**In Vitro Synergism of Trifluorothymidine and Ganciclovir against HSV-1**

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**PURPOSE.** To determine whether trifluorothymidine (TFT) and ganciclovir (GCV) are synergistic against herpes simplex virus type 1 (HSV-1).

**METHODS.** TFT and GCV activity against 12 strains of HSV-1 (including an acyclovir-resistant strain) was measured by plaque-forming unit (PFU) inhibition. Cellular toxicity was assessed with an MTT dye reduction assay. Synergism was determined by calculating fractional inhibitory concentration (FIC) indices based on PFU reduction.

**RESULTS.** Concentrations of TFT resulting in 50% inhibition of PFUs (IC50) of acyclovir-susceptible HSV-1 strains ranged from 3.07 ± 0.36 to 12.52 ± 0.61 μM. GCV IC50 values ranged from 0.40 ± 0.02 to 1.59 ± 0.14 μM. IC50 values of TFT and GCV against the acyclovir-resistant strain were 15.40 ± 3.17 and 93.00 ± 9.64 μM, respectively. Concentrations of TFT or GCV resulting in 50% cell cytotoxicity (CC50) were 0.99 ± 0.01 and 92.91 ± 8.92 μM, respectively. TFT and GCV combined (10:1) were 10 times more potent against all acyclovir-susceptible HSV-1 strains. For 8 of 12 HSV-1 strains, the IC50 of TFT and GCV combined was lower than the CC50 of either drug. For acyclovir-susceptible HSV-1 strains, TFT and GCV combined generated a FIC index of <0.5, suggesting strong synergism between the two drugs. The FIC value for TFT and GCV combined against the acyclovir-resistant HSV-1 strain was 0.84, indicating nonantagonism.

**CONCLUSIONS.** TFT and GCV are synergistic against acyclovir-susceptible HSV-1 at concentrations significantly less toxic than if each antiviral was used as a sole agent. (Invest Ophthalmol Vis Sci. 2011;52:830–833) DOI:10.1167/iovs.10-5671

Herpes simplex virus type 1 (HSV-1) is a common cause of acute and recurrent opthalmic disease worldwide, and is the leading cause of infectious blindness in the United States. HSV-1 infections of the cornea typically result in sight-threatening epithelial (keratitis) or stromal disease. Several antiviral drugs have shown efficacy in the treatment of experimental HSV-1 keratitis, including acyclovir, valacyclovir, cidofovir, trifluorothymidine (TFT), and ganciclovir (GCV).

Until recently, the only antiviral agent approved in the United States to treat acute HSV-1 keratitis was a 1.0% solution of TFT (Viroptic; King Pharmaceuticals, Bristol, TN). Topical acyclovir has not been approved in the United States for ophthalmic use. In September 2009, GCV (0.15% gel, Zirgan; Sirion Therapeutics, Tampa, FL) was approved by the US Food and Drug Administration as a second agent to treat HSV-1 corneal disease. This formulation of GCV has been used as an antitherapeutic ophthalmic medication in Europe for more than a decade.

Irreversibly inhibiting cellular thymidylate synthase after being phosphorylated by cellular and viral thymidine kinases (TK), TFT is clinically effective in treating acyclovir-resistant HSV-1 corneal disease. An acyclovir-resistant phenotype arises because of a mutation in viral TK or more rarely in the DNA polymerase of HSV-1. In contrast with TFT, GCV (which is phosphorylated only with viral TK) is ineffective against acyclovir-resistant HSV-1. Typical TFT can produce adverse effects such as a burning sensation on application, corneal edema, and increased intraocular pressure. GCV is reported to cause less discomfort (stinging, burning) or blurred vision.

TFT and GCV are clinically proven antitherapeutic agents formulated for ophthalmic use. Combining these two drugs might allow the use of less toxic TFT concentrations without sacrificing antiviral activity. In this study, a 10:1 combination of TFT and GCV had significant antiviral activity against acyclovir-susceptible HSV-1 strains at concentrations less toxic to cells than if each agent had been used alone. Combination therapy against an acyclovir-resistant HSV-1 strain was also successful in that significant antiviral activity was achieved with half the concentration required if each antiviral was used individually.

**MATERIALS AND METHODS**

**Viruses, Viral Culture, and Antivirals**

Three laboratory strains (McKrae, KOS, and TKG7+2G, an acyclovir-resistant TK mutant of KOS) and nine clinical isolates (VT7581, VT242, VT4688, VT5227, VT00694, VT7644, VT7632, VT53, and VT1736) were included in this study. Both KOS (acyclovir-susceptible) and its

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Synergism of TFT and GCV against HSV-1

TK mutant (acyclovir-resistant) were obtained from Donald M. Coen (Harvard Medical School, Boston, MA).

All clinical isolates were obtained from Gary H. Cohen (University of Pennsylvania School of Medicine, Philadelphia, PA). The clinical isolates were low passage and of oral origin. The reservoir of corneal infection for HSV-1 is most likely the mouth. Therefore, any viruses isolated from corneal lesions were originally inhabitants of the oral mucosa. HSV-1 can reach the cornea directly in droplets of oral secretions or by a “backdoor” mechanism via the trigeminal ganglion.

All HSV-1 strains were maintained on Vero cell (ATCC CCL81; American Type Culture Collection, Manassas, VA) monolayers grown to near confluence in 24-well plates at 37°C with 5% CO₂ in Earle’s minimal essential medium (EMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen). All strains were passaged once before being stored at −20°C.

TFT and GCV were purchased from Sigma-Aldrich Corp. (St. Louis, MO), dissolved in 10% dimethyl sulfoxide (Sigma), and stored at −20°C.

Toxicity of TFT and GCV

Vero cells were cultured as described above. Dilutions (1:1) of TFT (800 μM) or GCV (640 μM) in EMEM were added to wells, and after 5 days of incubation, 20 μL of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to each well. Plates were allowed to develop for 2 hours at room temperature in the dark. Plates were washed once with D-PBS (Gibco). The cells were solubilized in 200 μL of DMSO, and the absorbance measured at 560 nm on an ELISA plate reader (Synergy 2; Biotek Instruments, Winooski, VT). Concentrations of TFT or GCV resulting in 50% cell cytotoxicity (CC₅₀) were calculated using special software (CalcuSyn v.2.0; BioSoft). All assessments of toxicity were done in triplicate, and the CC₅₀ values are reported as the mean ± SE.

Table 1. Inhibitory Concentrations of TFT and GCV, as Single Agents or a Combination, Exhibiting a 50% Antiviral Effect (IC₅₀) with FIC Indices

<table>
<thead>
<tr>
<th>HSV-1 Strain</th>
<th>IC₅₀ TFT (μM)</th>
<th>IC₅₀ GCV (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single</td>
<td>Combo*</td>
</tr>
<tr>
<td>McKrae</td>
<td>12.52 ± 0.61</td>
<td>0.80 ± 0.04</td>
</tr>
<tr>
<td>KOS</td>
<td>7.10 ± 0.28</td>
<td>1.09 ± 0.06</td>
</tr>
<tr>
<td>VT7581</td>
<td>5.32 ± 0.45</td>
<td>0.94 ± 0.02</td>
</tr>
<tr>
<td>VT242</td>
<td>9.70 ± 0.83</td>
<td>1.05 ± 0.03</td>
</tr>
<tr>
<td>VT4688</td>
<td>3.07 ± 0.36</td>
<td>0.52 ± 0.03</td>
</tr>
<tr>
<td>VT5227†</td>
<td>9.64 ± 0.78</td>
<td>1.60 ± 0.04</td>
</tr>
<tr>
<td>VT00694</td>
<td>7.35 ± 0.48</td>
<td>0.89 ± 0.02</td>
</tr>
<tr>
<td>VT7644</td>
<td>8.65 ± 0.41</td>
<td>0.68 ± 0.01</td>
</tr>
<tr>
<td>VT7632</td>
<td>7.19 ± 0.45</td>
<td>0.58 ± 0.01</td>
</tr>
<tr>
<td>VT53</td>
<td>4.59 ± 0.39</td>
<td>0.61 ± 0.03</td>
</tr>
<tr>
<td>VT1736</td>
<td>6.91 ± 0.16</td>
<td>0.75 ± 0.01</td>
</tr>
<tr>
<td>TKG7+2G†</td>
<td>15.40 ± 3.17</td>
<td>4.20 ± 0.16</td>
</tr>
</tbody>
</table>

Synergism defined as a FIC index of ≤0.5, indifferent or nonantagonistic effect as a FIC index of >0.5 but ≤4 and antagonism as a FIC index of >4. Experiments were performed in triplicate. Values represent the mean value ± SEM.

† TFT (50 μM) combined with GCV (5 μM) except for TKG7+2G where GCV = 640 μM.
(92.91 ± 8.92 μM). However, when combined with TFT, the concentration of GCV required to reduce TKG+2G PFU by 50% was practically halved (53.77 ± 7.03 μM).

TFT was 100-fold more toxic to Vero cells on a molar basis when compared with GCV. The concentration of TFT that resulted in 50% Vero cell cytotoxicity (CC₅₀) was 0.99 ± 0.01 μM. In contrast with GCV where the CC₅₀ was less than the IC₅₀ for all HSV-1 strains except the acyclovir-resistant TK-deficient strain, the IC₅₀ values for TFT alone against all 12 strains of HSV-1 tested were more than threefold higher than the CC₅₀ of TFT (Table 1).

Fractional inhibitory concentration indices (Table 1) indicate that 50 μM TFT plus 5 μM GCV acted synergistically (FIC ≤ 0.5) in reducing PFU of all HSV-1 strains except for TKG7+2G. The combination of antivirals had a nonantagonistic effect (FIC = 0.84) on this virus.

**DISCUSSION**

In this study, TFT and GCV were evaluated for a synergistic antiviral effect in vitro against nine clinical and three laboratory strains of HSV-1. Topical TFT (1.0%) is the standard for treating HSV-1 keratitis in the United States. In patients with HSV-1 dendritic ulcers, a 1.0% TFT solution effectively treated 90% of cases, with activity comparable to antivirals such as vidarabine, bromovinyldeoxyuridine, and idoxuridine. Recently GCV has been approved in the United States as a topical medication for acute HSV-1 corneal disease. GCV is a nucleoside analog as effective as acyclovir but with less discomfort and ocular toxicity associated with the high concentrations of TFT required to reduce PFU counts by 50% was reduced by >70% when the drug was combined with GCV.

The fates of TFT and GCV once they enter a cell are shown in Figure 1. GCV is phosphorylated to GCV phosphate (GCV-P) by HSV-1 TK. The cellular enzymes thymidylate kinase and nucleoside diphosphokinase further phosphorylate GCV-P into ganciclovir triphosphate (GCV-TP). GCV-TP competitively inhibits incorporation of deoxyguanosine triphosphate (dGTP) into nascent viral DNA by HSV-1 DNA polymerase. Incorporation of GCV-TP into HSV-1 DNA ultimately terminates its elongation.

A combination of TFT and GCV could be synergistic in much the same way that antibacterial agents trimethoprim and sulfamethoxazole act synergistically against bacteria.

**Figure 1.** The fate of trifluorothymidine (TFT) and ganciclovir (GCV) in a cell. Antiviral agents and HSV-1 enzymes are in red, cellular metabolites and enzymes are in black. Synergism could arise from each antiviral agent targeting a different element in the synthesis of HSV-1 DNA. GCV is phosphorylated to GCV triphosphate (GCV-TP), which competes with cellular deoxyguanosine triphosphate (dGTP) as a substrate for HSV-1 DNA polymerase. Aberrant DNA synthesis occurs when GCV-TP is incorporated into nascent viral DNA by the viral polymerase. TFT is phosphorylated into TFT phosphate which then irreversibly inhibits thymidylate synthase, a key enzyme in supplying the cell with TTP for DNA synthesis and repair.
sulfamethoxazole are synergistic. Trimethoprim and sulfamethoxazole inhibit folic acid metabolism in bacteria at different points in the folate pathway. TFT and GCV inhibit HSV-1 DNA production in cells by targeting different elements involved in viral nucleic acid synthesis. There was no synergism (FIC = 0.84) of these two antivirals against strain TKG7 +2G, likely because of the reduced effectiveness of GCV. Acyclovir-resistant HSV-1 strains such as TKG7 +2G can complicate antiviral therapy.12,27 This resistance is associated with a mutation in the TK gene.11,28 Encountering an acyclovir-resistant corneal HSV-1 isolate is a relatively infrequent event in immunocompetent individuals, but such strains are more common in patients with AIDS.29 Although synergism was not demonstrated against TKG7 +2G, combining the two antivirals did reduce the IC50 values approximately 50% compared with the IC50 values obtained with each antiviral agent individually.

In conclusion, GCV and TFT are synergistic against acyclovir-susceptible HSV-1 strains in vitro. Although these in vitro results are very encouraging, these studies must be replicated in vivo using an animal model of HSV-1 before combination therapy can be adopted as an accepted clinical practice. Both of these antivirals are clinically effective as single agents in treating keratitis caused by acyclovir-susceptible HSV-1. However, combination therapy has the potential to overcome TFT-induced ocular toxicity as well as to effectively treat keratitis caused by acyclovir-resistant strains of HSV-1.

References