IJMS
Vol 27, No.4, December 2002

Original Article

Sodium Valproate-Induced Potentiation of Antiherpetic Effect of Acyclovir

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Abstract

Background: Sodium valproate (VPA), an anticonvalsant drug, has been reported to stimulate viral replication. A combination therapy with VPA and acyclovir (ACV) is used for the treatment of herpesvirus encephalitis, the commonest sporadic encephalitis of viral origin.

Objective: To determine a possible interaction between VPA and ACV leading to a modification of antiviral activity of ACV.

Methods: Cultured Hela cells were treated with 5μM of ACV and various concentrations of VPA followed by infection with herpes simplex virus type 1 (HSV-1). Virus replication was monitored by quantal assay. Further investigations comprised electron microscopy, immunoperoxidase and immunoblot procedures. Possible chemical interaction between VPA and ACV was studied by nuclear magnetic resonance (NMR) spectrometer.

Results: Combined treatment of infected cells with ACV and VPA revealed 50- to 250-fold potentiation of antiviral activity of ACV by increasing VPA concentrations. Examination by NMR spectrometer showed a strong chemical interaction between amino groups of ACV and carboxyl part of VPA.

Conclusion: The present in vitro studies should be paralleled by appropriate in vivo investigations, and if substantiated, a combination therapy with ACV and VPA may supersede single ACV therapy for herpesvirus encephalitis. Further studies are thus needed to establish which of VPA metabolites or newly-formed compounds is accountable for augmentation of antiviral effect of ACV.
Iran J Med Sci 2002; 27(4):180-187.

Keywords • Sodium valproate • acyclovir • nuclear magnetic resonance • immunopeoxidase • HSV-1

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Introduction

Combination of antivirals has been advocated for the treatment of viral infection and aims at reducing the emergence of drug resistance, namely triple-drug therapy in human immunodeficiency virus type 1 (HIV-1) infection.[1,2] Among several antiviral compounds with antiherpetic activities, acyclovir (ACV) is highly virus specific and well-tolerated by the host. The antiviral ac-

tivity of ACV becomes manifest following its phosphorylation by the viral and host enzymes.[3] The combined use of ACV with various compounds of similar or diverse biologic activities has been the subject of several investigations.[4-8] In the course of viral infection of the CNS in addition to the use of antiviral agents the chemotherapy by other compounds is undertaken to alleviate associated symptoms namely administration of sodium valproate (VPA), an anticonvalsant drug to prevent seizure disorders following the involvement of the CNS with HIV-1.[9] The stimulation of viral replication by VPA has been reported on HIV-I, human cytomegalovirus, poliovirus type 1 and measles virus.[9-11] The underlying mechanism of this stimulatory effect is not entirely clear but it has been suggested to result from transcriptional activation.[10] The combined therapeutic use of VPA and ACV in particular clinical settings constituted the basis for the present investigation. This study reports VPA induced potentiation of antiviral effect of ACV and a strong chemical interaction between VPA and ACV.

Materials and Methods

Cell culture and reagents

HeLa cells were grown in 96 microwell plates with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 7% Fetal bovine serum (GIBCO), 100 IU/ml of penicillin and 100 µg/ml of streptomycin.

Virus

Herpes simplex type 1 (HSV-1) was isolated from a lip lesion of a patient and identified by specific polyclonal and monoclonal (D&G) anti HSV-1 antibodies.

Sodium valproate (VPA) and Acyclovir (ACV)

VPA was supplied by Sigma U.S, dissolved in growth medium and diluted to appropriate concentrations (0.25, 0.5, 1, 2 and 4 mM). The cell viability was monitored using trypan blue exclusion dye test.[12]

ACV [9-(2-hydroxyethoxymethyl) guanine] (Acyclo-guanosine), powder, Union Quimic Farmaceutica, Barcelona Spain was generously supplied by Dr. Badri from Rooz Darou pharmaceutical Company Tehran, Iran. It was dissolved in 3% dimethyl sulfoxide (DMSO) in distilled water to prepare a stock solution of 12 mM, which was stored at -70 ºC until used.

Monoclonal and polyclonal antibodies

Mouse monoclonal antibodies against glycoproteins D,C,G and ICP35 of HSV-1 were supplied by Autogen Bioclear U.K. and were diluted 1:50 in phosphate buffered saline (PBS: pH=7.2) prior to use. Rabbit anti HSV-1 IgG fraction was supplied by DAKO, Denmark and used in immunoblot assay.

Antibody Conjugates

Peroxidase-labeled anti-mouse and anti-rabbit IgG conjugates were purchased from DAKO. The former was diluted 1:50 and the latter 1:750 for immunoperoxidase staining and immunoblot assay, respectively.

Infectivity assays

Virus titration was carried out in 96 microwell plates (Nunc) under 5% CO_2 and incubated at 36ºC. The infectivity titer was determined by quantal assay according to Reed & Muench.[13]
All the experiments were performed five times in quadruplicates.

Combined ACV and VPA treatment

Following exposure of cells to various doses of VPA for 24 h, the cell monolayers were infected with 100 µl of viral suspension (m.o.i. of $5x10^{-2}$) and after 2 h treated with 5µM of ACV and incubated at 36ºC for 48 h. They were then subjected to a single freeze-thaw cycle. After scraping the cells into PBS (pH 7.2), the contents of each series of wells were pooled and assayed for virus yield.

A modification of the above experiment was performed in which cell monolayers were treated with varying doses of VPA and 5µM of ACV for 24 and 4 hours, respectively. The cell lysate and supernate were then tested for their antiviral effects on cells infected 2 hours previously.

Immunoperoxidase Assay

Monolayers treated with VPA, ACV and a combination of both were infected with HSV-1 as described. At 18 h postinfection the cells were fixed in methanol and assayed by an indirect immunoperoxidase.[10] Viral glycoproteins D, C, G and ICP35 scafold proteins were detected using anti-mouse IgG.

Immunoblot procedure

For each treatment group, antigen was prepared by infection of HeLa cells with HSV-1 at moi of $5x10^{-2}$ and allowed to incubate at 36ºC for 48h for CPE to develop. The monolayers were then washed three times with PBS and the cells were scraped into PBS, pelleted and resuspended in distilled water. The cell suspension was treated with 10% Triton X-100 and 10% SDS to a final concentration of 0.5% and 0.1% respectively. The mixture was incubated on ice for 60 min and clarified by centrifugation at 350 g. for 5 min, aliquoted and stored at -70ºC until used. Prior to use protein concentration of the samples were determined by

the method of Bradford.[14] and adjusted to 4 mg/ml. The samples and a molecular marker (Sigma) were subjected to SDS-PAGE as described elsewhere[15] with 10% polyacrylamide gel.

Immunoblotting was carried out under 12 volts for 90 min as described using semi-dry electrophoretic transfer method.[16] with underlying modifications. Briefly, the proteins were transferred onto nitrocellulose sheet in a buffer containing 20% methanol, 0.19 M glycine and 0.025 M Tris. Following the transfer, the blots were stained with Ponceau S For 10 min, washed in distilled water, soaked for 12 h in TBST (10 mM Tris, 150 mM NaCl & 0.1% Tween 20) containing 3% bovine serum albumin to block nonspecific binding sites and washed 5 times in TBST. They were then submerged in rabbit anti-HSV-1 polyclonal antibody (DAKO) for 2 h at room temperature and washed 5 times with TBST. The nitrocellalose strips thus treated were soaked in peroxidase-labeled anti rabbit IgG (DAKO) for 90 min, washed 5 times in TBST, immersed in 3, 3`-diaminobenzidine chloride (DAB) in the presence of H_2O_2, washed in distilled water and dried.

Nuclear magnetic resonance (NMR)

NMR spectra of a mixture of 4mM VPA and 5µM ACV were recorded on a Brucker DPX-250 MH_z spectrometer using tetramethysilane (TMS) as internal standard with deuterated dimethyl sulfoxide (DMSO-d6) as solvent.

Electron microscopy (EM)

Electron microscopic examination was assigned to the study of infected monolayers treated with 4mM of VPA, 5 µM of ACV, a combination of VPA and ACV and similarly treated but uninfected cells. The monolayers were then fixed in 2.5% glutaraldehyde. Processing for EM was performed according to the method described by Robards and Wilson.[17] The ultrathin sections were stained with uranyl acetate and lead citrate prior to examination in a Philips TEM.10 EM. Approximately 50 cells were examined in each series.

Statistical analysis

The Kruskal-Wallis test[18,19] was applied to the results of infectivity titrations (Table 1) and the Chi-square test[20] for the determination of p values corresponding to enumeration of infected cells reactive in immunoperoxidase assays (Table 2)

Results

Infectivity assays

Table 1 represents the results of infectivity titration. The virus yield in the presence of 5µM of ACV (group 2) was 2.25 orders of magnitude less than virus control (group 1). Whereas 0.125 mM of VPA showed significant reduction of 2 orders of magnitude in virus yield (group 3), it did not increase the antiviral activity of ACV (group 4). However treatment of cells with higher concentrations of VPA alone (group 5, 7 & 9) did not significantly affect viral replication. Significant enhancement of antiviral activity of ACV by 1.75 to 2.4 orders of magnitude (50 to 250-fold) was demonstrated with higher doses of VPA. This potentiation of antiviral effect was in direct relationship with VPA concentrations when used in combination with ACV (group 8, 10 and 12). The antiviral activity of ACV was unaffected by simultaneous exposure of cells to both

Table 1: The effect of different concentrations of sodium VPA alone and in combination with 5 µM of ACV on HSV-1 replication in HeLa cells [a].

No.	Group	VPA (mM)	ACV (µM)	Virus yield [b] (Log_{10} $TCID_{50}$) (Mean±SEM)	p value [c]
1	Control 1	–	–	5.95±0.095	–
2	Control 2	–	5	3.70±0.24	0.008 [d]
3	VPA	0.125	–	4.00±0.33	0.009
4	Combined	0.125	5	3.65±0.23	0.831
5	VPA	0.5	–	5.05±0.26	0.014
6	Combined	0.5	5	2.90±0.13	0.033
7	VPA	1	–	5.45±0.20	0.068
8	Combined	1	5	1.95±0.094	0.008
9	VPA	2	–	6.70±0.31	0.110
10	Combined	2	5	1.60±0.061	0.008
11	VPA	4	–	6.65±0.19	0.025
12	Combined	4	5	1.30±0.15	0.009

[a] All experiments were performed 5 times in quadruplicates.
[b] The infectivity titers in cultured cells treated with various doses of VPA and a combination of VPA and ACV were compared with controls 1 and 2 respectively.
[c] Kruskal-Wallis test was used for determination of p values.
[d] p value corresponding to a comparison between controls 1 and 2.

Table 2: Immunoperoxidase staining of HSV-1 infected HeLa cells treated with ACV and a combination of ACV and VPA using four HSV-1 monoclonal antibodies.

			Average number of antigen positive cells/well			
Group	**VPA (mM)**	**ACV (µM)**	**gG**	**gD**	**gC**	**ICP_{35}**
Control 1	-	-	37	32	26	29
Control 2	-	5	21	19	20	21
Combined [a]	0.125	〃	19	18	18	20
〃	0.5	〃	16	14	16	19
〃	1	〃	12	10	11	16
〃	2	〃	[b] 8	[b] 7	10.5	12
〃	4	〃	[b] 4	[b] 4	[b] 5	[b] 8

[a] Combined treatments were compared with control 2.
[b] Statistically significant values ($p<0.05$).

VPA and ACV. An experiment was designed to examine cell lysate and supernate for their antiviral effect. In comparison with control (infected cells treated with ACV), cell lysate and supernate of monolayers treated with 2 & 4 mM of VPA plus ACV exhibited elevated antiviral activity (data not shown) but were similar with respect to one another.

Nuclear magnetic resonance (NMR)

The ^1H NMR spectrum of ACV in DMSO-d6 (Fig. 2) was compared with that for a mixture of ACV and VPA (Fig. 3). This comparison showed a strong and reproducible interaction between the carboxylate part of VPA and both –NH and $–NH_2$ groups of ACV in the mixture. This interaction not only changes the shape of signals of both –NH groups of ACV from sharp to very broad, but also changes the proton chemical shifts of both groups dramatically. The –NH group in ACV shifts from 10.66 ppm to nearly 7.7 ppm. The $–NH_2$ group of ACV also shifts from 6.5 ppm to 3.76 ppm. This remarkable change in the chemical shifts of both -NH groups could well account for the proposed interactions. The effect of this interaction on the hydrogen atoms attached to the carbon atoms is very little as expected. For example, the CH chemical shifts change from 7.81 to 7.74. Similarly, the protons of the methylene in $N–CH_2–O$ group shift from 5.35 ppm to 5.34 ppm.

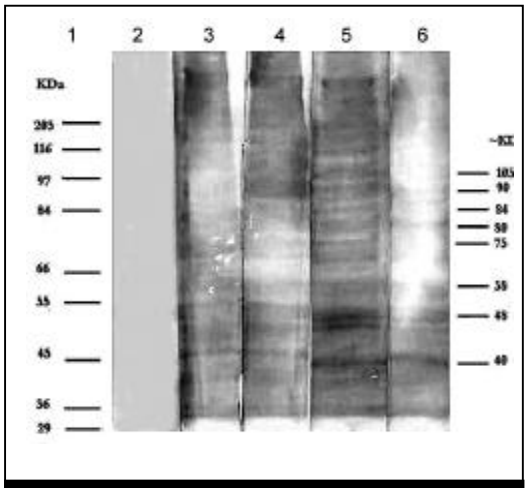

Fig 1: The immunoblot profle of antigens of HSV-1 infected HeLa cell monolayers treated with 4 mM of VPA and 5 µM of ACV; lane 1 molecular marker, lane 2 normal uninfected cells, lane 3 ACV-treated infected cells, lane 4 VPA-treated infected monolayer, lane 5 untreated infected cells and lane 6 virus infected cells treated with a combination of VPA and ACV. Note a more extensive deletion of hands in lane 6 compared with lane 3

Electron microscopy (EM)

Approximately 50 cells in each group were examined by EM. Compared with ACV-treated infected cells which exhibited few virions, those receiving a combination of ACV and VPA did not contain any or harboured rare virus particles. A fortuitous observation was distortion of the mitochondria occurring in cells one hour after treament with VPA. However these organelles appeared normal in monolayers treated with VPA for 24 hr.

Immunoperoxidase assay

The monoclonal antibodies against glycoproteins G,D,C and ICP35 of HSV-1 were studied on cells treated with various concentrations of VPA and 5 µM of ACV. The results displayed in Table 2 represent the average cell counts of two independent experiments. As demonstrated the statistically significant values for gG and gD positive cells were found with 2 and 4 mM of VPA in combined treatments. As for gC and ICP35 the corresponding values involved only 4 mM of VPA.

Immunoblotting

The immunoblot preparation of samples derived from cells infected with virus alone (lane 5) exhibited at least 12 bands ranging in molecular weights from ~42 to 105 kDa (Fig. 1). However a diffuse band appeared in the upper region from 116 to

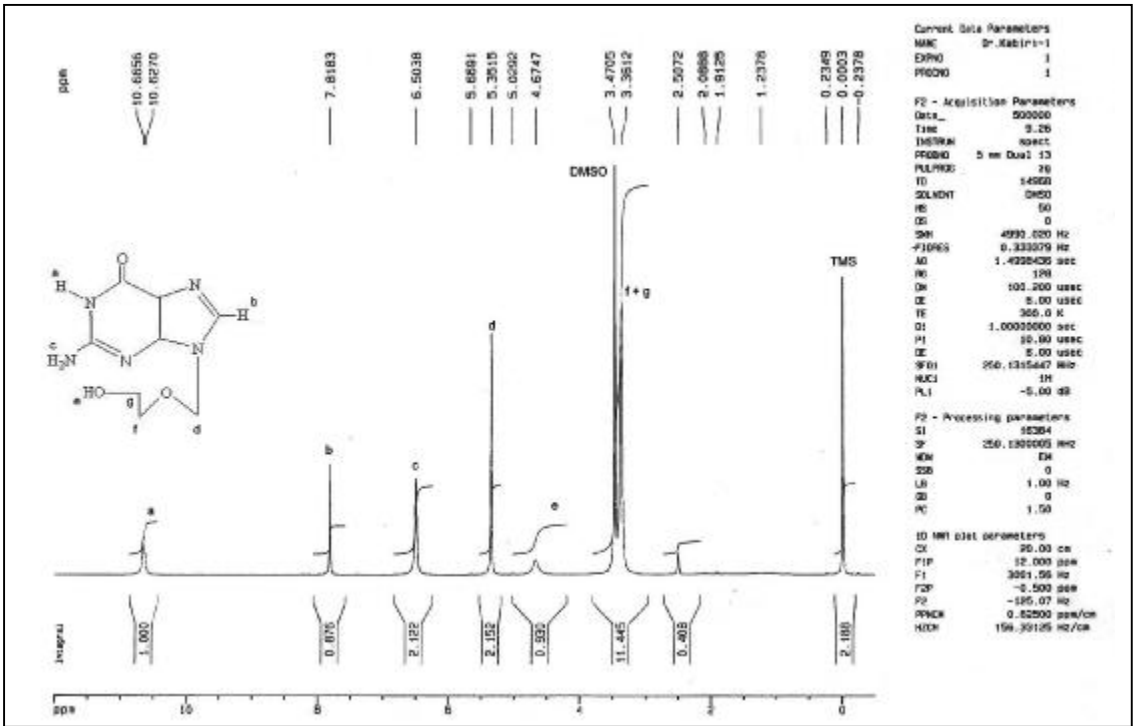

Fig 2: ^{1}H-NMR spectrum of ACV in DMSO-D6 using tetramethylsilane as internal standard.

over 205 kDa. Samples from infected cells treated either with ACV (lane 3) or a combination of ACV and VPA (lane 6) showed deletion of polypeptide bands. The latter was more prominent and extensive, comprising ~52 to over 205 kDa, and the former less pronounced and restricted within ~80 to 116 kDa. Partial clearance of bands between ~60 and ~80 kDa and a cluster of unresolved polypeptides from ~87 to over 205 kDa characterized infected cells treated with VPA alone (lane 4). The diminution of viral polypeptides between ~60 kDa and ~80 kDa was more pronounced in samples from infected cells treated with a combination of ACV and VPA (lane 6) than those exposed to VPA alone (lane 4). Samples of uninfected cells treated with ACV, VPA or a combination of both appeared similar to normal untreated cells (not shown).

Discussion

The mechanism by which ACV exerts its antiviral effect is via the phosphorylation by virus-specific TK. TK bearing viruses within the family herpesviridae include herpes simplex and varicella-zoster viruses that belong to the subfamily alphaherpesvirinae.[3] A potentiation of 1.75 to 2.4 orders of magnitude (50- to 250-fold) in antiviral activity of ACV was achieved when VPA was added to cell cultures in 1 to 4 mM 24 hours prior to infection with HSV-1 (Table 1). This inhibition of viral replication was in direct relationship with VPA concentration. The antiviral activity of ACV was unaffected by concurrent exposure of cells to both VPA and ACV. These observations imply that either intracellular metabolites of VPA, and not the intact VPA molecule, or a new compound arising from interaction of VPA with ACV may be responsible for amplification of antiviral effect.

In another experiment cell cultures were treated with VPA for 24 hours followed by 4 hours of exposure to ACV. The supernate and cell lysate were then tested for their antiviral activities on monolayers already infected with HSV-1 for 2 hours. The cell lysate and supernate corresponding to 2 and 4 mM of VPA in combination with ACV showed similar but higher antiviral activites than those exhibited by ACV-treated monolayers (data not shown). This suggests that conditions (putative VPA metabolites or newly-formed compound) favouring stimulation of antiviral effect were present in both internal and external cellular environments which were devoid of ACV triphosphate.

The mechanism for such a dramatic increase in antiviral activity 24 hours after exposure of cells to VPA is unclear but transcriptional activation by VPA metabolites reported by some investigators

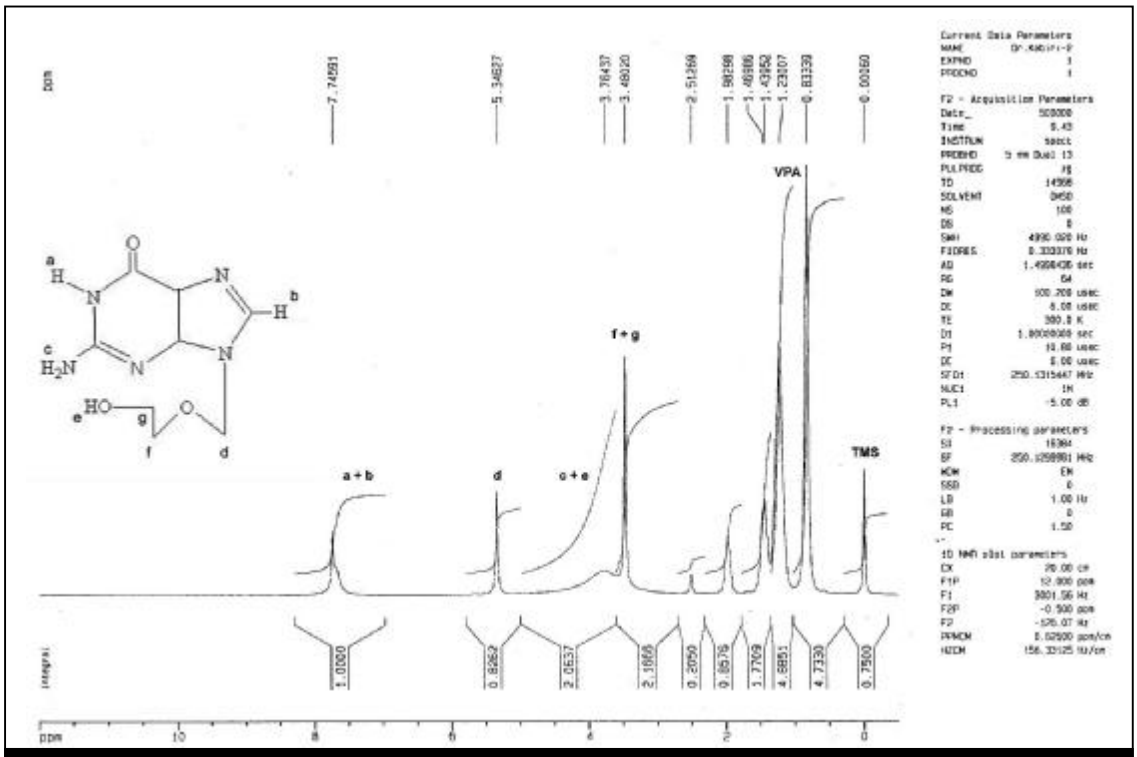

Fig 3: ^{1}H-NMR spectrum of the mixture of ACV and VPA in DMSO-D 6 using tetramethylsilane as internal standard.

provides a clue to augmentation of antiviral activity of ACV. It has been suggested that the stimulatory effect of VPA on the replication of HIV-1 and HCMV could operate through amplification of viral transcription.[9,10] An explanation based on a similar assumption is that the potentiation of ACV antiviral effect may result from an acceleration of viral specific TK transcription by VPA metabolites. This would increase the level of ACV triphosphate and hence its antiviral activity. Also account must be taken of the possibility of chemical interaction between VPA and ACV leading to the synthesis of a novel compound. The results obtained from our studies by nuclear magnetic resonance (Figs. 2 and 3) were in favour of such hypothesis. This study revealed a strong interaction (H-bonding) between carboxylate part of VPA and amino groups of ACV which implicates the emergence of a new compound. However, under our experimental conditions the influence of intracellular or extracellular factors namely pH may lead to dissociation of Na^{+} from valproate. The resulting valproic acid may then interact with amino group of ACV giving rise to a definitive novel compound with stronger antiviral effect than ACV. In this context the interesting report by Parmeggiani et al, on possible interaction between ACV and VPA is worthy of mention. In a child receiving a 6-day child receiving a 6-day ACV, phenytoin (PHT) and VPA they found a reduction of PHT and VPA plasma concentrations to subthrapeutic values.[21] Likewise, the potentiation of antiviral activity of ACV might be due to inhibition of viral DNA polymerase by either VPA metabolites or the newly formed compound.

The immunoblot procedure was performed to study antigenic profile of viral replication affected by ACV and a combinaion of ACV and VPA. As displayed in Fig. 1 at least 12 bands ranging in molecular weight from ~42 to over 205 kDa were demonstrated in preparation of cells infected with virus alone (lane 5). However polypeptide bands between ~90 to over 205 kDa were not discernible as separate bands in samples derived from VPA-treated infected cells. This might be due to the accumulation of polypeptides with close molecular weights. In marked contrast to ACV-treated monolayers (lane 3) samples derived from infected cells and treated with a combination of ACV and VPA exhibited extensive deletion of polypeptide bands from ~52 to over 205 kDa (lane 6). Because of unabated viral replication in cells treated with 4mM of VPA (Table 1) the low intensity and partial visibility of bands between ~60 and ~80 kDa (lane 4) may probably represent products of nonessential

genes namely UL 46, UL47, US8 and RL2. This hypothesis is consistant with the fact that nearly half of 75 genes of HSV-1 are not essential for viral replication in cultured cells.[22,23] These dispensable gene products might have regulatory functions or be involved in countering host defence mechanisms and their possible elimination or repressed synthesis expedite recovery from viral infection. In this context further experiments are needed to investigate the in vivo effect of combined ACV and VPA treatment on viral replication. The number of viral proteins detected by immunoblot method was fewer than 75 viral polypeptides synthesized by cells infected with HSV-1.[24] In this connection SDS-treated adenoassociated virus polypeptides revealed new antigens. Antisera against this preparation did not react with whole virions. However these antigens were present in cells prior to folding of polypeptides into virion subunits.[25]

Virion-associated ultrastructural features examined by electron microscopy were similar in infected cells treated with ACV and those receiving ACV and VPA. The results, with respect to the number of intracellular virions, were in agreement with those of infectivity, immunoblot and immunoperoxidase assays. Incidentally exposure of cells to VPA for one hour showed distortion of mitochondria. The effect was reversed 24 hours after VPA treatment. Similar studies are therefore suggested on monolayers one hour after VPA treatment.

Acknowledgements

This work was supported by research grant No. 79-950 of Shiraz University of Medical Sciences. The authors gratefully thank Dr. S.M. Sadjadi, Dr. E.Kamali, Mr. M. Karamian and Mrs. P. Habibi and Mr. S.H.R Tabatabai for their valuable cooperations.

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