Tranylcypromine Reduces Herpes Simplex Virus 1 Infection in Mice

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Herpes simplex virus 1 (HSV-1) infects the majority of the human population and establishes latency by maintaining viral genomes in neurons of sensory ganglia. Latent virus can undergo reactivation to cause recurrent infection. Both primary and recurrent infections can cause devastating diseases, including encephalitis and corneal blindness. Acyclovir is used to treat patients, but virus resistance to acyclovir is frequently reported. Recent in vitro findings reveal that pretreatment of cells with tranylcypromine (TCP), a drug widely used in the clinic to treat neurological disorders, restrains HSV-1 gene transcription by inhibiting the histone-modifying enzyme lysine-specific demethylase 1. The present study was designed to examine the anti-HSV-1 efficacy of TCP in vivo because of the paucity of reports on this issue. Using the murine model, we found that TCP decreased the severity of wild-type-virus-induced encephalitis and corneal blindness, infection with the acyclovir-resistant (thymidine kinase-negative) HSV-1 mutant, and tissue viral loads. Additionally, TCP blocked in vivo viral reactivation in trigeminal ganglia. These results support the therapeutic potential of TCP for controlling HSV-1 infection.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney (Vero) cells, mouse neuronal (N18) cells, human lung epithelial (A549) cells, and human rhabdomyosarcoma (RD) cells were maintained and propagated according to the instructions of the American Type Culture Collection. The wild-type HSV-1 strains 294.1 (34–36), RE (37), McKrae, and KOS were used for experiments. In addition, rLTRZ1 is a recombinant virus derived from KOS that lacks thymidine kinase activity due to an insertion within the gene (38). HSV-1 strains were propagated and titrated by plaque assay on Vero cell monolayers. Enterovirus 71 strain M2 was propagated and titrated on RD cell monolayers (39). The clinical isolate of type 3 adenovirus (kindly provided by Jen-Ren Wang in our college) was propagated and titrated on A549 cell monolayers. In vitro antiviral assay. N18 or A549 cell monolayers were infected with HSV-1 or enterovirus 71 at a multiplicity of infection (MOI) of 0.001 or adenovirus at one 50% tissue culture infectious dose for 1 h, treated with

Received 3 December 2013 Returned for modification 24 December 2013 Accepted 26 February 2014

Published ahead of print 3 March 2014

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Received: 26 February 2014

Published ahead of print: 3 March 2014

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with TCP (Sigma-Aldrich) or saline, and harvested 48 h postinfection (p.i.) unless otherwise indicated to determine viral titers by plaque assays.

**Cell proliferation assay.** N18 and A549 cell monolayers were incubated with saline or TCP for 48 h. Cell viability was assessed using cell counting kit 8 (Dojindo Molecular Technologies) according to the manufacturer’s instructions.

**Infection and treatment of mice with TCP.** All mouse experimental protocols were approved by the Laboratory Animal Committee of National Cheng Kung University. Six-week-old mice were anesthetized and infected with HSV-1 or mock infected with lysates of uninfected Vero cells topically on the right eye following scarification of the cornea with a needle 20 times. Male ICR mice were infected with $1 \times 10^6$ PFU/eye of tKLTRZ1 and treated with saline or TCP on the cornea (75 µg/eye) or both on the cornea and by intraperitoneal injection (10 mg/kg). Mice were given topical treatment 15 min before infection or 1 day p.i. to 3 days p.i. and systemic treatment 1 day before infection or 1 day p.i. to 3 days p.i. once daily. Male ICR mice were infected with $1 \times 10^7$ PFU/eye of 294.1, treated with TCP on the cornea immediately before infection and by intraperitoneal injection 1 day before infection to day 10 p.i., and monitored for body weight and survival. Female C57BL/6N mice infected with $5 \times 10^5$ PFU/eye of RE, treated with TCP on the cornea immediately before infection and by intraperitoneal injection 1 day before infection to day 9 p.i., and monitored for corneal opacity and angiogenesis as described in our previous reports (37, 40). The corneal opacity was graded on a scale of 0 to 5 as follows: 0, normal cornea; 1, mild corneal haze; 2, moderate corneal opacity or scarring; 3, severe corneal opacity, iris visible; 4, opaque cornea, iris invisible; and 5, necrotizing cornea with vascularization.

**RESULTS**

TCP treatment reduces the titers of wild-type and thymidine kinase-negative (TK−) HSV-1 strains in the human or mouse cell line. Pretreatment of human cell lines at least 4 h before infection was used to evaluate the anti-HSV efficacy of TCP in previous reports (24–26). Here, we assessed TCP treatment after infection using a mouse neuronal cell line (N18) and a human epithelial cell line (A549). N18 cells were infected with wild-type HSV-1 strain 294.1 (MOI = 0.001) for 1 h, treated with saline or different concentrations of TCP, and harvested 48 h.p.i. to determine viral titers. TCP at concentrations of 0.5 and 1 mM reduced HSV-1 titers in a dose-dependent manner (Fig. 1A), with a significant difference being found at the concentration of 1 mM compared with saline (P < 0.01, Mann-Whitney U test). In addition, TCP (1 mM) significantly decreased viral titers at all time points (24 to 72 h.p.i.), which were examined (Fig. 1B) (P < 0.05, Mann-Whitney U test). We next evaluated the antiviral efficacy of TCP on the virus whose replication cycle is not dependent on DNA. N18 cells infected with a neurotropic RNA virus, enterovirus 71 (MOI = 0.001), for 1 h were treated with TCP. TCP at concentrations of 0.5 and 1 mM failed to decrease enterovirus 71 titers at 48 h.p.i. (Fig. 1C).

In A549 cells, TCP at concentrations of 0.5 and 1 mM significantly reduced the titers of both HSV-1 294.1 and a clinical isolate of type 3 adenovirus in a dose-dependent manner at 48 h.p.i. compared with saline (Fig. 1D) (P < 0.01, Mann-Whitney U test). In addition, TCP (1 mM) significantly reduced the titers of two other wild-type HSV-1 strains (KOS and RE) and tKLTRZ1, which is the KOS-derived, TK− mutant resistant to acyclovir (38), compared with saline at 48 h.p.i. (Fig. 1E) (P < 0.01, Mann-Whitney U test). Collectively, the results for HSV-1, enterovirus 71, and adenovirus are consistent with the principle that TCP, which inhibits the histone-modifying enzyme LSD1, suppresses only the replication of DNA viruses. Our HSV-1 results show that TCP possesses...
antiviral activity against all the wild-type and TK− HSV-1 strains in all of the cell lines that were tested.

We examined the effect of TCP on cell proliferation by incubating uninfected cells in medium containing TCP for 48 h. TCP at concentrations of 0.5 and 1 mM failed to significantly affect the growth of N18 and A549 cells compared with saline as determined by cell proliferation assay (Fig. 1F and G) (**, P < 0.01, Mann-Whitney U test). TCP treatment (1 mM) reduced the proliferation of A549 and N18 cells by 13% and 2%, respectively, suggesting that A549 cells may be more sensitive to TCP than N18 cells.

**TCP treatment reduces the titers of TK− HSV-1 mutant in mice.** We then determined the anti-HSV-1 efficacy of TCP in vivo. ICR mice were infected with tkLTRZ1 on scarified corneas, as inoculation of HSV-1 via the abraded eye mimics human infection in some individuals. Mice were given TCP once per day topically on the cornea (75 μg/eye) immediately before infection and systemically by intraperitoneal injection (10 mg/kg) 1 day before infection to day 3 p.i. Mice in the control group received saline. Like most authentic thymidine kinase mutants, tkLTRZ1 replication is restricted to the initial inoculation site, as the virus is incompetent in replication in neurons and in neuronal spread to other tissues (42). Mouse eyes were harvested to determine viral titers. TCP pretreatment reduced viral titers in mouse eyes by 0.7 and 1.5 log units at days 1 and 3 p.i., respectively, with a significant difference found at day 3 p.i. compared with saline treatment (Fig. 2A) (**, P < 0.01, Mann-Whitney U test). We also tested the effect of TCP pretreatment only on the cornea, which reduced the viral titer in mouse eyes by 0.9 log at day 3 p.i. (Fig. 2A).

Previous *in vitro* studies showed that TCP pretreatment reduced the expression of HSV-1 immediate early genes, such as

![FIG 1](https://example.com/fig1.png)
**FIG 2** Effects of TCP treatment on the viral load and expression of viral and cellular genes in the mouse eye. ICR mice corneally infected with HSV-1 tkLTRZ1 were treated with saline or TCP on the cornea before infection or both on the cornea before infection and by intraperitoneal (i.p.) injection 1 day before infection to day 3 postinfection. (A) Viral titers in the eye at the indicated days postinfection. Levels of viral (ICP9 and ICP27) RNA (B) and cellular (Sp1 and TBP) RNA (C) in the eyes of mice treated with saline or TCP on the cornea plus by intraperitoneal injection were determined at day 3 postinfection. The RNA levels of saline-treated mice were set at 100%. Data are means and SE (error bars) for ≥4 samples per data point or group. *, P < 0.05; ***, P < 0.001 (Mann-Whitney U test).

ICP0 and ICP27, but failed to affect the expression of cellular genes, such as Sp1 and TBP (24, 26). We examined the influence of TCP pretreatment on the expression of viral and cellular genes in vivo. The eyes of infected mice treated with saline or TCP on the cornea and by intraperitoneal injection were harvested at day 3 p.i., and the levels of ICP0, ICP27, Sp1, and TBP transcripts and viral genomes were determined. By comparison with saline treatment, TCP treatment reduced the levels of ICP0 and ICP27 RNA per viral genome by 95% and 71%, respectively, with a significant difference found in the ICP0 level (Fig. 2B) (P < 0.05, Mann-Whitney U test). Additionally, TCP slightly decreased the expression of Sp1 and TBP by 23% and 14% (Fig. 2C) (P > 0.05). Lastly, we tested mice treated with TCP after infection. Mice infected with tkLTRZ1 were given TCP on the cornea and by intraperitoneal injection from days 1 to 3 p.i. TCP significantly reduced viral titers in mouse eyes by 0.8 and 1 log at days 2 and 3 p.i., respectively (Fig. 3) (P < 0.05, Mann-Whitney U test).

TCP treatment reduces HSV-1-induced lethality and tissue viral loads in mice. We tested the effect of TCP treatment in protecting mice from HSV-1-induced death. ICR mice were corneally infected with wild-type HSV-1 strain 294.1, which can induce encephalitis in mice (36). Infected mice were treated with saline on the cornea immediately before infection and by intraperitoneal injection from 1 day before infection to day 10 p.i. About 80% of saline-treated mice and 40% of TCP-treated mice showed severe signs of encephalitis, including hunched posture, lethargy, and ataxia. Compared with saline treatment, TCP treatment did not significantly affect the body weight of infected mice in the first 8 days but increased the body weight of infected mice 9 to 13 days p.i., when saline-treated mice succumbed to death (Fig. 4A). Of note, the final survival rate of TCP-treated mice (63%) was significantly higher than that of saline-treated mice (18%) by day 30 p.i. (Fig. 4B) (P < 0.05, log-rank test). Mouse eyes, trigeminal ganglia, and brains were harvested 1 to 9 days p.i. to measure viral titers, as HSV-1 replication was detected in these three tissues after corneal inoculation. The mean viral titers in these three tissues from TCP-treated mice were lower than those from saline-treated mice almost at all time points examined, with significant differences found at 1 or 2 time points in each tissue (Fig. 4C to E) (P < 0.05, Mann-Whitney U test). We examined the pathological changes in mouse brains harvested at day 8 p.i. Hematoxylin-and-eosin staining of the brain stem showed less inflammatory infiltrate and damage in infected mice treated with TCP than in infected mice treated with saline (Fig. 4F). Immunofluorescence staining of the brain stem showed less HSV-1 antigens and more neurons, as demonstrated by cells expressing NeuN (a neuron-specific marker), in infected mice treated with TCP than in infected mice treated with saline (Fig. 4G).

TCP treatment decreases the severity of HSV-1-induced corneal disease, eye viral load, and corneal angiogenesis and inflammatory infiltrate in mice. We tested the effect of TCP treatment on reducing the severity of HSV-1-induced stromal keratitis. C57BL/6N mice were corneally infected with wild-type HSV-1 strain RE, which can induce stromal keratitis to result in corneal blindness in mice (37, 40). Infected mice were treated with saline or TCP on the cornea immediately before infection and by intraperitoneal injection from 1 day before infection to day 9 p.i. All mice survived after infection. Corneas of infected mice were monitored for opacity and angiogenesis, two important features of herpetic stromal keratitis. Infected corneas of saline-treated mice developed symptoms progressively, with moderate opacity and visible irises (an opacity score of 2) by day 7 p.i., and became opaque and necrotizing with invisible irises (opacity scores of 4 to 5) from days 14 to 28 p.i. (Fig. 5A). TCP treatment significantly reduced the corneal opacity of infected mice, with mean scores of 2 from days 14 to 28 p.i. (P < 0.01, Wilcoxon signed-rank test). Abundant and extended neovessels were detected in the infected corneas of saline-treated mice, with a mean angiogenesis score significantly higher than that in the infected corneas of TCP-
FIG 4 TCP treatment reduces HSV-1-induced lethality and tissue viral loads of mice. The relative body weights (A) and survival rates (B) of ICR mice infected with HSV-1 294.1 and treated with saline (n = 11) or TCP (n = 11) were monitored at the indicated days postinfection. Viral titers in the eyes (C), trigeminal ganglia (D), and brains (E) of mice treated with saline or TCP were determined at the indicated days postinfection. Data are means ± SE (error bars) for 3 samples per data point. *, P < 0.05, and **, P < 0.01 (Mann-Whitney U test), compared with the saline-treated group (A) or with TCP-treated groups (C to E). *, P < 0.05 (log-rank test) (B). Brain stems of mock-infected or infected mice treated with saline or TCP were harvested 8 days postinfection and stained with hematoxylin and eosin (F) or antibodies against HSV-1 or NeuN (G). Original magnification, ×200 (G). Data are representative of at least 6 samples per group from two independent experiments.
TCP treatment reduces the severity of HSV-1-induced corneal disease, eye viral load, and corneal angiogenesis and inflammatory infiltrate in mice. C57BL/6N mice infected with HSV-1 RE were treated with saline (n = 19) or TCP (n = 17). (A and B) The corneal opacity scores of infected mice at the indicated days postinfection (A) and the corneal angiogenesis scores of infected mice at 21 days postinfection (B) are shown. *, P < 0.05, and **, P < 0.01 (Wilcoxon signed-rank test), compared with the TCP-treated group (A) or between the indicated groups (B). (C) Eyes of mock-infected and infected mice treated with saline or TCP were harvested 28 days postinfection and stained with hematoxylin and eosin. The corneal portion of representative samples is shown. (D) The corneas of infected mice treated with saline or TCP were harvested 28 days postinfection to quantify cells expressing CD31 by flow cytometry. Data are representative plots (left) and means plus SE (error bars) for 7 samples per data point. ***, P < 0.001 (Mann-Whitney U test), compared with the TCP-treated group.

We further assessed the opacity and neovascularization in corneas harvested at day 28 p.i. Results of hematoxylin and eosin staining showed that the infected corneas of saline-treated mice were thickened, with profound edema, inflammatory infiltrate, and vascularization with erythrocyte-filled vessels, especially in the stroma, compared with the infected corneas of TCP-treated mice (Fig. 5C). To further compare corneal angiogenesis, we quantified the number of cells positive for CD31, a marker specifically expressed on endothelial cells constituting the newly formed blood vessels (43). Flow-cytometric analysis showed that TCP treatment significantly reduced the mean number of CD31+ cells in infected corneas 6-fold at day 28 p.i. (Fig. 5D) (P < 0.05, Student’s t test). The results of opacity scores, angiogenesis scores, hematoxylin-and-eosin staining, and flow-cytometric analysis were consistent and collectively showed that TCP treatment reduced HSV-1-induced corneal disease and corneal angiogenesis and inflammatory infiltrate. Next, we determined the effect of TCP on viral replication in the eye and found that TCP treatment significantly reduced the eye viral titer by 1.4 log units at day 1 p.i. (Fig. 5E) (P < 0.001, Mann-Whitney U test). Previous reports showed that the level of acute HSV-1 replication positively affects the severity of stromal keratitis by initiating leukocyte influx and neovascularization in the mouse eye (37, 44).

TCP treatment reduces the in vivo reactivation of HSV-1 in mice. Previous studies used the ex vivo (explant) approach to test TCP treatment on HSV-1 reactivation (24–26). In the present study, we examined the effect of TCP treatment on blocking HSV-1 reactivation in vivo. Female C57BL/6N mice were inoculated with wild-type HSV-1 strain McKrae on the cornea. The virus established latency in mice at day 27 p.i., as demonstrated by the failure to detect infectious virus in any of 6 trigeminal ganglia harvested from infected mice by plaque assay. Latently infected mice were given saline or TCP at days 27 and 28 p.i. by intraperitoneal injection. Ten hours after the last saline or TCP treatment, mice were placed in a 43°C water bath for 10 min, because transient hyperthermia has been used to induce HSV reactivation in mouse trigeminal ganglia in vivo (45, 46). Mice were given saline or TCP 6 h after hyperthermic stimulation, and mouse trigeminal ganglia were harvested 19 h after hyperthermic stimulation to detect infectious virus. Figure 6 shows that trigeminal ganglia harvested from saline-treated mice yielded reactivated (infectious) virus with a frequency of 53% (10/19), and trigeminal ganglia harvested from TCP-treated mice yielded infectious virus with a frequency of 9% (1/11). TCP treatment significantly reduced HSV-1 reactivation in mouse trigeminal ganglia (P < 0.05, Fisher’s exact test). We also tested treatment of latently infected mice with TCP before or after hyperthermic stimulation. Trigeminal ganglia harvested from mice treated with TCP only before or after hyperthermic stimulation yielded infectious virus with frequencies of 33% (2/6) and 57% (4/7), respectively.

**DISCUSSION**

In this study, we provide evidence that TCP treatment of mice significantly reduces the severity of encephalitis and corneal blindness induced by wild-type viruses, viral reactivation from trigeminal ganglia, and infection by the TK− HSV-1 mutant. Furthermore, our in vitro results show that TCP treatment given after infection significantly decreases the titers of TK− mutant and sev-
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May 2014 Volume 58 Number 5 aac.asm.org

ever, their extensive use has led to the emergence of resistant mutant viruses. For acyclovir, the most effective anti-HSV-1 therapy, drug resistance is estimated to occur in ~6% of treated patients (20, 52). There is a clear need to develop novel antivirals. Agents targeting cellular factors to reduce viral infection may be another option to prevent drug resistance. In the future, studies selecting HSV-1 mutants resistant to TCP are needed to address whether virus can mutate to circumvent cellular inhibition and whether TCP would unlikely select for resistance mutations in host cell LSD1. TCP with the capacity to reduce LSD1 activity was originally designed to inhibit monoamine oxidases A and B for the treatment of severe psychiatric disorders, such as depression and anxiety (27–30, 33). The EC_{50} of TCP obtained in the present study (0.5 to 0.6 mM) is comparable to that obtained in a previous study (26) but is higher than that of acyclovir (3 to 9 μM) (33).

This has promoted a recent study to design chemicals with a high specificity for LSD1 (26), and one compound, with promising anti-HSV-1 activity and low EC_{50}s (3 to 10 μM), OG-L002, was found. However, until drugs such as OG-L002 are approved for human use, clinicians may consider the use of TCP or combinations thereof for treatment of patients with drug-resistant HSV-1.

ACKNOWLEDGMENTS

We thank Robert Anderson for critical reading of the manuscript. This work was supported by a grant from the National Science Council in Taiwan (102-2320-B-006-028-MY3).

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