ELSEVIER

Antiviral Research 39 (1998) 35–46

Antiviral Research

The antiviral activity of the ribonucleotide reductase inhibitor BILD 1351 SE in combination with acyclovir against HSV type-1 in cell culture

Carol Lawetz, Michel Liuzzi *

Department of Biological Sciences, Bio-Méga Research Division, Boehringer Ingelheim Ltd., 2100 Cunard Street, Laval, Quebec H7S 2G5, Canada

Received 8 December 1997; accepted 16 April 1998

Abstract

BILD 1351 SE is a selective peptidomimetic subunit association inhibitor of the herpes simplex virus (HSV) ribonucleotide reductase (RR) with potent antiviral activity both in cell culture assays and animal models of HSV disease. The ability of BILD 1351 SE to inhibit the replication of HSV-1 when used in combination with acyclovir (ACV) for the treatment of HSV infections was investigated in baby hamster kidney cells using a 96-well enzyme-linked immunosorbent assay. The effective concentrations to achieve 50% inhibition (EC_{50}) of virus replication by BILD 1351 SE in serum-starved and non serum-starved cells were 2 ± 0.9 and 4.1 ± 1.6 μM, respectively. The EC_{50} of ACV under both assay conditions was equal to 2.7 ± 0.9 μM when tested alone. However, upon addition of BILD 1351 SE, the antiviral activity of ACV was potentiated in a synergistic manner as determined by the isobole method. At a concentration of BILD 1351 SE that produced 30% inhibition of HSV-1 replication, the EC_{50} of ACV decreased by about 15-fold in confluent cells and 17-fold in serum-starved cells. Similar conclusions were reached when evaluating drug interactions by the median dose-effect. Assuming mutually non-exclusive conditions at a drug ratio of ACV/BILD 1351 SE of 1/2, synergy was demonstrated in confluent cells with a drug enhancement index at EC_{50} of 14 and a combination index of 0.25. None of the drug combinations tested showed increased cytotoxicity in comparison with each drug alone. These results are consistent with the expected mode of action of a selective HSV RR inhibitor and support the strategy of combining these inhibitors with ACV for improved therapy of HSV infections.

Keywords: Antiviral; ELISA; Herpes simplex; Ribonucleotide reductase; Synergy

* Corresponding author. Tel.: +1 450 6824640; fax: +1 450 6828434; e-mail: mliuzzi@bio-mega.boehringer-ingelheim.ca

0166-3542/98/$19.00
PII S0166-3542(98)00028-X

1. Introduction

BILD 1351 SE is a selective peptidomimetic inhibitor of the herpes simplex virus (HSV) ribonucleotide reductase (RR) (Moss et al., 1996). Like other related inhibitors, the structure of BILD 1351 SE (Fig. 1) is based on the C-terminal amino acid sequence of HSV RR small subunit (Liuzzi et al., 1994). These inhibitors bind to the HSV RR large subunit and prevent subunit association required for catalytic activity (Cohen et al., 1986; Dutia et al., 1986). BILD 1351 SE exhibits potent in vitro antiviral activity against HSV-1 and HSV-2 and in vivo antiviral activity in a murine model of HSV-1-induced keratitis when administered as an ophthalmic cream (Moss et al., 1996). Furthermore, this class of peptidomimetic HSV RR inhibitors has been shown to exert synergistic inhibition of HSV-2 replication in combination with acyclovir (ACV) in serum-starved cells (Liuzzi et al., 1994). Previous experiments in our laboratory to assess antiviral activity of RR inhibitors have been performed with a neutral red viral yield assay in serum-starved cells (Liuzzi et al., 1994) because HSV RR is not required for virus growth in replicating cells (Goldstein and Weller, 1988). In the present study we re-evaluated the antiviral activity of BILD 1351 SE in serum-starved and non serum-starved cells either alone or in combination with ACV. To facilitate drug interactions studies under various experimental conditions, we employed a robust ELISA assay and demonstrate that BILD 1351 SE exhibits antiviral activity in both starved and non-starved baby hamster kidney (BHK) cells with comparable potencies. In addition, significant synergy was found to exist for the combination of ACV and BILD 1351 SE in serum-starved as well as non serum-starved BHK cells. The specificity and potency of BILD 1351 SE together with the ability of this inhibitor to potentiate the antiviral activity of ACV suggest that BILD 1351 SE may be useful for the treatment of HSV disease either alone or in combination with ACV.

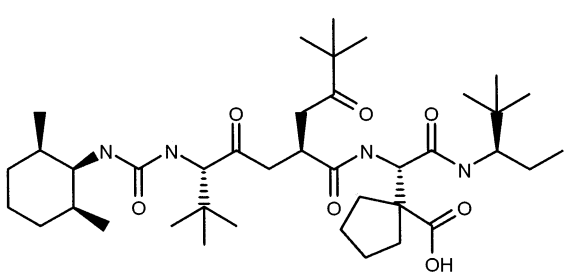

Fig. 1. Structure of the HSV RR inhibitor BILD 1351 SE.

2. Materials and methods

2.1. Cell culture and viruses

All cell culture reagents and media were obtained from Gibco BRL (Burlington, ON). The defined medium used for the antiviral assays consisted of F-12 medium/DMEM medium/BGjb medium, 6:3:1 (vol/vol) with 2 g/l BSA, 2.38 g/l HEPES, 50 mg/l garamycin, 100 μg/l cortisol, 1 μg/l insulin, 0.4 μg/l triiodothyromine, 0.2 μg/l parathyroid hormone, 10 μg/l glucagon, 0.1 μg/l epidermal growth factor, 0.2 μg/l fibroblast growth factor and 10 mg/l transferin (Brazeau et al., 1982). Cells were from American type culture collection (ATCC) (Rockville, MD). vero cells (African green monkey kidney cells) were grown in Dulbecco's modified eagle's medium (DMEM) supplemented with 8% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin sulfate and 100 μg/ml kanamycin sulfate. Baby hamster kidney (BHK)-21/C13 (ATCC CCL10) cells were grown in α-MEM medium instead of DMEM medium. All cells were grown at 37°C in an atmosphere of 5% CO_2. The laboratory strain of HSV-1 F HA14 used in this study has been described previously (Honess and Roizman, 1973). Virus stocks were routinely grown in vero cells and virus titers were determined by a standard plaque assay on confluent vero cells.

2.2. Reagents and antiviral compounds

Dimethylsulfoxide (DMSO), glutaraldehyde, tween 20, Triton X-100 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO). BILD 1351 SE was synthesized and purified as previ-

ously described (Moss et al., 1996). ACV was from Sigma. All compounds were dissolved in DMSO and then diluted with cell culture medium to yield 1% final DMSO. All stock compound solutions were filter sterilized through 0.22-μm millex-GV filters (Millipore, Bedford, MA).

2.3. Antiviral assays

Baby hamster kidney (BHK)-21/C13 cells were seeded in 96-well culture plates (Corning, Cambridge, MA) at a density of 3000 cells per well in α-MEM medium containing 8% (v/v) FBS, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate and 100 μg/ml kanamycin sulfate and incubated at 37°C with 5% CO_2 to reach 90% confluency. Cell monolayers were infected with HSV-1 strains F at a multiplicity of infection (MOI) of 0.05 in defined medium. After 1 h, the HSV-infected cells were rinsed with defined medium and then incubated for 24 h. The extent of replication was assessed by the ELISA assay or by a virus yield assay as described below. For inhibition studies by ACV and BILD 1351 SE or combinations of both, samples were assayed in 3-fold serial dilutions. When assessing the effect of compounds on the replication of HSV in serum-starved cells, BHK cells were seeded at a density of 5000 cells/well and incubated for 8 h. Then the concentration of FBS was reduced to 0.5% (v/v) and the cells were incubated for 3 additional days prior to infection with HSV-1 strain F.

2.4. ELISA

Cells were fixed with 0.063% glutaraldehyde in phosphate buffered saline (PBS) for 30 min and blocked with 0.5% casein in PBS for 1 h. Thereafter, mouse monoclonal antibody C11 that recognizes the HSV-1 late glycoprotein C (Trybala et al., 1994) was added to each well for 2 h. After washing the cells three times with PBS containing 0.05% Tween 20, the bound monoclonal antibody was detected using sheep anti-mouse IgG horseradish peroxidase (Amersham, Oakville, ON) for 1 h in the dark. The plates were washed three times with PBS and once with 0.1 M sodium citrate, pH 4.5. *o*-Phenylenediamine dihydrochloride (Pierce, Rockford, IL) was used as a substrate for 30 min in the dark and color development in individual wells was monitored at 450 nm using a Titertek model MCC/340 microplate spectrophotometer (ICN Flow, Montreal, QC). The percent inhibition of virus replication was calculated as follows: Percent inhibition $= 100 \times [1 - (OD_{450})_{\text{treated sample}}/(OD_{450})_{\text{control sample}}]$. EC_{50} values, (i.e. the concentration of compound to inhibit virus replication by 50%) were determined from plots of inhibition of virus replication as a function of compound concentration.

2.5. Virus yield reduction assay

For evaluation of total virus yield, HSV-1 strain F-infected cells were incubated in the presence of test compounds in 96-well plates exactly as described above for 24 h and duplicate rows of compound-treated cells were scraped into overlying cell culture media. Harvested cells and cell culture media were sonicated and stored frozen at -80°C. Infectious virus yield from harvested samples was determined in duplicate by standard plaque assay on monolayers of vero cells. A HSV-1 specific antiserum was applied to cells after adsorption. After incubation for 2 days, cells were fixed with 4% formaldehyde and stained with 2% crystal violet in 20% ethanol.

2.6. Cytotoxicity assay

The cytotoxicity of compounds for BHK cells was determined with a modified tetrazolium MTT assay (Denizot and Lang, 1986). BHK cells were treated exactly as described above for the 96-well antiviral assay. Instead of virus infection, cells were mock infected with sterile medium only. At the end of the incubation period, 20 μl of 5 mg/ml MTT in cell culture medium was added directly to the cell culture medium of each well (100 μl) and cells were incubated for an additional 3 h at 37°C. Medium was then removed from each well and 50 μl of 10% triton X-100 in 0.01 N HCl was added to dissolve the insoluble formazan product. The optical density at 570 nm was determined with a Titertek model MCC/340 microplate reader. The cytotoxic effect to each compound concentration

was expressed as the percentage of signal resulting from sham-treated cells. A minimal background from wells containing cells killed by the addition of 5 μl of 1 M HCl was subtracted prior to any calculations. The percent cell viability was calculated as follows: Percent cell viability = $100 \times (OD_{570})_{treated}/(OD_{570})_{control}$. The 50% cytotoxic concentration (i.e. CC_{50}) was derived from plots of percent viability as a function of compound concentration.

2.7. Drug combination assays

The antiviral activity of BILD 1351 SE in combination with ACV against HSV-1 strain F was assessed using the high throughput 96-well antiviral ELISA-based assay described above. The effect of the combination of ACV and BILD 1351 SE on HSV replication was evaluated by using the median-effect principle and the isobologram method (Suhnel, 1990; Chou, 1991b). In the isobologram method, selected concentrations of BILD 1351 SE (i.e. EC_5, EC_{10}, EC_{20} and EC_{30}) were tested in combination with various doses of ACV and average EC_{50} values of ACV were determined from duplicate dose-response curves. These values were then used to calculate FEC_{50} (ACV) which represents the ratio of the concentration of ACV required to inhibit HSV replication by 50% in the presence of a fixed concentration of BILD 1351 SE to the concentration of ACV required in the absence of BILD 1351 SE. The isobologram representation is generated by plotting FEC_{50} (ACV) as a function of the ratio of the concentration of BILD 1351 SE to the EC_{50} of BILD 1351 SE in the absence of ACV. In this representation, when experimental data points fall on the hypotenuse, the effects of the two drugs are additive. If the experimental data points fall below the theoretical line of non-interacting drugs, the effect of the two drugs is synergistic. Drug interactions were evaluated by the median-dose effect analysis using a computer dose-effect analysis software program to calculate the combination index (CI) at all effect levels (Chou, 1991a). BILD 1351 SE and ACV were mixed at different ratios (i.e. 5:1, 2:1, 1:1, 1:2 and 1:5) and then serially diluted at a constant ratio. Inhibition data and EC_{50} values for each combination ratio were derived from quadruplicate dose-response curves and the combination indexes (CI) were calculated assuming both mutually exclusive and non-exclusive conditions. A CI value of 1, <1 and >1 indicates additive, synergistic and antagonistic effects, respectively.

3. Results

3.1. Susceptibility testing of HSV-1 strain F using the ELISA antiviral assay

The effect of ACV or BILD 1351 SE alone on viral antigen production due to HSV-1 growth was determined from multiple dose-response curves. Representative examples are shown in Fig. 2. The effective concentration necessary to inhibit 50% of virus replication (EC_{50}) by ACV and BILD 1351 SE was found to be 2.7 ± 0.9 and 4.1 ± 1.6 μM in confluent BHK cells (Fig. 2A) and 2.7 ± 0.9 and 2.0 ± 0.9 μM in serum-starved BHK cells (Fig. 2B). The EC_{50} values of both compounds did not change as a function of MOI between 0.01 and 0.1, but increased significantly at a MOI of 1 (data not shown). These antiviral activities compared favorably with those obtained previously (Liuzzi et al., 1994). In the colorimetric viral yield assay, the EC_{50} of both compounds against HSV-1 strain F in serum-starved cells was equal to 2.0 μM (Liuzzi et al., 1994; Moss et al., 1996). In the viral yield assay using confluent cells, half maximum reduction of 5 $\log_{10}$ in progeny virus was achieved at 1.8 μM for ACV and 3.8 μM for BILD 1351 SE. The antiviral effect of BILD 1351 SE was not due to non-specific cytotoxicity since the CC_{50} of BILD 1351 SE in confluent mock-infected BHK cells was 129 ± 19 and 153 ± 22 μM in serum-starved BHK cells. The CC_{50} value for ACV was higher than 200 μM under both experimental conditions (Fig. 2). None of the drugs, either alone or in combination, reached a 50% cytotoxicity concentration in the dose ranges tested. No enhanced cytotoxicity to uninfected cells was seen for any of the combinations of compounds (data not shown).

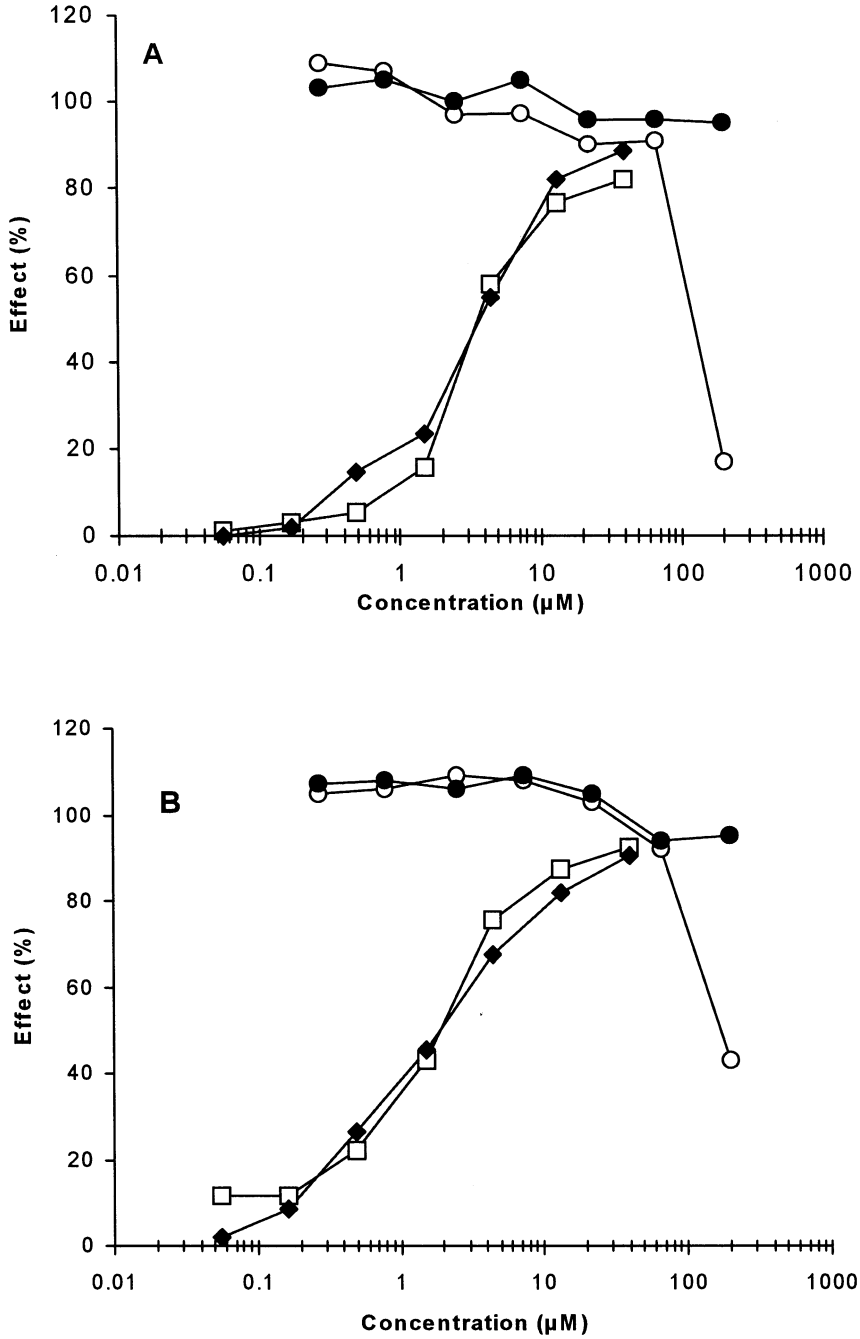

Fig. 2. Antiviral activity and cytotoxicity of BILD 1351 SE or ACV in confluent (A) and serum-starved (B) cells. Monolayers of BHK cells were mock-infected or infected at a MOI of 0.05 with HSV-1 strain F and then incubated in the presence of various concentrations of test compounds. The extent of HSV replication was determined with the ELISA assay and the cytotoxicity was determined with the MTT assay as outlined in Section 2. The results are presented as percent inhibition of HSV-1 replication (□, BILD 1351 SE; ◆, ACV) and percent cell viability (○, BILD 1351 SE; ●, ACV). Typical dose-response curves are shown. The average EC_{50} and CC_{50} values stated in the text were derived from at least ten independent determinations.

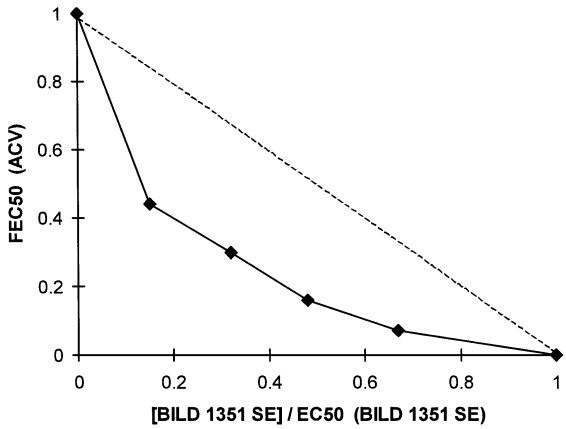

Fig. 3. Synergistic antiviral activity of ACV and BILD 1351 SE in confluent BHK cells. EC_{50} values were derived from the data shown in Table 1 and used to construct the isobologram. FEC_{50} (ACV) represents the ratio of the EC_{50} of ACV in the presence of a constant concentration of BILD 1351 SE to the EC_{50} of ACV alone. The x-axis represents the ratio of the fixed concentration of BILD 1351 SE to the EC_{50} of BILD 1351 SE alone. In this representation, displacement of the experimental data points to the left of the theoretical line for non-interacting inhibitors is indicative of synergistic behavior.

3.2. Potentiation of the antiherpetic activity of ACV by BILD 1351 SE

The antiviral activity of ACV in combination with BILD 1351 SE was evaluated in BHK cells using the ELISA-based antiviral assay. The effect of ACV in the presence of a constant amount of BILD 1351 SE ranging from 0.05–2 μM is shown in Table 1 from experiments using confluent BHK cells. To determine the type of drug interaction, EC_{50} values were calculated and used to construct the isobologram. A graphic illustration of the potentiation of ACV by BILD 1351 SE is shown in Fig. 3. Clearly, displacement of the curve to the left is indicative of synergistic interaction between ACV and BILD 1351 SE. Similar results were also observed when using serum-starved BHK cells (Table 2, Fig. 4). From the data obtained it can be calculated that the addition of EC_{30} concentration of BILD 1351 SE (i.e. about 2.0 and 1.5 μM BILD 1351 SE for confluent and starved cells, respectively) caused the EC_{50} of ACV to decrease by about 15-fold in confluent cells and about 17-fold in serum-starved cells.

The degree of synergy between BILD 1351 SE and ACV was also evaluated by the median-effect principle using the method of Chou (1991b). In this method, the concentrations of ACV and BILD 1351 SE were co-varied at a constant ratio and the data from each dose-response curve were used to calculate the combination index at all drug effect levels using a computer program (Chou, 1991a). Typical dose-responses resulting from serially diluting ACV and BILD 1351 SE at a constant ratio ranging from 5:1 to 1:5 are shown in Table 3 in the case of confluent BHK cells. Calculations of the combination index (CI) were based on the mutually non-exclusive assumption at all the combination ratios. A plot of CI as a function of F_a (given degree of effect in the combination) which describes the extent of synergism at each effect level is shown in Fig. 5. It is clearly visible that at high effects levels, the CI values are smaller than 1 at all combination ratios tested indicating synergy between ACV and BILD 1351 SE. The drug reduction index (DRI) of ACV at EC_{50}, which describes the fold reduction in dose due to synergism at 50% effect was 14 at the combination ratios of 1:2 with a CI value of 0.25. Synergy was also evident when the CI values were calculated by assuming mutually exclusive conditions (data not shown). Similar results were also observed when using serum-starved BHK cells

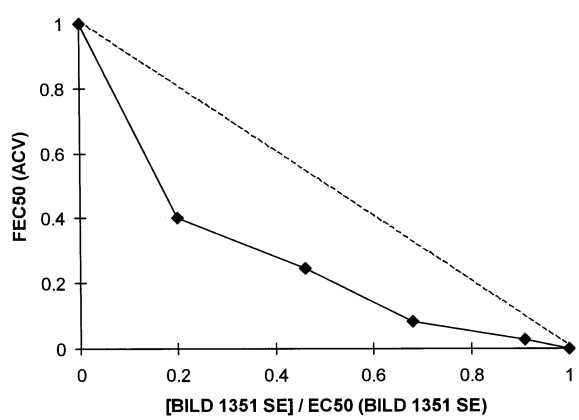

Fig. 4. Synergistic antiviral activity of ACV and BILD 1351 SE in serum-starved BHK cells. EC_{50} values were derived from the data shown in Table 2 and used to construct the isobologram as outlined in the legend to Fig. 3.

Table 1
Effect of BILD 1351 SE in combination with ACV on the replication of HSV-1 in confluent BHK cells

Concentration (μM)	% Inhibition of HSV-1 Replication					
	ACV alone	BILD 1351 SE alone	ACV+0.5 μM BILD 1351 SE	ACV+1 μM BILD 1351 SE	ACV+1.5 μM BILD 1351 SE	ACV+2 μM BILD 1351 SE
40	89.5	81	90.5[a]	93.7	94.7	95.8
13.3	77.9	74.7	83.2	87.4	88.9	92.6
4.4	56.8	60	73.7	84.2	87.4	93.7
1.5	25.2	11.6	47.4	60	70.5	85.3
0.49	8.4	1.1	13.2	34.7	47.4	74.7
0.165	−3.2	−.42	3.7	14.7	22.1	42.1
0.055	−9.5	−5.3	0	10.5	17.9	31.6
0.018	−5.3	−3	−1.1	7.4	14.7	22.1
0.006	−3.2	−1.1	3.2	7.4	17.9	26.3
0.002	−1.1	−1.1	−3.2	10.5	13.7	25.3

[a] The results are presented as percent inhibition as a function of the concentration of ACV.
Each value represents the average of duplicate determinations.

Table 2
Effect of BILD 1351 SE in combination with ACV on the replication of HSV-1 in serum-starved BHK cells

Concentration (μM)	% Inhibition of HSV-1 replication					
	ACV alone	BILD 1351 SE alone	ACV+0.5 μM BILD 1351 SE	ACV+1 μM BILD 1351 SE	ACV+1.5 μM BILD 1351 SE	ACV+2 μM BILD 1351 SE
40	89.5	91.6	93.7[a]	96.8	97.9	98.9
13.3	80.5	86.8	86.8	93.7	95.3	96.8
4.4	65.3	70.5	75.8	85.3	87.4	91.6
1.5	41.1	33.7	58.9	71.6	78.9	82.1
0.49	17.9	13.7	37.4	50.0	64.2	71.6
0.165	5.3	10.5	23.2	34.7	48.4	60.0
0.055	−0.5	8.4	13.7	26.8	37.9	50.5
0.018	−4.2	4.2	7.4	21.1	35.8	46.3
0.006	−5.3	3.2	6.3	18.9	34.7	43.2
0.002	−3.2	3.7	1.1	22.1	31.5	44.2

[a] The results are presented as percent inhibition as a function of the concentration of ACV.
Each value represents the average of duplicate determinations.

Table 3
Effect of serial dilution of BILD 1351 SE and ACV at a constant ratio on the replication of HSV-1 in confluent BHK cells

Concentration	Percentage inhibition of HSV-1 replication						
	ACV alone	BILD 1351 SE alone	ACV+BILD 1351 SE				
			Ratio 5:1	Ratio 2:1	Ratio 1:1	Ratio 1:2	Ratio 1:5
120			95.5[a]				98.2
60				95.6		96.4	
40	87.6	82	94.8		94.9		94.4
20				96.1		96.7	
13.3	76.8	78	93.4		93.3		90
6.67				90.3		93.1	
4.4	58.2	61.7	77.4		77.9		81.7
2.2				65.5		73.7	
1.44	25.5	18.4	43.3		32.6		42.9
0.74				28.1		36.3	
0.47	6	1.1	17.5		12.6		10.1
0.247				9.03		12.5	
0.16	−2.3	3.97	1		9.8		2.2
0.082				3.3		17.3	
0.052	−3.8	−0.8	−3.1		5.9		−1.7
0.027				1.4		8	
0.017	−6.6	−2.2	−6.1		5.9		−6.2

[a] The results are given as percent inhibition of HSV replication as a function of the total inhibitor concentration. Each value represents the average of four determinations.

(data not shown). Under these conditions, the CI and DRI of ACV at the 50% effect level for the drug combination ratio of 1:2 were calculated to be 0.6 and 7, respectively, assuming mutually non-exclusive drugs. Combined results demonstrate that BILD 1351 SE potentiates the antiviral activity of ACV in a synergistic manner.

4. Discussion

The purpose of this study was to investigate the efficacy of selective HSV RR inhibitors either alone or in combination with ACV under various cell culture conditions. Previous studies have shown that peptidomimetic RR inhibitors are effective against HSV-1 and HSV-2 replication in serum-starved BHK cells and that they potentiate the antiviral action of ACV. (Liuzzi et al., 1994; Moss et al., 1996). To re-evaluate the antiviral properties of this class of inhibitors, we employed an ELISA assay based on the detection of the HSV-1 glycoprotein C. Colorimetric detection of the late viral antigen gC is proportional to the amount of virus replication within each cell since the production of gC requires viral DNA replication (Roizman and Sears, 1993). Our results indicate that ACV is effective in this assay at concentrations similar to those reported using virus yield assays (this report, Liuzzi et al., 1994). It is noteworthy, however, that the EC_{50} values obtained for ACV appear about 5–10-fold higher than those reported by others using different antiviral assays (O'Brien and Campoli-Richards, 1989; Takeuchi et al., 1991). This divergence has been attributed to the presence of thymidine in the defined medium (Brazeau et al., 1982) which competes with ACV for the viral encoded thymidine kinase (Reardon and Spector, 1991). When performing the assay in α-MEM medium, the average EC_{50} of ACV dropped to about 0.4 μM (data not shown). Recently, this methodology has also been adapted for use with other herpesviruses or cell lines (e.g. ACV-resistant HSV-1)

(manuscript submitted), and as discussed below, the assay is useful to investigate the additive or possibly synergistic effects of antiviral agents.

With a reliable assay in hand, we have investigated the antiviral efficacy of selective HSV RR inhibitors using BILD 1351 SE as a prototype, either alone or in combination with ACV. BILD 1351 SE selectively inhibits the HSV RR since the structure of BILD 1351 SE is specifically based on the C-terminal amino acid sequence of HSV RR small subunit (Liuzzi et al., 1994) that lacks sequence homology with mammalian RR (Cosentino et al., 1991). BILD 1351 SE exhibits potent in vitro antiviral activity against HSV-1 and HSV-2 in serum-starved cells (Moss et al., 1996). Because HSV encode a RR that, unlike the mammalian RR is not subject to allosteric product inhibition (Erikson and Sjöberg, 1989), these viruses induce the synthesis of high levels of dNTP pools in infected cells (Jamieson and Bjursell, 1976; Daikoku et al., 1991; Liuzzi et al., 1994; Prichard and Shipman, 1995). Inhibition of HSV RR by a selective inhibitor such as BILD 1263 SE is associated with a marked reduction in the pool size of dGTP but does not affect the dNTP pools produced by the host RR (Liuzzi et al., 1994). ACV, a guanosine analogue is selectively phosphorylated by the HSV thymidine kinase in infected cells and further converted to the active inhibitor acyclovir-triphosphate (ACTP) by cellular enzymes to inhibit the HSV DNA polymerase and suppress viral DNA replication (Reardon and Spector, 1991). As a consequence of the inhibition of the viral DNA polymerase, the dGTP levels increase in HSV infected cells (Furman et al., 1982; Spector et al., 1989). Since dGTP competes with ACVTP for binding to DNA polymerase, a reduction of the HSV RR-induced dGTP pools should result in an increased effectiveness of ACV. Moreover, because of the presence of pre-existing higher dNTP pools in non serum-starved cells, a selective HSV RR inhibition should be less active in those cells. Our results indicate that BILD 1351 SE efficiently reduced HSV-1 replication in confluent cells with an EC_{50} of 4.1 ± 1.6 μM. This EC_{50} value is only 2-fold higher than that seen with serum-starved cells ($EC_{50} = 2.0 \pm 0.9$ μM), but statistically different as determined in a two-tailed unpaired Student's t test considering the large sample number ($n = 10$, $P < 0.005$). These results thus indicate that BILD 1351 SE is less effective in cells containing high levels of nucleotide pools and that pre-existing dNTP pools in confluent cells are not sufficient for optimal HSV-1 replication.

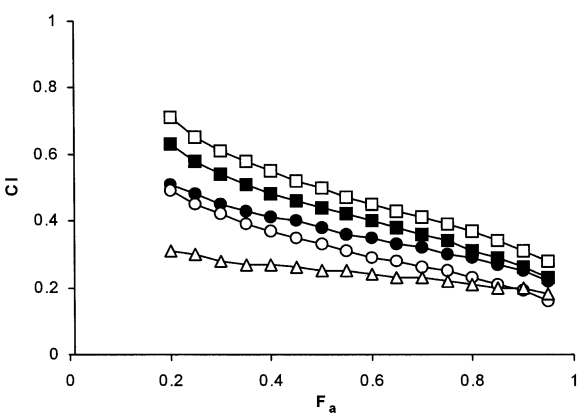

Fig. 5. F_a-CI plots for the combination of ACV and BILD 1351 SE at various ratios against HSV replication in confluent BHK cells. Data from Table 3 were used to calculate the combination index (CI) at all effect levels (F_a) for each drug ratio combination assuming mutually non-exclusive conditions using a computer program as outlined in Section 2. ●, ACV and BILD 1351 SE at a ratio 5:1; ○, ACV and BILD 1351 SE at a ratio 2:1; □, ACV and BILD 1351 SE at a ratio 1:1; △, ACV and BILD 1351 SE at a ratio 1:2; ■ ACV and BILD 1351 SE at a ratio 1:5.

The results in this study confirm previous reports that RR inhibitors potentiate the antiviral activity of ACV (Prichard and Shipman, 1990; Spector, 1993). Our results clearly demonstrate that the combination of ACV and BILD 1351 is synergistic against HSV-1 replication both in starved and non starved BHK cells. Interestingly, however, the degree of synergy as assessed by the isobologram method was about the same under both experimental conditions. Addition of EC_{30} concentration of BILD 1351 SE caused the EC_{50} of ACV to decrease by about 15-fold in confluent cells and about 17-fold in serum-starved cells. This observation implies that since the mechanism of potentiation of the antiviral activity of ACV involves increasing the ACVTP:dGTP ratio (Reardon and Spector, 1991; Spector, 1993), the

combinations of ACV and BILD 1351 SE achieve similar ratios under both experimental conditions. Synergy was also evident when evaluating drug interactions by the median dose-effect method assuming both mutually non-exclusive and mutually exclusive conditions. As clearly shown in Fig. 5 in the case of confluent cells, the CI values were smaller than 1 at all the drug combination ratios tested at the effect levels of 0.2 and higher. The highest synergy was seen at a drug ratio of 1:2 with a drug reduction index of 14, in good agreement with isobologram method. From these results we can expect that a selective HSV RR inhibitor should also be useful for enhancing the antiviral activity of ACV in actively replicating cells since the vast majority of the dNTP pools under these conditions are produced by the HSV RR (Daikoku et al., 1991; Prichard and Shipman, 1995).

In conclusion, peptidomimetic compounds such as BILD 1351 SE represent a new class of antiviral agents which may be clinically useful in the treatment of HSV disease. BILD 1351 SE appears as potent as the currently approved antiviral agent ACV in several in vitro antiviral assays against wild-type laboratory and clinical isolates of HSV-1. Like other inhibitors in this class, BILD 1351 SE inhibits the replication of HSV strains which are resistant to currently used antiviral agents (Liuzzi et al., 1994) and, as shown in this report, BILD 1351 SE potentiates the antiviral activity of ACV in cell culture assays. Recently, the potentiated antiviral effect of ACV by a selective HSV RR inhibitor has been demonstrated in vivo against ACV-resistant HSV-1 infections in athymic nude mice (manuscript submitted). Therefore, selective HSV RR inhibitors should be useful either alone or in combination with ACV to improve the outcome of HSV disease.

References

Brazeau, P., Ling, N., Bohlen, P., Esch, F., Ying, S.Y., Guillemin, R., 1982. Growth hormone releasing factor, somatocrinin, releases pituitary growth hormone in vivo. Proc. Natl. Acad. Sci. 79, 7909–7913.

Chou, J.H., 1991a. Quantitation of synergism and antagonism of two or more drugs by computerized analysis. In: Chou, T.C., Rideout, D.C. (Eds.), Synergism and Antagonism in Chemotherapy. Academic Press, New York, pp. 223–241.

Chou, T.C., 1991b. The median effect principle and the combination index for quantitation of synergism and antagonism. In: Chou, T.C., Rideout, D.C. (Eds.), Synergism and Antagonism in Chemotherapy. Academic Press, New York, pp. 61–102.

Cohen, E.A., Gaureau, P., Brazeau, P., Langelier, Y., 1986. Specific inhibition of herpesvirus ribonucleotide reductase by a nonapetide derived from the carboxy terminus of subunit 2. Nature 321, 441–443.

Cosentino, G., Lavallée, P., Rhakit, S., et al., 1991. Specific inhibition of ribonucleotide reductases by peptides corresponding to the C-terminal of their second subunit. Nature 69, 79–83.

Daikoku, T., Yamamoto, N., Maeno, K., Nishiyama, Y., 1991. Role of viral ribonucleotide reductase in the increase of dTTP pool size in herpes simplex virus-infected vero cells. J. Gen. Virol. 72, 1441–1444.

Denizot, F., Lang, R., 1986. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Methods 89, 271–277.

Dutia, B.M., Frame, M.C., Subak-Sharpe, J.H., Clark, W.N., Marsden, H.S., 1986. Specific inhibition of herpesvirus ribonucleotide reductase by synthetic peptides. Nature 321, 439–443.

Erikson, S., Sjöberg, B.M., 1989. Ribonucleotide reductase. In: Hervé, G. Jr. (Ed.), Allosteric Enzymes. CRC Press, Boca Raton, pp. 189–215.

Furman, P.A., Lambe, C.U., Nelson, D.J., 1982. Effect of acyclovir on the deoxyribonucleoside triphosphate pool levels in vero cells infected with HSV-1. Am. J. Med. 73, 14–17.

Goldstein, D.J., Weller, S.K., 1988. Factor(s) present in herpes simplex virus type-1-infected cells can compensate for the loss of the large subunit of the viral ribonucleotide reductase: charaterization of an ICP6 deletion mutant. Virology 166, 41–51.

Honess, R.W., Roizman, B., 1973. Proteins specified by herpes simplex virus XI. Identification and relative molar rates of synthesis of structural and nonstructural herpes virus polypeptides in the infected cell. J. Virol. 12, 1347–1365.

Jamieson, A.T., Bjursell, G., 1976. Deoxyribonucleoside triphosphate pools in herpes simplex type-1 infected cells. J. Gen. Virol. 31, 101–113.

Liuzzi, M., Déziel, R., Moss, N., et al., 1994. A potent peptidomimetic inhibitor of HSV ribonucleotide reductase with antiviral activity in vivo. Nature 372, 695–698.

Moss, N., Beaulieu, P., Duceppe, J.S., et al., 1996. Peptidomimetic inhibitors of herpes simplex virus ribonucleotide reductase with improved in vivo antiviral activity. J. Med. Chem. 39, 4173–4180.

O'Brien, J.J., Campoli-Richards, D.M., 1989. Acyclovir. An updated review of its antiviral activity, pharmacokinetic properties and therapeutic efficacy. Drugs 37, 233–309.

Prichard, M.N., Shipman, C. Jr., 1990. A three-dimensional model to analyze drug-drug interactions. Antivir. Res. 14, 181–206.

Prichard, M.N., Shipman, C. Jr., 1995. Ribonucleotide reductase: An important enzyme in the replication of herpes simplex virus type-1 and a target for antiviral chemotherapy. Chemotherapy 41, 384–395.

Reardon, J.E., Spector, T., 1991. Acyclovir: mechanism of action and potentiation by ribonucleotide reductase inhibitors. In: August, T. (Ed.), Advances in Pharmacology. Academic Press, New York, pp. 1–27.

Roizman, B., Sears, A.E., 1993. Herpes simplex viruses and their replication. In: Roizman, B., Whitley, R.J., Lopez, C. (Eds.), The Human Herpesviruses. Raven Press, New York, pp. 11–68.

Spector, T., 1993. 348U87: an inactivator of herpes virus ribonucleotide reductase that potentiates the antiviral activity of acyclovir. Drugs Future 18, 25–28.

Spector, T., Harrington, J.A., Morrison, R.W. Jr., Lambe, C.U., Nelson, D.J., Averett, D.R., Biron, K., Furman, P.A., 1989. 2-Acetyl-pyridine 5-[(dimethylamino)thiocarbonyl] thiocarbonohydrazone (A1110U), a potent inactivator of ribonucleotide reductases of herpes simplex and varicella zoster viruses and a potentiator of acyclovir. Proc. Natl. Acad. Sci. 86, 1051–1055.

Suhnel, J., 1990. Evaluation of synergism or antagonism for the combined action of antiviral agents. Antivir. Res. 13, 23–40.

Takeuchi, H., Baba, M., Shigeta, S., 1991. An application of tetrazolium (MTT) colorimetric assay for the screening of anti-herpes simplex virus compounds. J. Virol. Methods 33, 61–71.

Trybala, E., Bergstrom, T., Svennerholm, B., Jeansson, S., Glorioso, J.C., Olofsson, S., 1994. Localization of a functional site on herpes simplex virus type-1 glycoprotein C involved in binding to cell surface heparan sulphate. J. Gen. Virol. 75, 743–752.