In Vitro Synergism of Trifluorothymidine and Ganciclovir against HSV-1

Jeffery A. Hobden,[1,2] *Manish Kumar,*[2,3,4] *Herbert E. Kaufman,*[3,5,6] *Christian Clement,*[3] *Emily D. Varnell,*[3] *Partha S. Bhattacharjee,*[7] *and James M. Hill*[1,3,5,6]

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 (IC_{50}) of acyclovir-susceptible HSV-1 strains ranged from 3.07 ± 0.36 to 12.52 ± 0.61 μM. GCV IC_{50} values ranged from 0.40 ± 0.02 to 1.59 ± 0.14 μM. IC_{50} 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 (CC_{50}) 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 IC_{50} of TFT and GCV combined was lower than the CC_{50} 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 were used as a sole agent. (*Invest Ophthalmol Vis Sci.* 2011;52:830–833) DOI:10.1167/iovs.10-5671

From the Departments of [1]Microbiology, Immunology, and Parasitology, [3]Ophthalmology, and [5]Pharmacology, and the [6]Neuroscience Center of Excellence, Louisiana State University Health Sciences Center, New Orleans, Louisiana; and the [7]Department of Biology, Xavier University, New Orleans, Louisiana.

[2]Contributed equally to the work and therefore should be considered equivalent authors.

[4]Present affiliation: Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas.

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Corresponding author: James M. Hill, LSU Eye Center, 2020 Gravier Street, Suite B, New Orleans, LA, 70112; jhill@lsuhsc.edu.

Herpes simplex virus type 1 (HSV-1) is a common cause of acute and recurrent ophthalmic disease worldwide[1,2] and is the leading cause of infectious blindness in the United States.[1,3] HSV-1 infections of the cornea typically result in sight-threatening epithelial (keratitis) or stromal disease.[2,4,5] Several antiviral drugs have shown efficacy in the treatment of experimental HSV-1 keratitis, including acyclovir, valacyclovir, cidofovir, trifluorothymidine (TFT), and ganciclovir (GCV).[6–8]

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).[9] 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 antiherpetic ophthalmic medication in Europe for more than a decade.[10]

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.[4] An acyclovir-resistant phenotype arises because of a mutation in viral TK or more rarely in the DNA polymerase of HSV-1.[11] In contrast with TFT, GCV (which is phosphorylated only with viral TK) is ineffective against acyclovir-resistant HSV-1.[11] Topical TFT can produce adverse effects such as a burning sensation on application, corneal edema, and increased intraocular pressure.[12] GCV is reported to cause less discomfort (stinging, burning) or blurred vision.[12]

TFT and GCV are clinically proven antiherpetic 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,[13] 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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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.[14] 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.[15]

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% CO^2 in Earle's minimal essential medium (EMEM; Invitrogen, Carlsbad, CA) supplemented with L-glutamine and 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 (CC_{50}) were calculated using special software (CalcuSyn v.2.0; BioSoft, Cambridge, UK). All assessments of toxicity were done in triplicate, and the CC_{50} values are reported as the mean ± SE.

Antiviral Activity of TFT and GCV

Antiviral activity was assessed by a plaque reduction assay essentially as described by the Clinical and Laboratory Standard Institute (Wayne, PA).[16] Vero cells were grown as previously described, washed with D-PBS (Gibco), and absorbed with 200 plaque-forming units (PFUs) of a HSV-1 strain for 45 minutes. The medium was removed and wells filled with 1:1 serial dilutions of TFT (50 μM) or GCV (5 μM for all strains except TKG7+2G where [GCV] = 640 μM). Each 24-well plate contained both negative (cells only) and positive (cells, virus, and no antiviral) control wells.

After 48 hours of incubation, cells were fixed with 10% buffered formalin and stained with 0.8% crystal violet (w/v; Sigma) in 50% ethanol for 5 minutes. Plates were washed and air dried before counting plaques with the aid of a dissecting microscope. The antiviral effect for each treatment was calculated using the following formula: $(P_{[+]control} - P_{[test]})/P_{[+]control}$, where $P_{[+]control}$ represents the average number of plaques in positive control wells and $P_{[test]}$ represents the average number of plaques in drug-treated wells. The inhibitory concentrations of TFT or GCV that yielded a 50% reduction in the number of plaques (IC_{50}) were calculated (CalcuSyn software; BioSoft). All assessments of antiviral activity were done in triplicate and the IC_{50} values are reported as the mean ± SE.

Antiviral Activity of TFT and GCV Combined

Combinations of TFT (50 μM) and GCV (5 μM for all strains except TKG7+2G where [GCV] = 640 μM) were tested with a plaque reduction assay, as previously described. These concentrations of TFT and GCV were selected because they represented the minimum amount of drug that resulted in a >99% reduction in PFUs.

To evaluate the efficacy of combinations in reducing PFUs, a fractional inhibitory concentration (FIC) index was calculated using the following formula: FIC = (IC_{50} of drug A in combination/IC_{50} of drug A alone) + (IC_{50} of drug B in combination/ IC_{50} of drug B alone).[17,18] Synergism is defined with a FIC value ≤ 0.5, indifference, or nonantagonism with a FIC > 0.5 but ≤ 4, and antagonism as a FIC > 4.

Results

Excluding the acyclovir-resistant mutant of HSV-1 constructed in a laboratory, the origin of a strain made little difference in its susceptibility to TFT or GCV, either alone or when combined. The CC_{50} value for GCV was 92.91 ± 8.92 μM. This concentration of GCV, which elicited a cytotoxic effect in half the Vero cells in a given population, is more than a hundred-fold higher than concentrations of GCV alone or combined with TFT (Table 1) required to reduce PFU formation by half (i.e., the IC_{50} value) in all clinical and laboratory strains of HSV-1 tested, with the exception of the acyclovir-resistant TK- mutant strain TKG+2G. The IC_{50} of GCV as a single agent against strain TKG7+2G (93.00 ± 9.64 μM) is essentially identical with its CC_{50}

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

	IC_{50} TFT (μM)		IC_{50} GCV (μM)		
HSV-1 Strain	**Single**	**Combo***	**Single**	**Combo***	**FIC**
McKrae	12.52 ± 0.61	0.80 ± 0.04	0.58 ± 0.04	0.08 ± 0.02	0.18 ± 0.01
KOS	7.10 ± 0.28	1.09 ± 0.06	0.86 ± 0.04	0.11 ± 0.01	0.23 ± 0.03
VT7581	5.32 ± 0.45	0.94 ± 0.02	0.49 ± 0.04	0.09 ± 0.03	0.34 ± 0.04
VT242	9.70 ± 0.83	1.05 ± 0.03	1.02 ± 0.07	0.11 ± 0.03	0.18 ± 0.02
VT4688	3.07 ± 0.36	0.52 ± 0.03	0.45 ± 0.05	0.05 ± 0.01	0.28 ± 0.01
VT5227	9.64 ± 0.78	1.60 ± 0.04	1.59 ± 0.14	0.16 ± 0.06	0.26 ± 0.02
VT00694	7.33 ± 0.48	0.89 ± 0.02	1.03 ± 0.04	0.08 ± 0.01	0.16 ± 0.03
VT7644	8.65 ± 0.41	0.68 ± 0.01	1.05 ± 0.03	0.07 ± 0.01	0.18 ± 0.02
VT7632	7.19 ± 0.45	0.58 ± 0.01	0.41 ± 0.04	0.06 ± 0.01	0.19 ± 0.03
VT53	4.39 ± 0.39	0.61 ± 0.03	0.40 ± 0.02	0.05 ± 0.01	0.40 ± 0.06
VT1736	6.91 ± 0.16	0.75 ± 0.01	0.75 ± 0.03	0.05 ± 0.01	0.31 ± 0.05
TKG7+2G†	15.40 ± 3.17	4.20 ± 0.16	93.00 ± 9.64	53.77 ± 7.03	0.84 ± 0.11

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.

† Thymidine kinase negative mutant of KOS.

(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 (CC_{50}) was 0.99 ± 0.01 μM. In contrast with GCV where the CC_{50} was less than the IC_{50} for all HSV-1 strains except the acyclovir-resistant TK-deficient strain, the IC_{50} values for TFT alone against all 12 strains of HSV-1 tested were more than threefold higher than the CC_{50} 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.[7,9,19] 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.[20–22] Recently GCV (0.15%) 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 (stinging, burning) or blurred vision after application of the drug.[12]

TFT was effective in vitro against all 12 clinical and laboratory strains but at concentrations much higher than GCV (Table 1). The IC_{50} values of GCV were similar against all HSV-1 strains except for the acyclovir-resistant, TK-deletion mutant of KOS (TKG7+2G), where, not unexpectedly, the IC_{50} was two magnitudes higher than its parent strain (Table 1). The efficacy of GCV against a HSV-1 isolate is based on the presence of a functional viral TK.[23] In contrast, TFT was effective against all HSV-1 strains tested, including strain TKG7+2G.

TFT acts synergistically against HSV with other compounds such as docetaxel,[4] docosanol,[24] and interferon alpha,[25] whereas GCV acts synergistically with cyclocytidine.[26] However, these agents are not used to topically treat HSV-1 keratitis in humans. In this study, the antiviral efficacy of a combination of TFT and GCV against HSV-1 was examined using a 10:1 ratio of these drugs, and a significant synergistic interaction was documented (Table 1). The FIC indices of these two antivirals were <0.5. With the exception of the acyclovir-resistant strain TKG7+2G, this synergism was observed against both laboratory and clinical strains of HSV-1. Coincidentally, the 10:1 ratio of antivirals exhibiting synergism in this in vitro study is similar to what would exist if current commercially available preparations of these drugs were used (a 1.0% TFT solution and 0.15% GCV ointment). The most tangible benefit of TFT and GCV acting synergistically against HSV-1 would be the reduction of ocular toxicity associated with the high concentrations of TFT needed to exert an antiviral effect. In this study, the amount 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.

Treatment of cells with TFT results in the production of aberrant host and viral DNA, RNA, and proteins. TFT is phosphorylated to TFT phosphate (TFT-P) by both HSV-1 and cellular TK. TFT-P irreversibly inhibits thymidylate synthase, a key enzyme in supplying the cell with TTP for DNA synthesis and repair.

A combination of TFT and GCV could be synergistic in much the same way that antibacterial agents trimethoprim and

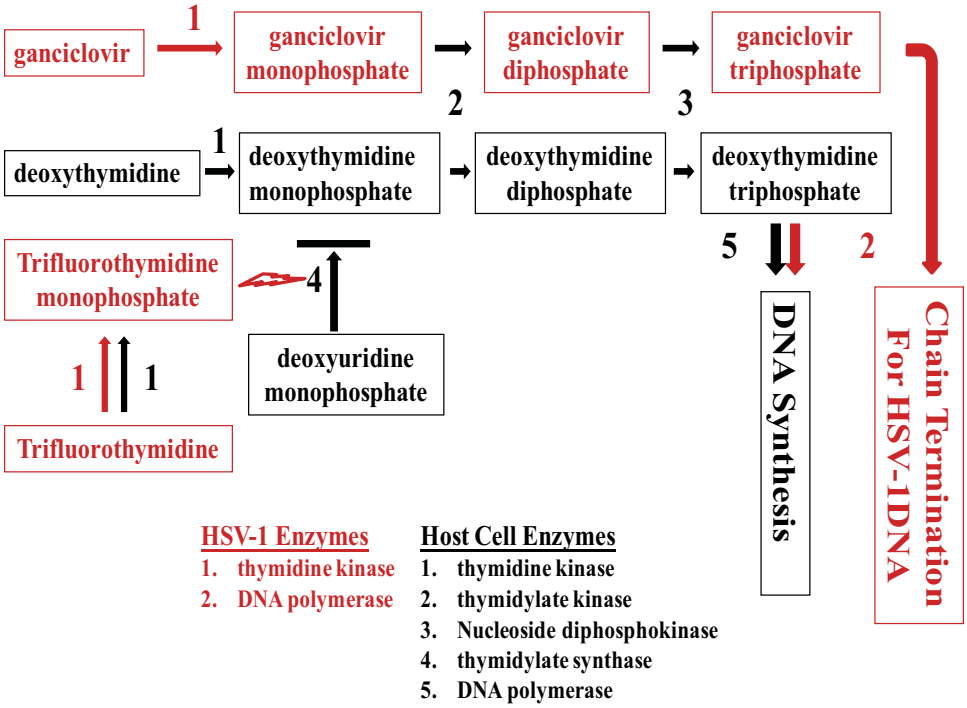

Figure 1. The fate of trifluorothymidine (TFT) and ganciclovir (GCV) in a cell. Antiviral agents and HSV-1 enzymes are in *red*, cellular metabolites and enzymes are in *black*. Synergism could arise from each antiviral agent targeting a different element in the synthesis of HSV-1 DNA. GCV is phosphorylated 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 cellular thymidylate synthase, an enzyme responsible for maintaining adequate concentrations of TTP for DNA synthesis and repair.

sulfamethoxazole are syngergistic. 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 IC_{50} values approximately 50% compared with the IC_{50} 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.

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