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Interactions among antiviral drugs acting late in the replication cycle of human cytomegalovirus

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Abstract

This study describes the extent of cross-resistance and interactions for selected inhibitors of human cytomegalovirus (HCMV) DNA synthesis and DNA processing. HCMV isolates resistant to the benzimidazole D-ribonucleoside viral DNA processing inhibitors TCRB and BDCRB were sensitive to BAY 38-4766, a non-nucleoside inhibitor of viral DNA processing. This indicates that these two drug types have distinct interactions with the products of HCMV genes UL56 and UL89 required for viral DNA cleavage and packaging. These virus isolates also were sensitive to ganciclovir (GCV) but slightly resistant to the L-benzimidazole ribonucleoside viral DNA synthesis inhibitor 1263W94. Virus resistant to 1263W94 remained sensitive to BDCRB, GCV, and BAY 38-4766. Examination of drug–drug interactions in cell culture assays measuring inhibition of HCMV replication revealed strong synergism for the combination of BDCRB with 1263W94, and for combinations of 1263W94 with cidofovir (CDV) and foscarnet (PFA), but not with GCV. Combinations of GCV with CDV and PFA were synergistic as well. The combination of GCV with 1263W94 showed additive antiviral interactions, whereas, a combination of BAY 38-4766 with GCV showed antagonism. Interaction of BDCRB with BAY 38-4766 showed a mixed pattern of synergy and antagonism. The antiviral synergy observed between GCV and PFA or CDV serves to validate clinical combination therapies for these drugs. Antagonism seen for BAY 38-4766 with GCV indicates that these two drugs are unlikely to be useful for combination therapies. Notably, 1263W94 demonstrated greater synergy in combination with PFA or CDV than did GCV, suggesting some promise for this benzimidazole L-riboside in such combination therapies.

Keywords: Benzimidazole ribonucleoside; HCMV; 1263W94; Drug interactions

1. Introduction

Human cytomegalovirus (HCMV) causes significant morbidity and mortality in immunocompromised populations. It is an opportunistic

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disease in AIDS and bone marrow transplant patients and is often a factor in their death. HCMV is also a leading cause of birth defects such as deafness and mental retardation (Alford and Britt, 1993). More recently, HCMV infection has been implicated in increased risk of organ transplant rejection and in coronary artery disease restenosis following angioplasty (O'Connor et al., 2001).

Current systemic therapies for HCMV infection include nucleoside analogs ganciclovir (GCV) (Crumpacker, 1996) and cidofovir (CDV) (Hitchcock et al., 1996), and the pyrophosphate analog foscarnet (PFA) (Chrisp and Clissold, 1991) (Fig. 1). These drugs suffer from disadvantages including poor oral bioavailability and certain toxicities. At therapeutic doses GCV is toxic to bone marrow progenitor cells, and CDV and PFA each produce renal toxicity (Deray et al., 1989; Kendle and Fan-Havard, 1998). All three drugs have the same mode of action: inhibition of viral DNA polymerase (Field and Biron, 1991). Following the phosphorylation of GCV to its monophosphate by the viral protein pUL97, GCV is phosphorylated to its triphosphate which acts as an inhibitor of HCMV DNA polymerase (pUL54) (Sullivan et al., 1992). CDV diphosphate and PFA share the same molecular target as GCV triphosphate. Consequently, drug-resistant strains of HCMV have been found for GCV, CDV, and PFA, and the emergence of cross-resistant strains has been described in clinical settings (Field and Biron, 1991; Erice, 1999). Thus, there is an unmet need for less toxic and more orally bioavailable drugs with molecular targets not shared with those currently in use.

In 1995 we reported that 2-bromo-5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole (BDCRB) and the 2-chloro analog (TCRB) are potent and selective inhibitors of HCMV replication (Fig. 1) (Townsend et al., 1995). These compounds have a novel mechanism of action which does not involve the inhibition of DNA synthesis. Rather, the benzimidazole D-ribonucleosides prevent the cleavage of high molecular weight viral DNA concatemers to monomeric genomic lengths

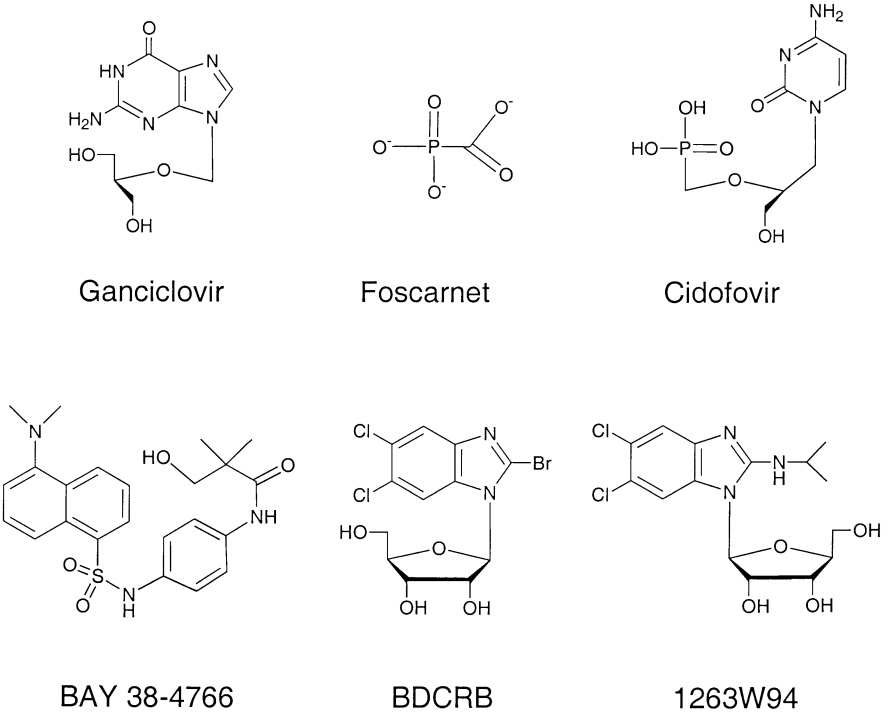

Fig. 1. Structures of compounds used in this study. These drugs were synthesized, purchased, or gifts as described in Section 2.

(Underwood et al., 1998). Resistance to these compounds has been mapped to HCMV genes UL56 and UL89 (Underwood et al., 1998; Krosky et al., 1998). These are only two of at least six viral genes (UL104, UL89, UL77, UL56, UL52, UL51) identified as required for viral DNA cleavage and packaging due to homology with bacteriophage and herpes simplex virus genes (Chee et al., 1990; Krosky et al., 2000; and references therein). pUL56 has been independently identified as a viral enzyme that binds the pac motif and possesses specific endonuclease and ATPase activity (Bogner et al., 1998; Hwang and Bogner, 2002). Nuclease activity also has been identified in pUL89 (Scheffczik et al., 2002).

Although TCRB and BDCRB are excellent inhibitors of HCMV in vitro, they are less effective in vivo due to rapid metabolic cleavage of the sugar from the heterocycle (Good et al., 1994; Townsend et al., 1999). Synthetic efforts to improve upon this limitation have produced, among other promising compounds, 1263W94 (maribavir; Fig. 1). 1263W94 is a β-L-ribosyl analog of BDCRB with an isopropylamine in the 2-position that is a potent and selective inhibitor of HCMV replication (Koszalka et al., 1996; Biron et al., 2002). However, its mechanism of action is different from that of BDCRB and involves inhibition of viral DNA synthesis. A mutation in UL97 has been shown to be responsible for resistance to this compound (Biron et al., 2002). Its development has progressed to a phase I/II clinical trial where it significantly reduced CMV shedding in HIV-infected individuals (Drew et al., 1998).

Similar to the D-benzimidazole ribonucleosides, the non-nucleosidic compound BAY 38-4766 (Fig. 1) is a potent and selective inhibitor of HCMV replication via inhibition of viral DNA processing(Reefschlaeger et al., 2001; Weber et al., 2001). Remarkably, resistance to BAY 38-4766 and BDCRB maps to similar positions in the UL56 and UL89 genes (Krosky et al., 1998; Bueger et al., 2001) even though the two compounds are chemically unrelated. Thus, we hypothesized that these compounds might display cross-resistance. Similarly, because 1263W94 and GCV are both inhibitors of DNA synthesis and resistance to both compounds maps to UL97 (Erice, 1999; Biron et al., 2002), we were prompted to determine the extent of cross-resistance for these compounds as well.

Drug interactions are particularly important considerations in combination antiviral therapies. Highly active anti-retroviral therapy (HAART) protocols for the treatment of HIV infections currently utilize three or more drugs in combination (Matsushita, 2000). Likewise, the goal for treatment of viral diseases is to aggressively treat infection and suppress the emergence of drug-resistant populations of virus. This can be accomplished if the antiviral effects of drugs used in combination are at least additive without a concomitant increase in cytotoxicity. Drugs which show in vitro antagonism in combination probably can be eliminated from combination therapies. Thus, we sought to determine the extent of drug-drug interactions for BDCRB, 1263W94, BAY 38-4766, along with the currently FDA-approved drugs for the treatment of HCMV infections: GCV, CDV, and PFA.

2. Materials and methods

2.1. Compounds

TCRB and BDCRB were synthesized in the laboratories of L.B. Townsend (Townsend et al. 1995). 1263W94 was synthesized at Burroughs Wellcome Co. (Koszalka et al., 1996; Chulay et al., 1999) and was provided through the courtesy of Dr Biron. BAY 38-4766 was synthesized by a method similar to that reported in the literature (Bender et al., 1999) and was characterized by ^{1}H-NMR and mass spectrometry. GCV was kindly provided by Hoffman La Roche (Palo Alto, CA). CDV was a gift from Dr MJM Hitchcock of Gilead Sciences (Foster City, CA) and trisodium PFA was purchased from Sigma (St. Louis, MO). Stock solutions of all compounds were prepared at 10 mg/ml in dimethylsulfoxide (except for PFA, which was prepared at the same concentration in sterile water) and stored at −20 °C. Compounds were added to cultures so that the resulting

concentrations of dimethylsulfoxide never exceeded 0.05% by volume.

2.2. Cell culture procedures

The routine growth of normal human diploid cells was performed in minimal essential media with Earle's salts [MEM(E)] with 10% fetal bovine serum (FBS). Two such lines were used, human foreskin fibroblasts (HFF) derived in our laboratory and MRC-5 cells, a human embryonic lung cell line obtained from American Type Culture Collection (ATCC CCL 171). Cells were routinely passaged at 1:2 dilutions according to conventional procedures using 0.05% trypsin with 0.02% EDTA in HEPES buffered saline (Shipman, 1969; Turk et al., 1987).

2.3. Virus strains and virological procedures

The Towne strain, plaque-purified isolate P_O, of HCMV was kindly provided by Dr MF Stinski, University of Iowa. HCMV strains D10, and C4 were selected for resistance to TCRB from wild-type Towne, and r56 derived from C4 as described by us (Underwood et al., 1998; Krosky et al., 1998). The AD169 strain of HCMV was obtained from ATCC and plaque purified in the laboratory of Dr KK Biron, Burroughs Wellcome, Co. Strain 2916^r (resistant to 1263W94) was derived from wild-type AD169 and was kindly provided by Dr Biron (Biron et al., 2002). Stocks of HCMV were prepared by infecting HFF cells at a multiplicity of infection (MOI) of 0.01 plaque forming units (PFU) per cell according to a procedure described previously (Turk et al., 1987). Virus titers were determined using monolayer cultures of HFF cells, also as described previously (Prichard et al., 1990).

2.4. Antiviral activity assays

2.4.1. HCMV plaque reduction assay

HFF cells at 85 000 cells per well in 24-well cluster plates were infected with HCMV at 100 PFU per well in MEM(E) with 10% FBS. Following an initial 1 h adsorption, medium containing selected drug dilutions and 0.5% methylcellulose was added. All drug concentrations were tested in at least triplicate using eight 1:3 dilutions from a starting concentration of 100 μM. After incubation at 37 °C for 8–10 days in an atmosphere of 5% CO_2, cell sheets were fixed, stained with crystal violet and plaques were enumerated under light microscopy. Drug effects were calculated as a percentage of reduction in number of plaques in the presence of each drug concentration compared with the number observed in the absence of drug.

2.4.2. HCMV yield reduction assay

HFF were planted at 12 500 cells per well in 96-well plates, incubated overnight, medium removed and the cultures were inoculated with HCMV at an MOI of 0.1 PFU per cell as described above. After virus adsorption, fresh medium containing test compounds in duplicate using eight 1:3 dilutions from a starting concentration of 100 μM in a total volume of 300 μl. Addition of medium alone served as virus (positive) controls. Plates were incubated at 37 °C for 7 days, subjected to one cycle of freezing and thawing; duplicate 100 μl aliquots from each of the 12-wells of a given column were transferred to the first column of a fresh 96-well monolayer culture of HFF cells. Contents were mixed and serially diluted 1:3 across the remaining seven columns of the secondary plate. Cultures were incubated, plaques were enumerated, and titers calculated as described above.

2.4.3. HCMV ELISA

ELISA analysis of HCMV was performed using an assay described by us (Renau et al., 1996). MRC-5 were planted at 12 500 cells per well in 96-well plates, incubated overnight, medium removed and the cultures were inoculated with HCMV (AD169) at an MOI of 0.002 PFU per cell as described above. After virus adsorption, fresh medium containing test compounds in duplicate using eight 1:3 dilutions from a starting concentration of 100 μM to a total volume of 300 μl. Addition of medium alone served as virus (positive) controls. Plates were incubated at 37 °C for 7 days and fixed with 95% ethanol. Wells were blocked then treated with 50 μl of a 1:100 dilution of a mouse antibody to HCMV (DAKO#M0757; Carpinteria, CA). Following washes, 50 μl of a

1:1000 dilution of horse radish peroxidase-conjugated rabbit anti-mouse antibody (DAKO#P0260) was added to each well, incubated for 2 h, and washed to remove unbound antibody. 150 μl of a 0.1 mg/ml solution of 3,3′,5,5′-tetramethylbenzidine (Sigma) in citrate-phosphate buffer, pH 5.0 was added to each well, developed for 15 min, and quenched with 50 μl 2 M H_2SO_4. Absorbance was determined at 450/570 nm in a Bio-Tek EL 312 microplate reader. Background was subtracted from control wells.

2.5. Data analysis

Dose response relationships were used to quantify drug effects by linearly regressing the percent inhibition of parameters derived in the preceding assays (except for yield experiments) against log drug concentrations. For yield experiments, the log of viral titer was plotted against the log drug concentration. About 50% inhibitory concentrations (IC_{50}'s) and 90% inhibitory concentrations (IC_{90}'s, yield experiments) were calculated from the linear portions of the regression lines.

2.6. Analysis of drug interactions

Drug combination assays were performed using the HCMV ELISA procedure described above and a dye technique for cytotoxicity (Prichard et al., 1991). The three-dimensional method (MacSynergy^{TM} II) developed by Prichard and Shipman (1990) was used to analyze drug-drug interactions. Briefly, data derived from three to six replicate plates were used to construct dose response surfaces. Theoretical additive interactions were calculated from the dose–response curves for each drug used alone. This calculated surface was subtracted from the experimentally determined dose–response surface to reveal regions of non-additive activity. Interpretation of data is as follows: If the resulting surface appeared as a horizontal plane at 0% inhibition, the interactions between the two drugs were additive. Depressions in the plane indicate antagonism, whereas, peaks above the plane indicate synergistic interactions between the two drugs. Confidence intervals (95%) around each of the points that defined the dose–response surface were calculated from the replicate data to provide limits for the experimental dose–response surface. If the upper confidence limits of the experimental data were less than the lower confidence intervals for the calculated additive surface, then antagonism was considered significant at that confidence level. Conversely, if the lower confidence limits of the experimental data were greater than the upper limits for the calculated additive surface, the synergy was considered significant. Finally, if the calculated additive surface was contained within the confidence limits, the interaction was considered to be additive. The amount of drug–drug interactions was quantified by integrating the area above or below the theoretical plane of additivity, thereby, giving 'volumes' of synergy or antagonism.

3. Results

3.1. Antiviral cross-resistance

The susceptibility of BDCRB-resistant viruses to BAY 38-4766, BDCRB, GCV, and 1263W94 was evaluated by plaque-reduction assay (Table 1). The viruses used in this study have been described previously (Underwood et al., 1998; Krosky et al., 1998). D10 has a mutation in UL89 (D344E), r56 contains a mutation in UL56 (Q204R), and C4 contains both mutations. As expected, all three mutant virus strains were resistant to BDCRB, with C4 displaying greater resistance to BDCRB than either mutation alone (Table 1). These viruses remained susceptible to GCV and surprisingly also to BAY 38-4766. Also unexpected, some cross-resistance to 1263W94 (2–3-fold) was noted. This cross-resistance also was observed in yield reduction assays, with 1263W94 displaying $\sim$10-fold greater IC_{90} values against C4 as compared with Towne (Table 1). Interestingly, 1263W94 displayed poor antiviral activity in plaque reduction assays against Towne virus (Table 1). Although we do not have an explanation for this effect, we have speculated that it may be due to greater DNA synthesis in the Towne strain of HCMV (Biron, 1995, personal communication).

Table 1
Sensitivity of BDCRB-resistant viruses to BAY 38-4766, 1263W94, and GCV[a]

Compound	Towne (wild-type)	D10[b] (UL89 D344E)	r56[b] (UL56 Q204R)	C4[b] (both mutations)
Plaque reduction IC_{50} (μM) for virus strains				
BDCRB	2.7±0.8	20.3±3.9**[c]	17±2.0**	33±5.0**
BAY 38-4766	1.3±0.9	2.4±0.6	1.9±0.2	2.1±0.6
1263W94	34±7	72±5**	86±15**	100±3**
GCV	2.3±0.7	2.7±1.2	2.6±0.3	2.8±0.8
Yield reduction IC_{90} (μM) for virus strains				
BDCRB	0.9±0.2	ND[d]	ND	23±5.0[e]
1263W94	0.3±0.25	ND	ND	2.9±1.0[e]
GCV	1.6±0.2	ND	ND	1.6±0.2

[a] IC_{50} (plaque reduction) and IC_{90} (yield reduction) values are reported as the mean±standard deviation (S.D.) of experiments performed in at least triplicate.
[b] HCMV strain D10 is described in Underwood et al. (1998) and strains r56 and C4 are described in Krosky et al. (1998).
[c] For the comparison of antiviral drug susceptibilities of wild-type vs. drug-resistant strains, $P < 0.05$ is indicated by *, and $P < 0.005$ is indicated by ** as determined by the Student's t-test function of MICROSOFT EXCEL.
[d] ND, not determined.
[e] Data were analyzed as individual lines with error bars from the mean±S.D. of three to four experiments rather than calculating the IC_{90} values for each individual experiment. The dose–response curves for C4 were statistically different from those for Towne on a 99% confidence interval.

The susceptibilities of a 1263W94-resistant virus to BDCRB, BAY 38-4766, and GCV also were determined (Table 2). This virus bears a mutation in UL97 (L397R) which has been shown to confer resistance to 1263W94 (Biron et al., 2002). There were no indications of cross-resistance to this 1263W94-resistant virus for the compounds evaluated.

3.2. Antiviral drug interactions

The inter-relationships among how these drugs act also were studied by examining the combined effects of two compounds on HCMV replication as measured by an HCMV ELISA. Fig. 2a presents a three-dimensional surface plot which shows that BDCRB was marginally synergistic in combination with GCV (volume of interaction = +53 $\mu M^2\%$). Since these two compounds have different mechanisms of action but involve the sequential steps of viral DNA synthesis (GCV) and DNA processing (BDCRB), the sum of their antiviral effects could be expected to be additive or greater. In contrast, BDCRB demonstrated much greater synergistic viral inhibition in combination with 1263W94 (Fig. 2b; volume of interaction = + 348 $\mu M^2\%$). Not only was the amount of synergy large, it also occurred at low concentrations of each drug (Fig. 2b), suggesting an important biochemical basis for this interaction. As

Table 2
Sensitivity of 1263W94-resistant HCMV to BDCRB, BAY 38-4766, and GCV[a]

Compound	Towne (wild-type)	AD169 (wild-type)	2916r[b] (UL97 L397R)
Yield reduction IC_{90} (μM) for virus strains			
1263W94	0.4±0.1	2.0±0.4	46±5.0
BDCRB	0.5±0.1	0.7±0.1	0.9±0.1
BAY 38-4766	1.3±0.4	1.1±0.1	0.3±0.1
GCV	1.1±0.5	1.2±0.3	1.0±0.1

[a] IC_{90} (yield reduction) values are reported as the mean±S.D. of experiments performed in at least triplicate.
[b] HCMV strain 2916r is described in Biron et al. (2002) and was derived from AD169.

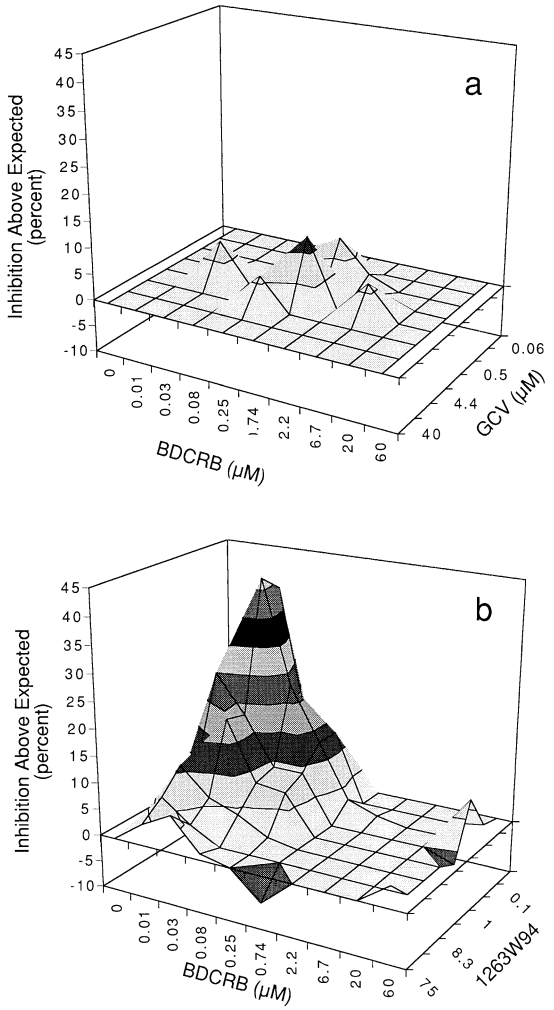

Fig. 2. Drug–drug HCMV inhibitory interactions between BDCRB and two DNA synthesis inhibitors. The extent of viral inhibition arising from drug combinations was assessed and is presented as a three-dimensional surface. *X*- and *Y*-axes give drug concentrations in μM. The *Z*-axis is the difference between the experimental dose–response surface and an additive surface calculated as described in Section 2. Peaks above the horizontal plane at 0% inhibition difference describe synergy, valleys below this plane describe antagonism. The amounts of synergy or antagonism are quantified by the volume of the peaks and valleys ($\mu M^2\%$ units) at 95% confidence intervals. (a) BDCRB with GCV. (b) BDCRB with 1263W94.

1263W94 is also an inhibitor of viral DNA synthesis, and we had found some cross-resistance between these compounds, this amount of synergy was surprising.

Since synergy of viral inhibition coupled with equivalent synergy of cytotoxicity results in a zero sum gain, we also measured the cytotoxicity of selected combinations of antiviral drugs. Combinations of BDCRB and GCV showed slightly synergistic cytotoxicity (volume of interaction = $+66\ \mu M^2\%$, data not shown). In contrast BDCRB in combination with 1263W94 displayed additive cytotoxicity (volume of interaction = $+23\ \mu M^2\%$, data not shown). We interpret these data to mean that the minor synergy seen with the HCMV ELISA for the combination of BDCRB and GCV was due to increased cytotoxicity, but the substantial synergy observed with the HCMV ELISA for BDCRB and 1263W94 was caused by inhibition of viral functions.

Interactions between other combinations of experimental compounds are summarized in Fig. 3. Combinations of the two viral DNA processing inhibitors BAY 38-4766 and BDCRB showed synergy at low concentrations (volume of interaction = $+83\ \mu M^2\%$) and antagonism at higher concentrations (volume of interaction = $-190\ \mu M^2\%$). Combinations of BDCRB and BAY 38-4766 also displayed some antagonism in the cytotoxicity assay (volume of interaction = $-64\ \mu M^2\%$). BAY 38-4766 demonstrated a large amount of antagonism in combination with GCV (volume of interaction = $-350\ \mu M^2\%$), but a minimal amount of synergy in combination with 1263W94 (volume of interaction = $+48\ \mu M^2\%$). 1263W94 displayed a large amount of synergy in combination with viral DNA polymerase inhibitors PFA (volume of interaction = $+331\ \mu M^2\%$) and CDV (volume of interaction = $+247\ \mu M^2\%$), but additivity in combination with GCV (volume of interaction = $+22\ \mu M^2\%$), also a viral DNA polymerase inhibitor. However, combinations of GCV and 1263W94 resulted in a moderate amount of synergy when measuring cytotoxicity (volume of interaction = $+68\ \mu M^2\%$).

The volume of interaction between marketed drugs CDV and GCV was $+212\ \mu M^2\%$ (Fig. 4a) and between CDV and PFA $+154\ \mu M^2\%$ (Fig. 4b) indicating a large and biologically relevant amount of synergy for combination therapy with these drugs.

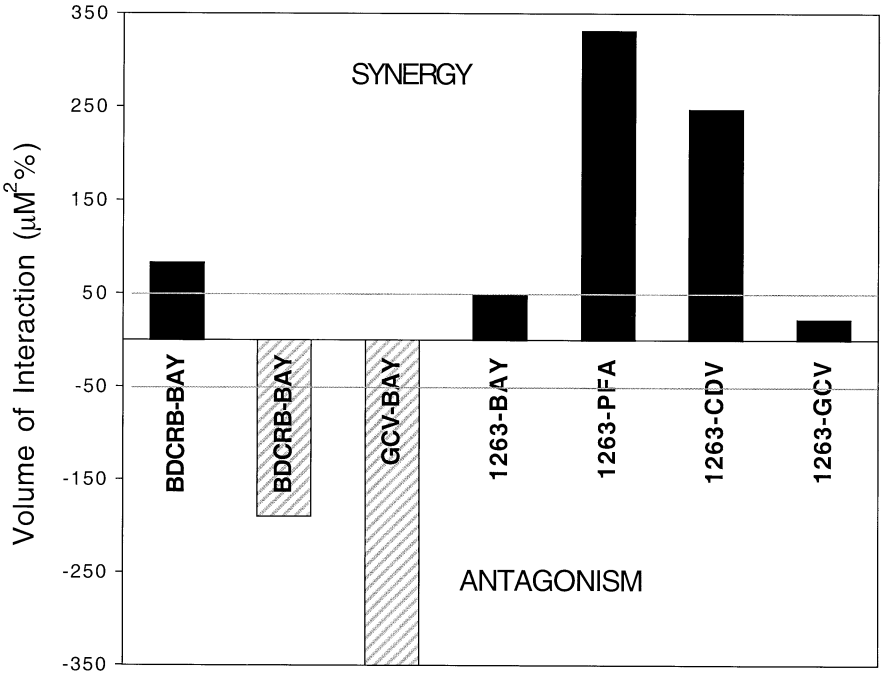

Fig. 3. Quantitation of drug–drug interactions with experimental compounds. Data were derived as described in Fig. 2 and amounts of synergy or antagonism are presented as vertical bars. The horizontal lines drawn at ± 50 $\mu M^2\%$ indicates the absolute valves of the boundary of volumes of interaction considered to be significant.

4. Discussion

In this study we have examined the extent of cross-resistance and interactions among late-acting antiviral drugs in order to gain new insights into their mechanisms of action against HCMV. BDCRB and BAY 38-4766 are both inhibitors of viral DNA processing. Resistance to both compounds maps to very similar regions of the same viral genes (UL56 positions 202 and 208 for BAY 38-4766 compared with 204 for BDCRB; UL89 positions 358 and 344, respectively, for these compounds). Since we did not observe cross-resistance between these two compounds, they are unlikely to have the same putative physical binding site to HCMV UL89 or UL56 proteins despite great similarities in mutation loci responsible for drug resistance. Similar results were reported with BDCRB for an HCMV isolate resistant to BAY 38-4766 (Bueger et al., 2001).

The exact roles of the HCMV UL56 and UL89 proteins in viral replication remain somewhat speculative. The HSV and pseudorabies virus homologs of UL89 and UL56 are UL15 and UL28, respectively (Chee et al., 1990). pUL15 functions as a nuclear chaperone for the putative viral DNA terminase pUL28 (Kozlowski et al., 1998, 1999; Abbotts et al., 2000). Since resistance to BDCRB and BAY 38-4766 maps to both UL89 and UL56, it is tempting to speculate that these compounds inhibit the intranuclear translocation of the putative HCMV DNA terminase UL56 (Bogner et al., 1998) by its chaperone UL89. However, it has been demonstrated that pUL56 has its own nuclear localization signal and does not require a nuclear chaperone (Giesen et al., 2000). In addition, pUL89 also has nuclease activity (Scheffczik et al., 2002), thereby, further reducing the possibility that it is a chaperone for pUL56. It does not necessarily follow that the

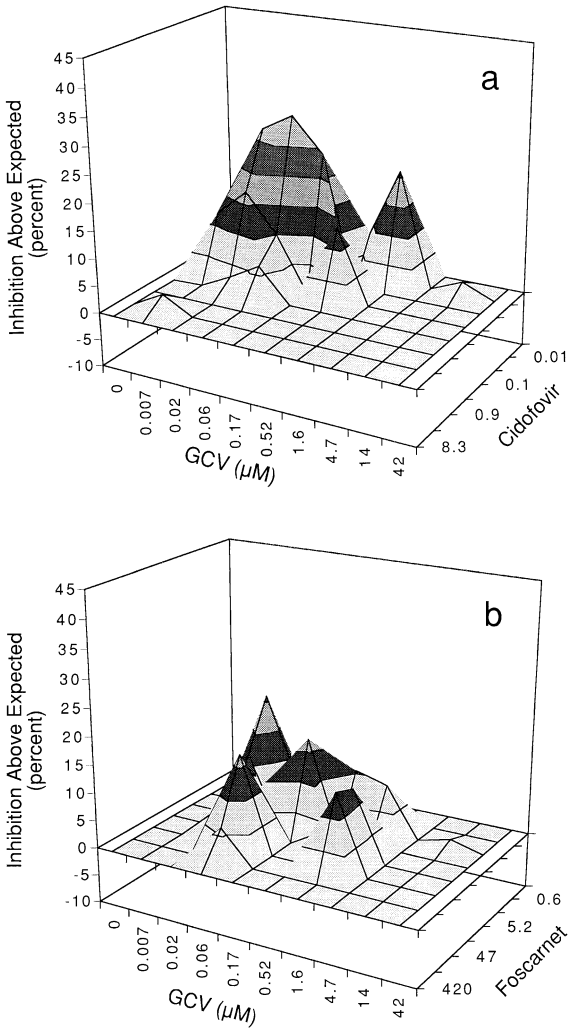

Fig. 4. Drug–drug HCMV inhibitory interactions between GCV and two DNA synthesis inhibitors. The extent of viral inhibition arising from drug combinations was assessed and is presented as described in the legend to Fig. 2. (a) GCV with CDV. (b) GCV with PFA.

observation of resistance to two viral gene products means that the two viral gene products must directly and physically interact. For example, resistance to GCV maps to UL97 and UL54 (Erice, 1999), two gene products which have distinct temporal expression profiles.

The lack of cross-resistance between GCV and BDCRB is easier to understand. These two compounds have different mechanisms of action and affect different viral gene products. However, we were surprised to see even slight cross-resistance between BDCRB and 1263W94, since BDCRB is a DNA processing inhibitor whereas 1263W94 is a DNA synthesis inhibitor even though its mode of action is not analogous to GCV. One possible explanation for this cross-resistance is that 1263W94 possesses a second mechanism of action. Following the inhibition of viral DNA synthesis, 1263W94 may inhibit the processing of residually synthesized viral DNA in a manner analogous to BDCRB. Alternatively, because 1263W94 inhibits pUL97 and it has been suggested that pUL97 plays a direct role in HCMV encapsidation (Wolf et al., 2001), inhibition of this process could be a second mechanism for 1263W94.

1263W94 displayed significant synergy in combination with viral DNA polymerase inhibitors CDV and PFA, but the effects were markedly lower in combination with GCV. 1263W94 inhibits the activity of pUL97 (Biron et al., 2002), the protein that phosphorylates GCV (Sullivan et al., 1992) which is required to produce the active form of GCV, its triphosphate. This requirement is not shared with CDV and PFA. Thus, inhibition of pUL97 by 1263W94 could be responsible for the lack of synergy between 1263W94 and GCV, as compared with 1263W94 and CDV or PFA. However, the mutations in UL97 conferring resistance to GCV (Erice, 1999) are located in a region distinct from the mutation shown to confer resistance to 1263W94 (Biron et al., 2002). 1263W94 also demonstrated synergistic interactions with BDCRB and BAY 38-4766. We suggest that these results would occur because inhibition of both DNA synthesis and DNA processing could be expected to yield greater viral inhibition than blocking either process alone. However, we find it interesting to observe a much greater amount of synergy between BDCRB and 1263W94 than between BAY 38-4766 and 1263W94 since we have shown cross-resistance with the former compounds but not the latter. We do not have an explanation for these results, and we note that although there is a great deal of discussion of mathematical models for measuring

drug interactions, and therapeutic considerations in clinical settings, very little consideration of the biochemical implications of synergism and/or antagonism in drug interactions is in the literature concerning these topics (Berenbaum, 1989). Likewise, we do not have an explanation for the observation of both synergy and antagonism seen between BAY 38-4766 and BDCRB. This interactive profile is rare in the literature but not unknown. The strong antagonism between GCV and BAY 38-4766 is a harbinger that combination therapy with these compounds may not be wise in a clinical setting.

We found significant synergy for GCV in combination with CDV and PFA. We note, however, that literature reports describing the interactions of these drugs are at odds with each other. It has been alternatively reported that GCV and PFA are additive (Freitas et al., 1989), synergistic (Manischewitz et al., 1990), and partially synergistic (Snoeck et al., 1992). Similarly, the interactions of GCV and CDV have been reported to be partially synergistic (Snoeck et al., 1992) and synergistic at low concentrations, but antagonistic at high concentrations (Yang et al., 1990). Our results demonstrate antiviral synergy for combinations of GCV with CDV or PFA and are consistent with reports of the utility of these drug combinations in the clinical treatment of HCMV infections which did not respond to monotherapy (Coker et al., 1991; Nelson et al., 1991; Kuppermann et al., 1993; Butler et al., 1992; Dieterich et al., 1993). We did not evaluate the in vitro drug-drug interactions of CDV in combination with PFA since co-administration of these drugs is contra-indicated due to renal toxicity (Kendle and Fan-Havard, 1998). The additivity we observed for 1263W94 in combination with GCV and its synergy when combined with CDV are consistent with a previous study employing a different methodology (Selleseth et al., 1997). However, these investigators reported additivity for a combination of 1263W94 with CDV, whereas, we found synergy. Interestingly, in our assays, 1263W94 displayed a greater amount of synergy with CDV and PFA than did GCV, indicating that 1263W94 may prove clinically more useful than GCV in combination therapies with these drugs.

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