. Our results showed that increased Rac1 activity facilitated HSV-1 replication and inhibition of Rac1 activity by NSC23766 and Ehop016 significantly reduced HSV-1 replication. Thus, we identified NSC23766 and Ehop016 as possessing potent anti-HSV-1 activities by suppressing the Rac1 activity, suggesting that Rac1 is a potential target for treating HSV-1-related diseases.

Key words ras-related C3 botulinum toxin substrate 1 activity; NSC23766; Ehop016; herpes simplex virus-1 infection

INTRODUCTION

Herpes simplex virus-1 (HSV-1) is a highly contagious human neurotropic virus. Conferring to the 2012 statistics of WHO, approximately 3700 million people below the age of 50 were diagnosed with HSV-1 globally. The modes of HSV-1 transmission include vertical transmission from mother to fetus and direct contact with infected individuals. HSV-1 causes lifelong latent infection as it can exist in trigeminal ganglia. HSV-1 infection also results in serious outcomes such as keratitis and encephalitis which can lead to infectious blindness or even death.

Herpes simplex virus keratitis (HSK) is a common clinical manifestation resulting in corneal blindness as well as a main cause for corneal transplantation in the U.S.A. Approximately half a million people suffer from HSV-related ocular diseases per year in the U.S.A. In high income countries, herpetic eye disease has been the main cause of corneal scarring and secondary visual loss. Although HSV can infect the posterior and anterior eye segments, corneal epithelial infection is the most common. HSV can survive latently in the sensory ganglion after primary infection, and the virus reactivation results in a lysis of ocular tissue, which leads to a dendritic ulcer. Many antiviral agents, especially nucleoside analog acyclovir (ACV), have been used to treat HSV-1 infection. However, studies found that viral shedding frequently occurred during antiviral therapy, and available treatments did not sufficiently suppress the viral load even in immunocompetent hosts.

On the other hand, the emergence of drug-resistant HSV-1 strains is also a great challenge for HSV-1 treatment. Therefore, new therapeutics with distinct mechanisms are urgently needed.

Ras-related C3 botulinum toxin substrate 1 (Rac1) belongs to Rho guanosine 5'-triphosphatase (GTPTase) family of small G proteins; it is universally expressed and plays a vital role in multiple cellular signaling pathways that regulate cell proliferation, apoptosis, motility, vesicle trafficking, cell polarity, gene transcription, and redox signaling. Rac1 has guanosine diphosphate (GDP)-bound inactive form and guanosine triphosphate (GTP)-bound active form and acts as a molecular switch by transforming between these two forms. During the process of virus infection, Rac1 is a vital regulator of actin remodeling through which viruses can regulate vesicular trafficking to support virion surfing, receptor clustering, and virus-containing vesicle internalization. Although it is reported that Rac1 signaling acts in early HSV-1 infection in epithelial Madin–Darby canine kidney strain II (MDCKII) cells, whether Rac1 participates in the HSV-1-induced pathology of HSK remains unclear.

Here, we used HCEC, a human corneal epithelial cell, and HaCaT, a human skin keratinocyte, to evaluate the role of Rac1 activity during HSV-1 infection in vitro and showed that Rac1 activity was considerably up-regulated in HSV-1-infected cells and the viral replication was inhibited by two Rac1 inhibitors NSC23766 and Ehop016.

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MATERIALS AND METHODS

**Cells and Virus**  Vero (African green monkey kidney) cells, HeLa (human cervical carcinoma) cells, and HCEC (human corneal epithelial cell line) were bought from American Type Culture Collection (ATCC) (Manassas, VA, U.S.A.), human skin keratinocyte HaCaT cells were brought from Cell Line Service (Eppelheim, Germany). All cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (Atlanta, GA, U.S.A.) at 37°C with 5% CO₂. HSV-1 strain was a gift from Professor Ergun Li, Nanjing University, propagated on Vero cells and standard plaque assay was used to calculate the titers. 21)

**Chemicals, Reagents, Plasmids and Instruments**  NSC23766 (Cat#: 733767-34-5) was obtained from Med Chem Express (NJ, U.S.A.) and dissolved in double distilled water at a concentration of 10 mM for storage. Ehop016 (Cat#: 1380432-32-5) was obtained from Selleck Chemicals (TX, U.S.A.) and was dissolved in dimethylsulfoxide (DMSO) at a concentration of 50 mM for storage. pcMV3-Rac1-HA plasmid (Cat#: HG10553-CY) was purchased from Sino Biological (Beijing, China). IRDye 800 goat-anti-mouse immunoglobulin G (IgG) and IRDye 680 goat-anti-rabbit IgG were bought from LI-COR Odyssey Infrared Imager. The bands intensity was measured using ImageJ software.

**Western Blotting and In-Cell Western**  Cells were lysed using RIPA lysis buffer (50 mM Tris–HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 5 mM NaF, 0.25% sodium deoxycholate, 1% NP40, 0.4% sodium dodecyl sulfate (SDS), 5 mM sodium orthovanadate, 0.1% sodium phenylmethylsulfonyl fluoride (PMSF)) with protease inhibitor, and phosphatase inhibitor on ice for 30 min, following centrifugation at 12000 × g for 10 min at 4°C. Next, we measured the concentration of total protein from the supernatant by bicinchoninic acid (BCA) protein assay kit. Sixty micrograms protein per sample was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and the proteins were transferred to polyvinylidene difluoride (PVDF) membranes bought from Millpore (Billerica, MA, U.S.A.). The membranes were blocked, and followed by staining with primary antibodies at 4°C overnight. The membranes were then incubated with IRDye 800 goat-anti-mouse IgG or IRDye 680 goat-anti-rabbit IgG (1:10000) for 1 h at room temperature and imaged with LI-COR Odyssey Infrared Imager.

**Pull-Down Assay**  Pull-down assay was conducted to examine Rac1 activity. The level of Rac1-GTP was measured by Rac1 interactive binding domain of p21-activated kinase (PAK) following the previous published protocol. 22) Cell lysates with the same amount of total protein (60 µg) were incubated with GST-tagged PAK (p21-activated kinase)-PBD agarose beads (Cytoskeleton, PAK02) for GTP-bound Rac1 at 4°C for overnight and GAPDH was used as a basic control. On 2nd day, we washed thrice the beads with lysis buffer prior to SDS-PAGE (12%) and consequently relocated into PVDF membranes. Following blocking, the membranes were next incubated with primary antibodies at 4°C overnight. Next, we incubated the membranes with IRDye 800 goat-anti-mouse IgG (1:10000 dilution) for 1 h at room temperature and imaged with LI-COR Odyssey Infrared Imager.

**In Vitro Antiviral Assay**  The antiviral effect of NSC23766 and Ehop016 was evaluated through In-Cell Western assay. 23) Briefly, Vero cells were cultured until they reached 80% confluence in 96-well plates. We serially diluted the testing compounds and added them to the cells. After being incubated for 30 min at 37°C, we used the HSV-1 at an MOI = 3 to infect the cells, containing the test compound. Following incubation for 24 h, we discarded the supernatant, and then determined the HSV-1 gD-1 expression by In-Cell Western assay. Viral inhibition (%) was measured following the formula: \[ \frac{1 - \text{fluorescence}_{\text{gD-1}}}{\text{fluorescence}_{\text{control}}} \times 100. \] "fluorescence_{gD-1}" represents gD-1 expression of the infected cells with drug treatment, and "fluorescence_{control}" represents gD-1 expression of the infected cells without the drug. The 50% effective concentration (EC_{50}) for antiviral effect was described as the concentration of antiviral compound inhibiting 50% of the virus-induced infection compared to the virus infection without compound treatment.

**In Vitro Cytotoxicity Assay**  The cell cytotoxicity of NSC23766 or Ehop016 was determined by a cell counting kit (Kumamoto, Japan) following the manufacturer’s protocol. In brief, 2 × 10^4 cells were spread into each well of 96-well plates and grown for 24 h prior to serial concentrations of NSC23766 and Ehop016 were supplemented in triplicate. Following 24 h, the supernatant containing compounds was removed and medium containing 10 µL Cell Counting Kit-8 (CCK-8) mixture was added per well, and the culture plate was incubated for 2 h at 37°C. Absorbance at 450 nm was determined by a TECAN Infinite M200 microplate reader (Männedorf, Switzerland). Cell viability was measured by the below formula: Cell viability (%) = \[ \frac{OD \text{ (experiment)}}{OD \text{ (control)}} \times 100. \]

**Cell Transfection Assay**  HeLa cells were transiently transfected in 6-well plate with 1 µg/well or 2 µg/well plasmid pcMV3-Rac1-HA by Lipofectamine 3000 transfection reagent from Life Technologies (Carlsbad, CA, U.S.A.) when cell grew to 40–60%. The cells were further cultured for 24 h, and added HSV-1 at an MOI = 1 into the well. After cell culture for another 24 h, the supernatant was discarded and proteins were detected by Western blot analysis.

**Plaque Assay**  The antiviral activity of NSC23766 or Ehop016 in vitro was also examined through titrating the infectious virions in NSC23766 or Ehop016-treated cells as reported previously. 24, 25) Briefly, Vero cells were infected with HSV-1 (MOI = 3) in the presence of indicated concentration of NSC23766 or Ehop016. After 24 h, cells were repeatedly fro-
zen and thawed three times to release virions. Then the medium with the virions was diluted and dispensed on Vero cells. Viral titer was calculated by counting the plaque amount.

**Statistical Analyses** Statistical analysis was carried out using GraphPad Prisms 6.0 software. Results were presented as means ± standard deviation (S.D.) with at least three biological replicates. The student’s t-test analysis was executed to compare the means of two groups or one-way ANOVA was used for more than two groups. Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001.

**RESULTS**

**Rac1 Activity Contributes to HSV-1 Replication** The ratio of GTP-bound active form of Rac1 (Rac1-GTP) to total Rac1 was considered as a measurement for Rac1 activity. To determine the role of Rac1 activity after HSV-1 infection, HeLa cells were infected with HSV-1 at various MOIs and we found that Rac1 activity dramatically increased by 70% at an MOI of 1 in the infected cells as compared to the uninfected cells, as shown by pull-down and Western blot assays (Figs. 1A, C). Furthermore, our result showed that Rac1 activity increased approximately 60 and 70% after transfecting the cells with Rac1-HA plasmid at the dose of 1 and 2 µg, respectively (Figs. 1B, D). Meanwhile, the expression of viral late protein gD-1 significantly increased after the transfection of Rac1-HA plasmid (Figs. 1B, E), indicating that overexpression of Rac1 facilitated viral replication. Then, to investigate the effect on

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**Fig. 1.** Rac1 Activity Was Necessary for Efficient HSV-1 Replication

(A) HeLa cells were infected with HSV-1 at various MOIs for 24h. Rac1-GTP was detected by pull-down and Western blotting assay. The level of Rac1-GTP was measured by Rac1 interactive binding domain of PAK. Cell lysates with the same amount of total protein (60 µg) were incubated with GST-tagged PAK (p21-activated kinase)-PBD agarose beads (Cytoskeleton, PAK02) for GTP-bound Rac1 at 4 °C for overnight and GAPDH was used as a basic control. The ratio of Rac1-GTP/total Rac1 was measured in arbitrary units. (B) Rac1-HA plasmids in a dose-dependent were transfected into HeLa cells for 24h and the cells were then infected with HSV-1 (MOI = 1) for 24h. The level of Rac1 activity was evaluated by pull-down and Western blotting assay. (C–E) Quantification analysis of Rac1-GTP/total Rac1 and gD-1/GAPDH. (F) Quantification analysis of plaque assay after Rac1-HA over-expressing. The blot shown is an exemplary representative of triplicate experiments. Data shown are means of results from three independent experiments. Error bars, S.D. Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001.
the progeny virus after the transfection of Rac1 plasmid, we performed a plaque assay and found that the upregulation of Rac1 activity promoted HSV-1 replication (Fig. 1F). Thus, Rac1 activity positively regulated HSV-1 replication.

**NSC23766 and Ehop016 Inhibit HSV-1 Replication**

Previous literatures reported that both NSC23766 and Ehop016, chemical structures shown in Figs. 2A and B, are inhibitors of Rac1 activity.25,26) We used these two agents to investigate if inhibition of Rac1 would result in the suppression of HSV-1 replication. To investigate the antiviral activities, we firstly determined their cytotoxicity in Vero, HCEC, HeLa and HaCaT cells for 24h and found that Ehop016 exhibited a higher cytotoxicity than NSC23766 (Figs. 3A, B) with a 50% cytotoxicity concentration (CC50) at 11.41 μM while NSC23766 at 2019.00 μM in Vero cells. Furthermore, HCEC, HeLa and HaCaT cells seemed to be more sensitive to NSC23766 and Ehop016 at a high concentration. We chose the non-cytotoxic concentrations of 50 to 500 μM for NSC23766 and 0.3125 to 5 μM for Ehop016 to evaluate anti-HSV-1 effect by In-Cell Western assay. The results showed that NSC23766 and Ehop016 both suppressed the HSV-1 replication in a dose-dependent way (Figs. 3C–F) with an EC50 of 381.50 μM and Ehop016 both suppressed the HSV-1 replication in a dose-dependent way. The results showed that NSC23766 and Ehop016 both exhibited a higher cytotoxicity than NSC23766 (Figs. 3A, B) with a 50% cytotoxicity concentration (CC50) at 11.41 μM while NSC23766 at 2019.00 μM in Vero cells. Furthermore, HCEC, HeLa and HaCaT cells at various MOIs (Figs. 4A–D) and NSC23766 suppressed HSV-1 replication. To investigate the antiviral activities, we performed a plaque assay and found that they both markedly reduced the virus titer in a dose-dependent manner. Treatment with NSC23766 at 500 μM produced a 2.2 log reduction in virus titer while treatment with Ehop016 at 2.50 μM produced a 2.6 log reduction in virus titer (Figs. 3H, I).

**NSC23766/Ehop016 Suppresses Viral Protein Expression by Decreasing Rac1 Activity**

HSK or blisters formed on exposed skin and mucosae was the main symptom of HSV-1 infection27) and epithelial cells were the primary target cells exposed skin and mucosae was the main symptom of HSV-1 infection. We asked whether NSC23766 and Ehop016 would suppress HSV-1 infection by plaque assay and found that they both markedly reduced the virus titer in a dose-dependent manner. Treatment with NSC23766 at 500 μM produced a 2.2 log reduction in virus titer while treatment with Ehop016 at 2.50 μM produced a 2.6 log reduction in virus titer (Figs. 3H, I). The results showed that HSV-1 infection of the skin and cornea, in vitro is a human skin keratinocyte, and both are well established models for HSV-1 infection of the skin and cornea, respectively.28,29) Thus, we investigated the anti-HSV-1 effects of NSC23766 and Ehop016 in HCEC and HaCaT cells, respectively. The results showed that HSV-1 could replicate in HCEC and HaCaT cells at various MOIs (Figs. 4A–D) and NSC23766 and Ehop016 both exhibited a dose-dependent inhibition of the production of viral early protein ICP4-1 and late protein gD-1 in these cells (Figs. 4E–L). NSC23766 caused approximately 70% reduction of production of ICP4-1 and 100% reduction of production of gD-1 at 75 μM in infected HCEC (Figs. 4E, F). NSC23766 at the same concentration reduced approximately 90% production of ICP4-1 and 80% production of gD-1 in infected HaCaT cells (Figs. 4G, H). At the concentration of 2.5 μM, Ehop016 caused approximately 50% reduction of production of ICP4-1 and 60% reduction of production of gD-1 in HSV-1-infected HCEC (Figs. 4I, J) and approximately 70% reduction of production of ICP4-1 and 40% reduction of production of gD-1 in HSV-1-infected HaCaT cells (Figs. 4K, L), respectively.

**DISCUSSION**

Rac1 is an established effector of receptors and an important hub in signaling networks critical for tumorigenesis, metastasis and virus infection.30) In the GTP-bound active form, Rac1 interacts with effector molecules to initiate signaling, while in the GDP-bound inactive form the signaling is attenuated. Rac1 was also reported to be involved in numerous human diseases, including cardiovascular disease,31) arthritis,32) kidney disorders,33,34) infectious diseases,35) pathological inflammatory responses,36,37) and neurodegenerative disorders.32,38) Besides, Rac1 particularly played a critical role during many virus infections. Porcine reproductive and respiratory syndrome virus (PRRSV) infection triggered Rac1 activation and Rac1 inhibitors could significantly suppress the virus entry.39) African swine fever virus (ASFV) acti-
vated Rac1 GTPase during internalization in Vero cells and transfecting cells with a dominant-negative plasmid for Rac1 (pcDNA-Rac1 N17) constrained ASFV infection. 39,40) Vaccinia virus entered host by modulating Rac1 activation. 41) Hepatitis B virus (HBV) replication activated Rac1 by mediating HBX protein in HepG2 cells. 42) Rotavirus and enterovirus 71 (EV71) also employed Rac1 activity to support their replication.43,44) In addition, Rac1 also served as a key regulator of actin dynamics and Rac1 participated in the viral life cycle of HSV-1 by modulating actin cytoskeleton remodeling. 19) In previous reports, both Hoppe et al. and Petermann et al. showed that the early stage of HSV-1 infection leded to a temporary increase of Rac1 activity at 15 and 30 min post infection (p.i.), followed by a reduced activity level at 60 min and a rebound in activity level at 120 min. 18,45) However, there was no further evaluation of longer time infection. In the present study, we found that Rac1 activity was still increased at 24 h p.i. (Fig. 1A). In Hoppe’s study, constitutively active L61Rac1 mutants expressing in MDCKII cells with increased Rac1 activity reduced 58% of HSV-1 infectivity. However, wild-type and inactive Rac1 did not affect HSV-1 infection. In addition, Petermann et al. further showed that over-expression of constitutively activate Rac1 mutants in HaCaT cells led to a decrease of HSV-1 infection, while wild-type Rac1 over-expression and knockdown of Rac1 did not affect HSV-1 infection, indicating that HSV-1-induced temporary activation of Rac1 activity may have no functional significance. In our study, increased Rac1 activity positively regulated the viral late protein gD-1 (Fig. 1B). We also observed that both

| Rac1 inhibitor | CC50 | EC50 | SI |
|---------------|------|------|----|
| NSC23766      | 2019.00<sup>a</sup> | 381.50<sup>a</sup> | 5.29<sup>a</sup> |
|               | 205.80<sup>a</sup> | 37.56<sup>a</sup> | 5.48<sup>a</sup> |
|               | 206.50<sup>b</sup> | 25.32<sup>b</sup> | 8.16<sup>b</sup> |
|               | 303.50<sup>c</sup> | 59.31<sup>c</sup> | 5.12<sup>c</sup> |
| Ehop016       | 11.41<sup>c</sup> | 2.29<sup>c</sup> | 4.98<sup>c</sup> |
|               | 13.85<sup>c</sup> | 2.23<sup>c</sup> | 6.21<sup>c</sup> |
|               | 5.92<sup>c</sup> | 0.87<sup>c</sup> | 6.8<sup>c</sup> |
|               | 12.10<sup>c</sup> | 0.84<sup>c</sup> | 14.4<sup>c</sup> |

The CC50 and EC50 were acquired via GraphPad Prism 5 analysis. SI = CC50/EC50. 
<sup>a</sup>Vero; <sup>b</sup>HaCaT; <sup>c</sup>HCEC; <sup>d</sup>HeLa.

in addition, Rac1 also served as a key regulator of actin dynamics and Rac1 participated in the viral life cycle of HSV-1 by modulating actin cytoskeleton remodeling. 39,40) In addition, Rac1 activity positively regulated the viral late protein gD-1 (Fig. 1B). We also observed that both

Fig. 3. NSC23766/Ehop016 Inhibited HSV-1 Infection in a Dose-Dependent Manner

(A, B) Vero, HCEC, HeLa and HaCaT cells were treated with NSC23766 and Ehop016 for 24 h, respectively. CCK-8 kit was used to evaluate the cell viability. The effect of NSC23766 (C) and Ehop016 (E) on HSV-1 gD-1 expression was measured by In-Cell Western assay. Firstly, Vero cells were inoculated with serially diluted NSC23766 and Ehop016 for 30 min in 96-well plate, respectively. Then the compound-treated-Vero cells were infected with HSV-1 (MOI = 3) for 24 h. HSV-1 gD-1 protein was detected through In-Cell Western assay. (D, F) Quantification analysis of anti-HSV-1 effects of NSC23766 (D) and Ehop016 (F). Values are shown as percentages of the untreated cells. (G) Western blotting analysis of Ehop016 on HSV-1 gD-1 protein expression. Top panel: HSV-1 gD-1. Bottom panel: GAPDH. (H, I) Effect of NSC23766 and Ehop016 on HSV-1 titer. The blot shown is an exemplary representative from three independent experiments. Data presented are means of the results from three independent experiments. Error bars, S.D. Statistical significance: * p < 0.05, ** p < 0.01, *** p < 0.001. (Color figure can be accessed in the online version.)

Table 1. Cell Viability (CC<sub>50</sub>), Anti-HSV-1 Activity (EC<sub>50</sub>) and the Selectivity Index (SI) of NSC23766 and Ehop016, Respectively
Fig. 4 NSC23766/Ehop016 Existed Anti-HSV-1 Activities in Epithelial Cells

(A, C) HCEC and HaCaT cells were infected with HSV-1 at various MOIs for 24h. Western blotting analysis of the viral early protein ICP4-1 and viral late protein gD-1 and GAPDH from uninfected and HSV-1-infected cells were shown. (B, D) The quantification analysis of ICP4-1 and gD-1 relative to GAPDH comparing to uninfected cells were shown. NSC23766 showed anti-HSV-1 activities in HSV-1-infected HCEC (E, F) and HaCaT cells (G, H). Ehop016 inhibited HSV-1 infection in HCEC (I, J) and HaCaT cells (K, L). HCEC/HaCaT cells in 6-well plate were inoculated with HSV-1 (MOI = 3) with serially diluted NSC23766 and Ehop016, following 24h, the supernatant was removed and the total proteins were collected, and the viral early protein ICP4-1 and late protein gD-1 expression were detected by Western blotting analysis. Top panel: HSV-1 ICP4-1; middle panel: HSV-1 gD-1; bottom panel: GAPDH. The blot shown is an exemplary representative from three independent experiments. Data represented as means from three independent experiments. Error bars, S.D. #/##/###: compared with mock group, */**/***: compared with HSV-1 group. Statistical significance: #/## p < 0.05, ###/#### p < 0.001.
NSC23766 and Ehop016 suppressed the infection of HSV-1 in a dose-dependent way in vitro (Figs. 3–5). In addition, Rac1 inhibitors reduced viral protein ICP4-1 and gD-1 expression by decreasing the level of Rac1-GTP/total Rac1 (Fig. 5). Therefore, we provided evidence that Rac1 activity was positively correlated with HSV-1 replication and HSV-1 replication relied on high Rac1 activity. The discrepancy between ours and Hoppe et al. and Petermann et al. may be due to the differing observation time points. Hoppe et al. and Petermann et al. suggested that the initial infection of HSV-1 was not
limited by a reduction in Rac1. However, because Rac1 can promote microtubules growth and retrograde flow, the reduction of Rac1 activity may inhibit the transport and delivery of virions and this may explain why the replication of HSV-1 was inhibited when Rac1 activity decreased. We thus inferred that Rac1 activity was correlated with HSV-1-infected diseases and Rac1 is an effective therapeutic target for HSV-1/HSK and NSC23766/Ehp016-like agents would be potential candidates to treat HSK.

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Conflict of Interest The authors declare no conflict of interest.

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