EFFECT OF METAL COMPLEXES OF ACYCLOVIR AND ITS ACETYLATED DERIVATIVE ON HERPES SIMPLEX VIRUS 1 AND HERPES SIMPLEX VIRUS 2 REPLICATION

T. VARADINOVA[1*], N. VILHELMOVA[1], F. BADENAS[2], A. TERRÓN[2], J. FIOL[2], A. GARCIA-RASO[2], P. GENOVA[1]

[1]Laboratory of Virology, Faculty of Biology, Sofia University, 8 Dragan Tzankov Blvd, 1164 Sofia, Bulgaria; [2]Department of Chemistry, Balear University, Palma de Mallorca, Spain

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Summary. – The effect of zinc, nickel, cobalt and cadmium complexes of acyclovir (ACV) and its *O*-acetylated derivative (Ac-ACV) on the replication of wild type (*wt*) and ACV-resistant (ACV^R) strains of Herpes simplex virus 1 (HSV-1) and Herpes simplex virus 2 (HSV-2) was examined. According to cytotoxicity, these compounds followed the order Ni-ACV chloride > Cd-ACV 3 Ni-ACV nitrate > ACV = Zn-ACV nitrate = Ac-ACV = Zn-Ac-ACV > Zn-ACV chloride> Co-ACV. Besides Ac-ACV, the only active complexes in inhibiting virus replication were Zn-ACV nitrate and Zn-Ac-ACV, which effectively suppressed the growth of both *wt* and ACV^R strains of HSV-1 and HSV-2. The most active and most selective inhibitor of the growth of ACV^R strains of HSV-1 and HSV-2 was Ac-ACV; its EC_{50} and SI were 100 and 10 times higher than those of ACV, respectively. Zn-Ac-ACV was less active than Ac-ACV, obviously due to the stability of the complex. Zn-ACV nitrate was active against both *wt* and ACV^R strains of HSV-1; its activity and selectivity were100 and 75 times higher than those of ACV, respectively. Ac-ACV and Zn-Ac-ACV suppressed the pre-mitotic arrest caused by HSV-1 infection during the first 2 hrs of infection and later on restored the cell division.

Key words: Herpes simplex virus 1; Herpes simplex virus 2; acyclovir; acetylated acyclovir; metal complexes; antiviral activity

Introduction

It is known that HSV-1 and HSV-2 cause a broad spectrum of diseases and belong to major causes of morbidity and mortality in patients with transplanted organs, AIDS and cancer (Corey, 1986, 2002; Schacker *et al.,* 1998). Although a series of clinical problems are developing in HIV-1 and/or HIV-2 (HIV-1/2) infected patients suffering for longer periods of time, in particular the co-morbidity for HIV-1/2 with other sexually transmitted diseases, most often HSV-2 infections (Ahmed *et al*., 2003; Heng *et al*., 1994; Mertz *et al*., 1992; Russell *et al*., 2001; Severson and Tyring, 1999). Moreover, HSV-1 and/or HSV-2 (HSV-1/2) increase HIV-1/2 replication as much as 3.4-fold, while genital herpes is a risk factor for HIV-1/2 infection and may facilitate the transmission of AIDS (Heng *et al*., 1994; Mbopi-Keou *et al*., 2000; McClelland *et al*., 2002; Serwadda *et al*., 2003; Wright *et al*., 2003). Genital herpes is the most frequent sexually transmitted disease among HIV-1/2-positive persons (O'Farrell *et al*., 1994). However, epidemiological surveys have revealed a rise in the prevalence of herpes infections during recent years in HIV-1/2-negative individuals as well (Corey 2002; Hook *et al*., 1992; Mole *et al*., 1997). A significant role in transmission HSV-1/2 in 70% of patients plays asymptomatic virus shedding (Breinig *et al*.,

*E-mail: tvaradinova@biofac.uni-sofia.bg; fax: +3592-86566 41.

Abbreviations: ACV = acyclovir; ACV^R = ACV-resistant; BS = bovine serum; CC_{50} = concentration inhibiting cell viability by 50%; CPE = cytopathic effect; DMEM = Dulbecco's Minimum Essential Medium; EC_{50} = concentration inhibiting virus yield by 50%; HIV-1 = Human immunodeficiency virus 1; HIV-2 = Human immunodeficiency virus 2; HIV-1/2 = HIV-1 and/or HIV-2; HSV-1 = Herpes simplex virus; HSV-2 = Herpes simplex virus 2; HSV-1/2 = HSV-1 and/or HSV-2; MI = mitotic index; MNC = maximal non-cytotoxic concentration; MOI = multiplicity of infection; p.i. = post infection; TK = thymidine kinase; *wt* = wild type

1990; Langenberg *et al*., 1989; Mertz *et al.*, 1992). HIV-1/2-positive individuals have been shown to benefit from an improved survival rate if treated with nucleoside-based drug ACV and its oral prodrug forms valacyclovir or pencyclovir. Thus, ACV is still the standard drug in the control of HSV-1 infections and is approved as the drug of choice for the treatment of primary and recurrent HSV-1/2 disease (Blair *et al*., 1997; Elion, 1989). As a prodrug, ACV must be phosphorylated by viral thymidine kinase (TK) and subsequently by cellular kinases to inhibit competitively viral DNA polymerase and terminate the elongation of viral DNA chain.

Strategies for preventing further HSV-1/2 transmission include approaches to protect the susceptible host through use of pre-emptive antivirals such as ACV. However, upon systematic administration and inappropriate under-dosing, resistant mutants appear with relatively high frequency and their main source is an immunocompromised individual (Blair *et al.*, 1997; Field, 2001; Bacon *et al*., 2003). The most common causes of resistance are mutations in thymidine kinase (TK*)* gene (Bacon *et al*., 2003; Blair *et al.*, 1997; Villareal, 2001); 95–96% of ACV-resistant (ACV^R) HSV-1/2 isolates are TK-deficient (TK^N) or TK-partial (TK^P), the remaining isolates being usually TK-altered (TK^A), unable to phosphorylate the pro-drug but not the thymidine (Bacon *et al*., 2003; Pottage and Kessler, 1995; Villareal, 2001). Thus, as the suitability and selectivity of the drug diminish, higher doses have to be administered and adverse effects are more likely to appear.

Metals play an essential role in the regulation of many processes in living systems including resistance to stress and infections. On the other hand, viruses, HSV-1/2 in particular, disturb the homeostasis of essential metals. We have found earlier that 1 hr after HSV-1 infection the host cell expels its zinc (Varadinova *et al*., 1993). For this reason, by disturbing the homeostasis of essential metals, HSV-1 infection destroys the host resistance to other pathogens, viruses including. The failure of treatments could be solved by using metal-based compounds. Related to this idea is the well known anti-HSV-1/2 activity of zinc salts (Gupta and Ropp*,* 1976; Fridlender *et al.,* 1978; Kumel *et al*., 1990). Complexes of zinc with picollinic and aspartic acids inhibit key steps of HSV-1 replication and restore physiological levels of Zn^{2+} ions in HSV-1 infected cells (Varadinova *et al*., 1990, 1993). Moreover, these Zn^{2+} complexes show a synergistic activity against *wt* and ACV^R HSV-1 strains if combined with the pavine alkaloid (-)-thalimonine (Angelova, 2000).

However, it is well known that metals can increase the biological activity of organic drugs. For these reasons, a number of Cu^{2+}, Pt^{2+}, Co^{2+}, Ni^{2+} , Zn^{2+} and Cd^{2+} complexes of ACV have been synthesized and chemically characterized (Cavallo *et al*., 1991; Cini *et al*., 2000; Garcia-Rasso *et al*., 1999; Grabner *et al*., 1998; Herrero *et al*., 2001; Turel *et al*., 1998, Barceló-Oliver *et al.*, 2004). In all the cases the metal ion is bound to ACV through N(7) of the purine ring. Some of them, like complexes of Pt^{2+} and of Cu^{2+}, were substantially more potent against HSV-1/2 replication than ACV alone (Coluccia *et al.*, 1995; Panteva *et al*., 1998). Moreover, Cu^{2+}-ACV nitrate (Turel *et al*., 1998) was also effective against ACV^R HSV-1 mutants (Panteva *et al*., 1998).

In this study, a comparative evaluation of the inhibitory activity of Ni^{2+}, Cd^{2+}, Co^{2+} and Zn^{2+} complexes of ACV (Garcia-Rasso *et al*., 1999; Herrero *et al*., 2001) and Ac-ACV on the replication of *wt* and ACV^R strains of HSV-1 and HSV-2 was performed. Also the effects of the metal complexes on cell division during *wt* HSV-1 infection were investigated.

Materials and Methods

Preparation of Ac-ACV and metal complexes of ACV. Metal complexes of ACV (Table 1) were prepared according to Garcia-Raso *et al*. (1999). Ac-ACV (Fig. 1) was prepared as follows: 2.5 g of ACV was mixed with 13 ml of acetic anhydride and 15 ml of pyridine and the mixture was stirred for 24 hrs at room temperature. The precipitate was collected by paper filtration, washed with cold water and acetone and dried. The yield of Ac-ACV was 75–80%. Ac-ACV was thermically stable. Zn-(Ac-ACV) was prepared similarly to ACV (Garcia-Rasso *et al*., 1999).

Cells and viruses. MDBK cells were grown at 37°C in the Dulbecco's Minimum Essential Medium (DMEM, Sigma) supplemented with 10% of bovine serum (BS) and antibiotics (10% BS medium), while 5% BS medium was employed for maintaining the cells in experiments. *wt* HSV-1 Victoria strain, *wt* HSV-2 Bja strain, ACV^R HSV-1 R-100 mutant in TK gene (TK^A) and ACV^R HSV-2 PU mutant in *tk* gene (TK^N) were employed. To prepare virus stocks the viruses were grown in MDBK cells. Cultures were harvested at full cytopathic effect (CPE), freeze thawed and stored at -70°C.

Methods of determining cell growth kinetics, cell viability, MNC and CC_{50}. Stock solutions (100 μmol/l) of the tested compounds in DMSO were used for preparation of serial tenfold dilutions (100–0.00001 μmol/l) in the 5% BS medium. The cells were seeded into 96-well tissue culture plates at a concentration of 10^4 cells/ml/well and cultured at 37°C in 5% CO_2. Confluent monolayers were washed, covered with tenfold dilutions of the compounds in the 5% BS medium and cultured at 37°C for 48 hrs (for *wt* strains) or 72 hrs (for ACV^R mutants). The cells grown in a compound-free medium served as controls. CPE was read by microscopy of unstained as well as Trypan Blue-stained cells. Each experiment was done in duplicate. Based on the data from cell survival assay, dose-response curves were constructed by linearly regressing drug concentrations against the percent inhibition of cell viability values for the cell control. The MNC and CC_{50} values for each compound were calculated from the dose-response curves. The maximum concentration that altered neither the morphology of cells nor the cell survival rate was taken for MNC.

Assay of antiviral activity of the tested compounds was done on the basis of their effects on the infectious virus titer. Cell mo-

nolayers in 96-well tissue culture plates were washed and covered with tenfold dilutions of particular virus. After 1hr of virus adsorption, the inoculum was removed and the cells were further incubated with tenfold dilutions (starting with MNC) of the tested compounds in the 5% BS medium at 37°C for 48 hrs (for *wt* strains) or 72 hrs (for ACV^R mutants). CPE and virus titers (log $TCID_{50}$/0.1ml) were determined for each well. Dose-response curves were constructed by linearly regressing compound concentrations against the percent inhibition of virus yield values.

Effective compound concentrations required to inhibit virus yields by 50% (EC_{50}) were calculated from the regression lines. In order to be able to compare the compounds on the basis of their selective inhibition of virus replication *vs.* cytotoxicity, selective indices (SI) were calculated as CC_{50}/EC_{50} ratios.

Cell division assay. Cell monolayers on cover slips were washed and infected with *wt* HSV-1 Victoria strain at a multiplicity of infection (MOI) of 1 $TCID_{50}$ per cell. After 1 hr (time 0) of virus adsorption the cells were covered with the 5% BS medium containing the tested compounds in MNC. After 2, 4, 6 and 8 hrs the cells were fixed with a formaldehyde solution (10 ml formaldehyde, 70 ml acetate buffer pH 4.0 and 0.64 g NaCl) and stained with hematoxylin-eosin. The number of cells in different phases of division including abnormal mitotic forms per 1000 cells was counted and expressed in promiles (‰). Mitotic index (MI) represented total number of dividing cells per interval. The following controls were used: (i) uninfected cells kept in a compound-free medium (cell control), (ii) HSV-1 infected cells kept in a compound-free medium (viral control) and (iii) uninfected cells kept in a medium containing particular compound. Each experiment was performed in duplicate. In order to be able to compare data from various time intervals the growth media from uninfected cells were changed for compound-free media (cell control) or media containing particular compound at time 0.

Results and Discussion

Cytotoxicity of the compounds

A dose-dependent reduction of cell surveillance was found with the tested compounds (data not shown). The results of the experiments on the cytotoxicity of the compounds are shown in Table 1. It is evident that the cytotoxicity of ACV, Ac-ACV and their metal complexes was time-independent and the compounds were stabile for the time observed. Based on MNC data the cytotoxicity decreased in the following order: Ni-ACV chloride > Cd-ACV 3 Ni-ACV nitrate > ACV = Zn-ACV nitrate = Ac-ACV = Zn-Ac-ACV > Zn-ACV chloride> Co-ACV.

It is evident that the Ni and Cd complexes were most cytotoxic; they were up to 50 times more cytotoxic than ACV. Their high cytotoxicity was apparently due to significant genotoxicity of Ni^{2+} and Cd^{2+} ions and induction of apoptosis/necrosis (Hong *et al*., 1997; Ishido *et al*., 2002; Lynn *et al*., 1997; O'Halloran, 1993; Zhou *et al*., 1999). Moreover, by intercalating into DNA, Cd^{2+} ions cause irreversible chain breakage (Misra *et al*., 1998), while Ni^{2+} ions induce generation of highly cytotoxic reactive oxygen intermediates in the cytoplasm (Hong *et al.*, 1997; Lynn *et al*., 1997).

A less cytotoxic group was represented by Zn-ACV chloride and Co-ACV, as their MNCs were up to 20 times higher than that of ACV. On the other hand, the cytotoxicity of Ac-ACV, Zn-Ac-ACV and Zn-ACV nitrate was similar to that of ACV. These data show that the acetylation had no effect on the cytotoxicity of ACV, while Zn^{2+}, Co^{2+} and chloride ions reduced it. This could be explained by essential role of Zn^{2+} and Co^{2+} ions in various biological processes. In this regard, cobalt is essential for vitamin B12 (Howard-Lock, 1987). Zinc also participates in a huge variety of processes and structures (expression and transcription of the genome, innate and immune defense, stabilization of nuclear receptors and plasma membrane etc. (Coleman *et al.,* 1992; Cunningham *et al*., 1990; Dattani *et al*., 1993; Nekludova and Pablo, 1994; Schmiedeskamp and Klevit, 1994; Suzuki and Yagi, 1994)).

Taken together, the results presented above are in accord with the well-known fact that the bioactivity of particular complex is predetermined by specific activities of both metal and ligand component of the complex.

Antiviral activity of the compounds

To evaluate the antiviral activity of the compounds we analyzed their influence on multi-step growth of HSV-1 and HSV-2. As these experiments were performed with the aim to overcome ACV resistance, *wt* as well as mutants resistant to ACV (ACV^R) with different *tk* gene mutations were employed. EC_{50} was indicative for the antiviral activity, while SI for the selectivity. The results showed (Table 1) that ACV in concentrations 0.0002–0.02 μmol/l effectively inhibited the replication of *wt* HSV-1 strain Victoria , while w*t* HSV-2 strain BJA was 10 times less sensitive to ACV and the selectivity of ACV for the BJA strain was 12.5 times lower than that for *wt* Victoria strain (Fig. 1). The fact that the *wt* HSV-2 strain BJA was a low responder to ACV was apparently due to low activity of the virus-specific TK (data not shown). ACV was also neither an effective (Table 1) nor a selective (Fig. 2) inhibitor of the replication of TK mutants R-100 and PU. The resistance of these virus mutants to ACV was confirmed by EC_{50} values equal to MNC and SI values of 1×10^3 that were below the critical value of 3×10^3.

Of the remaining compounds tested three were found to suppress virus replication in a virus-specific manner: Zn-ACV nitrate, Ac-ACV and Zn-Ac-ACV (Table 1, in bold), Zn-ACV nitrate, being as cytotoxic as ACV, was a 10 and 100 times more effective inhibitor of the growth of *wt* HSV-1 strain Victoria and HSV-1 ACV^R R-100 mutant, respectively, than ACV. Moreover, the selectivity of Zn-ACV nitrate for

Table 1. Cytotoxicity (CC_{50} and MNC) and antiviral activity (EC_{50}) of ACV, Ac-ACV and their metal complexes

Compound formula	Compound -abbreviated name	48 hrs p.i.				72 hrs p.i.			
		CC_{50}	MNC	EC_{50}		CC_{50}	MNC	EC_{50}	
				Victoria	Bja			R-100	Pu
ACV	ACV	25	0.02	0.0002	0.002	20	0.02	0.02	0.02
$[Zn(ACV)Cl_2].H_2O$	**Zn-ACV chloride**	**30**	**0.1**	**0.005**	**0.01**	**28**	**0.2**	**0.01**	**0.1**
$[Zn(ACV)(NO_3)_2].4H_2O$	Zn-ACV nitrate	12	0.02	0.00002	0.002	15	0.02	0.0002	0.002
$[Ni(ACV)_2(H_2O)_4]Cl_2.2ACV$	Ni-ACV chloride	7	0.0004	0.0001	0.0004	12	0.0001	0.0001	0.0001
$[Ni(ACV)(NO_3)_2].5H_2O$	Ni-ACV nitrate	17	0.007	0.001	0.001	18	0.001	0.001	0.001
$[Cd(ACV)Cl_2].H_2O$	Cd-ACV	6	0.002	0.0002	0.002	9	0.002	0.002	0.002
$[Co(ACV)_2(H_2O)_4]Cl_2.2ACV$	Co-ACV	44	0.2	0.01	0.1	46	0.2	0.1	0.1
Ac-ACV	**Ac-ACV**	**13**	**0.02**	**0.0002**	**0.0002**	**20**	**0.02**	**0.0002**	**0.0002**
$[Zn(Ac\text{-}ACV)_2Cl_2(H_2O)]$	**Zn-Ac-ACV**	**17**	**0.02**	**0.0001**	**0.001**	**10**	**0.02**	**0.001**	**0.001**

All the values are in μmol/l.

the R-100 mutant (TK^A) was 75 times higher as compared to ACV. However, the inhibitory activity of Zn-ACV nitrate against both *wt* and ACV^R HSV-2 strains was similar to that of ACV. These data show that the antiviral activity of Zn-ACV nitrate was virus-specific. Ac-ACV effectively inhibited the replication of both *wt* and ACV^R HSV-2 but not that of *wt* HSV-1. Thus Ac-ACV was an up to 10 times more active (Table 1) and selective (Fig. 1) inhibitor of *wt* HSV-2 than ACV. Furthermore, Ac-ACV overcame the ACV resistance by as much as 100 times and, more importantly, the efficacy (Table 1) and the selectivity (Fig. 2) of Ac-ACV were independent on the nature of ACV resistance of the virus mutants R-100 and PU, i.e. whether they were TK^N (PU) or TK^A (R-100). On the other hand, the antiviral activity and selectivity of Zn Ac-ACV against the ACV^R virus mutants were 5 and 10 times lower, respectively, than those of Ac-ACV (Table 1 and Fig. 2), but it was up to 20 times higher than those of ACV. Of all the compounds tested Zn-ACV chloride, Ni-ACV chloride, Ni-ACV nitrate, Cd-ACV and Co-ACV were less active than ACV. Thus the antiviral activity of Zn-ACV chloride against *wt* and ACV^R HSV-1 and HSV-2 strains was up to 4 times lower than that of ACV. Ni-ACV chloride, Ni-ACV nitrate, Cd-ACV and Co-ACV suppressed the replication of both HSV-1 and HSV-2 in concentrations equal to MNC.

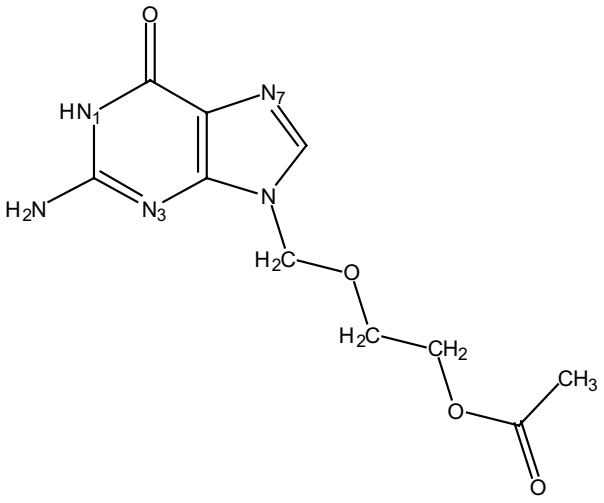


Fig. 1

Structure of Ac-ACV

Structure-activity relationships

Besides being virus/strain specific, the antiviral activity of metal complexes of ACV was also influenced by their structure. The structure-activity relationship was manifested by the fact that out of six complexes tested five, namely Zn-ACV chloride, Ni-ACV chloride, Ni-ACV nitrate, Cd-ACVand Co-ACV were not active against *wt* as well as ACV^R HSV-1 and HSV-2. Based on X-ray diffraction, ^{1}H-NMR, ^{13}C-NMR and calorimetric studies Zn-ACV chloride, Ni-ACV chloride, Cd-ACV and Co-ACV have the metal bound to the N(7) of the guanine ring of ACV (Garcia-Raso *et al.*, 1999). Ni-ACV chloride, Cd-ACV and Co-ACV have a distorted octahedral geometry, but the first two have a monomeric, while the third has a polymeric structure in solid state. Formation of a complex between ACV and Ni^{2+} or Co^{2+} ions is accompanied by establishment of hydrogen bonds stabilizing the monomer and/or the crystal structure. Both Ni complexes of ACV differing in the anion (chloride vs. nitrate) were not active viral inhibitors. On the other hand, of the two Zn complexes of ACV also differing in the anion only (chloride vs. nitrate), the nitrate but not chloride containing complex was an efficient inhibitor of virus growth. It is possible that the lack of antiviral activity in the six of nine compounds tested was due to their specific structure preventing dissociation of ACV from the complex.

The significant antiviral activity of Ac-ACV (Table 1 and Fig. 2) and its ability to overcome the ACV resistance were obviously due to the acetylation of the main structure. It seems that Ac-ACV utilizes a different target/route of action

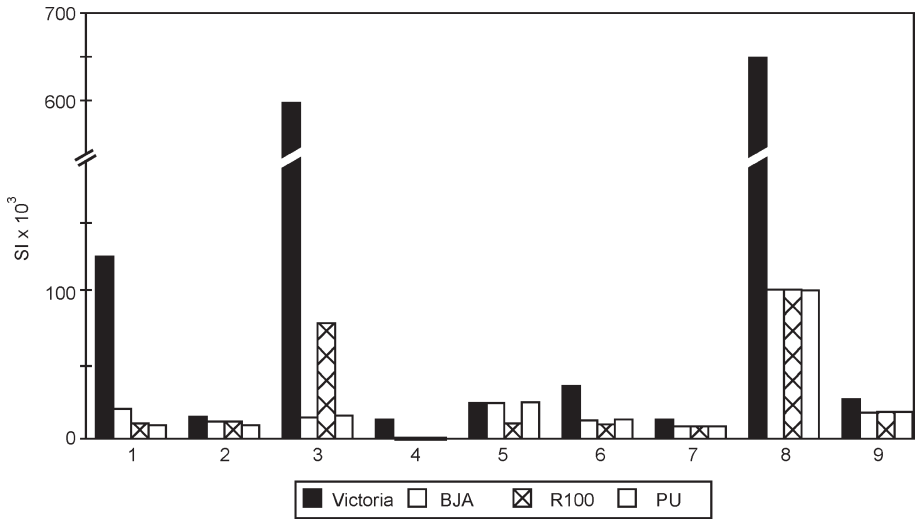


Fig. 2

Selective indices (SIs) of ACV, Ac-ACV and their metal complexes

compared to ACV and bypasses in this way the route of action of ACV. This idea is supported by the fact that Zn-Ac-ACV but not Zn-ACV chloride was active in antiviral action. On the contrary, Zn-Ac-ACV was less active than Ac-ACV (Table 1 and Fig. 2). This suggests that the decreased activity of Zn-Ac-ACV could be due to its specific structural properties. Experiments directed to the elucidation of the mode of action of Ac-ACV are in progress.

All the facts mentioned above indicate that the lack of antiviral activity of Ni, Cd and Co complexes of ACV and the decreased antiviral activity of Zn complexes of ACV and Ac-ACV as compared to ACV and Ac-ACV, respectively, was obviously due to stability of the complexes.

Effect of the compounds on cell division

The next step in our investigations was directed to the effect of the compounds on the division of uninfected and HSV-1-infected cells, because, to date, there were no such reports concerning ACV or its derivatives. A special attention was paid to abnormal mitoses accompanying the treatment with the compounds and the infection with *wt* HSV-1 strain Victoria.

In uninfected cells treated with the compounds, a typical dynamics of MI (Fig. 3A1) and an increasing number of dividing cells until the 6th hr were observed (Table 2). The latter phenomenon was apparently caused by changing culture medium. No more than 2‰ of abnormal metaphases (metaphases with lacking chromosomes) were detected at the 6th hr. Because ACV remains in uninfected cells in the form of biologically inactive precursor (prodrug), no changes in MI and in the number of cells in different mitotic phases were found with ACV. However, the vesicles in the cytoplasm observed between the 2nd and the 4th hr with all the compounds suggest that they enter the cells by endocytosis.

With Ac-ACV, both the number of metaphases at the 2nd hr and MI at the 6th hr increased, apparently due to an up to 2 times higher number of cells in all mitotic phases. However, at the 8th hr, the cell division restored. Obviously, Ac-ACV, due to the acetylation of the main structure, induces temporary and reversible changes in the division of uninfected cells.

Regarding the metal complexes of ACV, both Zn complexes that were as cytotoxic as ACV did not affect the cell division (Table 3 and Fig. 3A2). With Zn-Ac-ACV, MI sharply increased at the 4th hr due to an increased number of pro-, meta- and telophases and restored later. The cytotoxicity of Ni and Cd complexes was reflected in their effect on the cell division. Thus, at the 4th hr, the most cytotoxic Ni-ACV chloride induced metaphase arrest, manifested by an increased number of metaphases. An increased incidence of metaphases with lacking chromosomes was observed too. On the contrary, with Ni-ACV nitrate, MI increased at the 4th hr apparently due to sharply elevated numbers of pro-, meta- and telophases (Fig. 3A3 and Table 2). A prolonged exposure of the cells to Ni-ACV complexes resulted in colchicine-like metaphases, pycnosis, condensation of chromatin and blabbing of nuclei. With Cd-ACV, changes in MI, mitotic phases and cell morphology were similar to those with Ni-ACV chloride (Fig. 3A3 and Table 2). These data show that, soon after entering the cell, Ni and Cd complexes of ACV induced irreversible changes in the cell cycle followed by rapid destruction of chromatin and cell death. All these facts are in accord with the reports

Table 2. Effect of ACV and Ac-ACV and their metal complexes on the division of uninfected MDBK cells

Compound	Hr	Prophases	Metaphases	Anaphases	Telophases	Abnormal forms[f]
Control	2	14 ± 7.4	16 ± 7.9	2 ± 2.8	6 ± 4.6	0
	4	8 ± 5.6	16 ± 7.9	4 ± 4.0	6 ± 4.6	0
	6	10 ± 6.3	30 ± 10.6	4 ± 4.0	10 ± 6.3	2 ± 2.8
	8	4 ± 4.0	4 ± 4.0	0	2 ± 2.8	0
ACV	2[a]	12 ± 6.9	16 ± 7.9	0	6 ± 4.6	0
	4	16 ± 7.9	22 ± 9.3	2 ± 2.8	8 ± 5.6	0
	6	6 ± 4.6	24 ± 9.6	8 ± 5.6	22 ± 9.3	6 ± 4.6
	8	8 ± 5.6	20 ± 8.8	2 ± 2.8	8 ± 5.6	2 ± 2.8
Zn-ACV chloride	2[a]	14 ± 7.4	36 ± 11.8	2 ± 2.8	6 ± 4.6	0
	4	12 ± 6.9	26 ± 10.0	2 ± 2.8	10 ± 6.3	0
	6	10 ± 6.3	6 ± 4.6[g]	4 ± 4.0	18 ± 8.4	0
	8	12 ± 6.9	28 ± 10.4	4 ± 4.0	8 ± 5.6	0
Zn-ACV nitrate	2[a]	16 ± 7.9	14 ± 7.4	0	10 ± 6.3	0
	4	10 ± 6.3	22 ± 9.3	8 ± 5.6	16 ± 7.9	6 ± 4.6
	6	36 ± 11.8[g]	18 ± 8.4	10 ± 6.3	12 ± 6.9	0
	8	12 ± 6.9	10 ± 6.3	0	4 ± 4.0	0
Ni-ACV chloride	2	12 ± 6.9	10 ± 6.3	0	2 ± 2.8	0
	4[a]	6 ± 4.6	42 ± 12.6[g]	4 ± 4.0	4 ± 4.0	8 ± 5.6[g]
	6[a, c, e]	20 ± 8.8	10 ± 6.3	4 ± 4.0	10 ± 6.3	8 ± 5.6
	8[c, d, e]	12 ± 6.9	6 ± 4.6	0	8 ± 5.6	0
Ni-ACV nitrate	2[a]	2 ± 2.8[g]	4 ± 4.0	0	6 ± 4.6	0
	4[a, b]	30 ± 10.6[g]	46 ± 12.8[g]	4 ± 4.0	22 ± 9.3[g]	6 ± 4.6[g]
	6[a, b, d]	6 ± 4.6	26 ± 10.0	10 ± 6.3	10 ± 6.3	12 ± 6.9
	8[b, d, e]	2 ± 2.8	2 ± 2.8	0	2 ± 2.8	0
Cd-ACV	2[a, e]	6 ± 4.6	18 ± 8.4	2 ± 2.8	2 ± 2.8	22 ± 9.3[g]
	4[a, e]	20 ± 8.8	84 ± 19.3[g]	10 ± 6.3	12 ± 6.9	22 ± 9.3[g]
	6[a, b, d, e]	8 ± 5.6	22 ± 9.3	6 ± 4.6	4 ± 4.0	10 ± 6.3
	8[b, d, e]	4 ± 4.0	4 ± 4.0	4 ± 4.0	0	0
Co-ACV	2[a]	10 ± 6.3	2 ± 2.8	0	2 ± 2.8	2 ± 2.8
	4[a]	12 ± 6.9	8 ± 5.6	0	8 ± 5.6	6 ± 4.6
	6[a]	20 ± 8.8	38 ± 12.1	4 ± 4.0	34 ± 11.4[g]	6 ± 4.6
	8[a, b]	6 ± 4.6	8 ± 5.6	0	12 ± 6.9	4 ± 4.0
Ac-ACV	2[a]	8 ± 5.6	36 ± 11.8	0	8 ± 5.6	0
	4	6 ± 4.6	22 ± 9.3	4 ± 4.0	18 ± 8.4	6 ± 4.6
	6	20 ± 8.8	44 ± 12.8	12 ± 6.9	16 ± 7.9	16 ± 7.9
	8	14 ± 7.4	12 ± 6.9	0	6 ± 4.6	4 ± 4.0
Zn-Ac-ACV	2	10 ± 6.3	2 ± 2.8	0	2 ± 2.8	0
	4[a]	34 ± 11.4[g]	82 ± 19.0[g]	8 ± 5.6	22 ± 9.3[g]	0
	6	18 ± 8.4	36 ± 11.8	4 ± 4.0	6 ± 4.6	4 ± 4.0
	8	8 ± 5.6	12 ± 6.9	0	2 ± 2.8	0

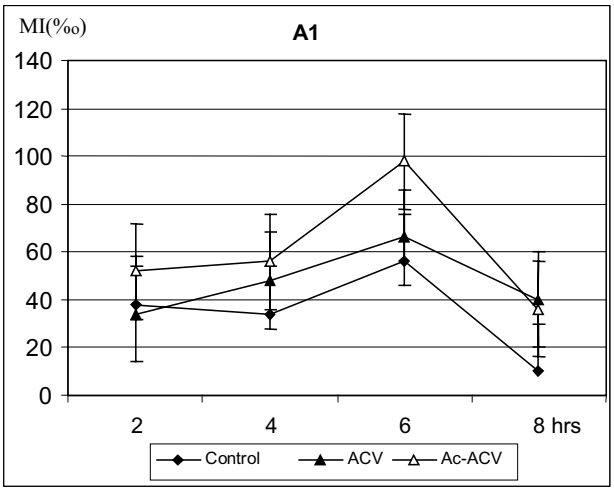
All the compounds were applied in MNC. [a]Vacuolization of cytoplasm. [b]Granulation of cytoplasm. [c]Pycnosis. [d]Blabbing. [e]Conensation of chromatin. [f]Colchicine-like metaphases, multigroup mitoses and metaphases with lacking chromosomes. [g]Statistically significant differences from the control.

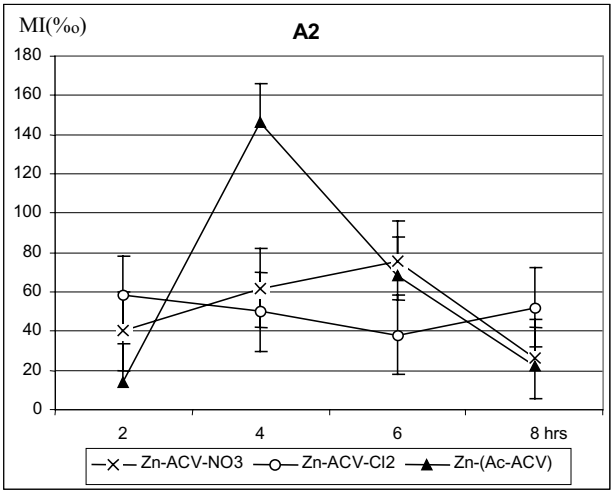
showing significant genotoxicity and carcinogenicity of Ni and Cd (Hong *et al*., 1997; Ishido *et al*., 2002; Lynn *et al*., 1997; Misra *et al*., 1998; O'Halloran, 1993; Zhou *et al*., 1999).

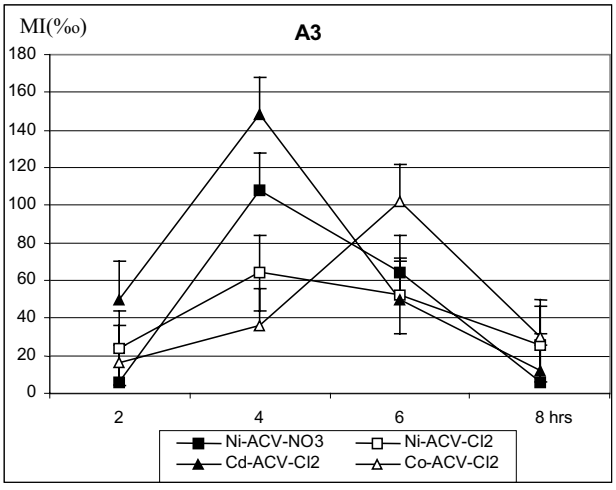
Co-ACV caused an increase of MI at the 6th hr (Fig. 3A3) due to an elevated the number of telophases (Table 2). An increased incidence of colchicine-like mitoses during all the period of observation was found. These findings suggest, by inducing telophase arrest, destruction of chromosomes and mitotic apparatus, Co-ACV suppressed cytokinesis.

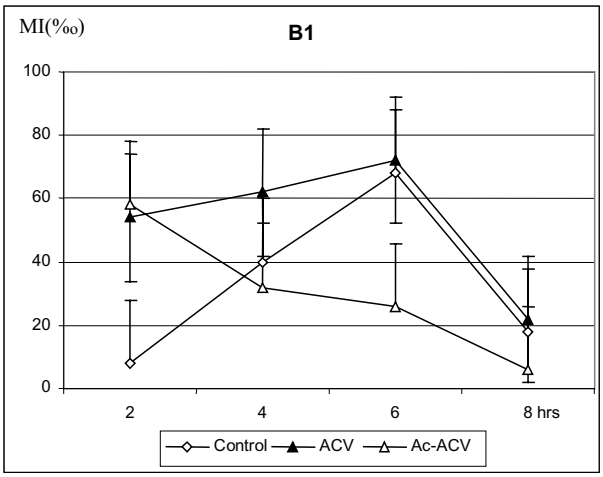
Regarding the effect of the virus infection alone on the cell division, MI was reduced at the 2nd hr in HSV-1 strain Victoria-infected cells (Fig. 3B1), apparently due to sharply decreased numbers of pro- and metaphases (Table 3). This shows that soon after entering the cell the virus induces a pre-mitotic arrest. Also colchicine-like metaphases between the 6th and the 8th hr p.i. were observed. Besides, the virus replication was accompanied by typical cytopathology, namely destruction of the nucleoli at the 4th hr p.i. and nuclear membrane reduplication and condensation of the chromatin between the 6th and 8th hr p.i.

As Ni, Cd and Co complexes of ACV exerted significant and irreversible changes in the cell morphology and division, they were excluded from testing on infected cells.









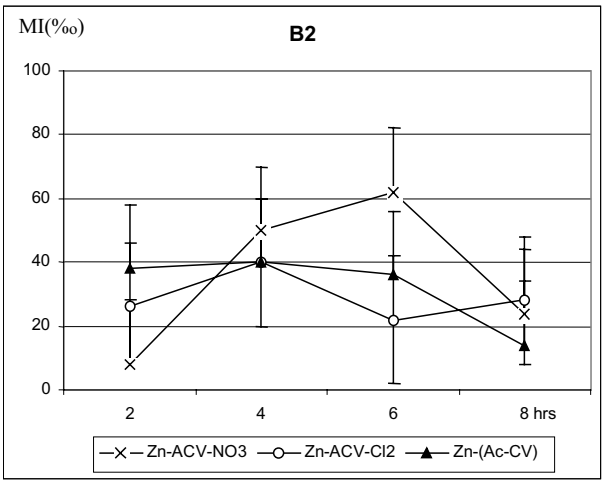


Fig. 3

Effect of ACV, Ac-ACV and their metal complexes on mitotic index (MI) of uninfected (A) and HSV-1-infected (B) MDBK cells

Regarding the effect of the compounds on the division of HSV-1-infected cells, ACV in MNC (0.02 μmol/l), was unable to reverse the toxic viral effect on the structure of chromosomes manifested by an increased number of abnormal forms, predominantly colchicine-like metaphases (Table 3 and Fig. 3B1). A normal cell division was obtained with Ac-ACV, Zn-ACV chloride and Zn-Ac-ACV at the 2nd hr p.i. (Fig. 3B2). Only with Zn-ACV nitrate the proportion of metaphases was equal to that in viral control and lower than that in cell control. However, an increased vacuolization was observed with Zn complexes of ACV at the 8th hr p.i.

In conclusion, the data presented above show that Ac-ACV and Zn Ac-ACV (i) were active and selective inhibitors of the growth of HSV-1 and HSV-2, (ii) were able to overcome the ACV resistance, and (iii) suppressed the pre-mitotic arrest induced by HSV-1 in the immediate early stage of infection.

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Table 3. Effect of ACV, Ac-ACV and their Zn complexes on the division of HSV-1-infected MDBK cells

Compound	Hr p.i.	Prophases	Metaphases	Anaphases	Telophases	Abnormal forms[f]
Control	2	4 ± 4.0[g]	2 ± 2.8[g]	0	2 ± 2.8	0
	4[a]	8 ± 5.6	20 ± 8.8	2 ± 2.8	10 ± 6.3	0
	6[a, e]	6 ± 4.6	28 ± 10.4	14 ± 7.4	16 ± 7.9	4 ± 4.0
	8[a, e]	6 ± 4.6	6 ± 4.6	0	4 ± 4.0	2 ± 2.8
ACV	2[a]	16 ± 7.9	26 ± 10.0[d]	2 ± 2.8	4 ± 4.0	6 ± 4.6[g]
(0.02 μmol/l)	4	20 ± 8.8	32 ± 11.0	0	8 ± 5.6	2 ± 2.8
	6	8 ± 5.6	36 ± 11.8	6 ± 4.6	12 ± 6.9	10 ± 6.3
	8	4 ± 4.0	12 ± 6.9	0	4 ± 4.0	2 ± 2.8
Zn-ACV chloride	2[a]	14 ± 7.6	20 ± 8.8[d]	0	4 ± 4.0	0
(0.01 μmol/l)	4	4 ± 4.0	20 ± 8.8	4 ± 4.0	8 ± 5.6	4 ± 4.0
	6[a]	6 ± 4.6	18 ± 8.4	0	6 ± 4.6	6 ± 4.6
	8[a, c]	2 ± 2.8	8 ± 5.6	0	4 ± 4.0	0
Zn-ACV nitrate	2	6 ± 4.6	2 ± 2.8[d]	0	0	0
(0.02 μmol/l)	4[a]	10 ± 6.3	20 ± 8.8	4 ± 4.0	16 ± 7.9	0
	6[a]	10 ± 6.3	26 ± 10.0	10 ± 6.3	14 ± 7.4	2 ± 2.8
	8[a, c]	8 ± 5.6	8 ± 5.6	2 ± 2.8	6 ± 4.6	0
Ac-ACV	2[a]	6 ± 4.6	36 ± 11.8[d]	4 ± 4.0	10 ± 6.3	2 ± 2.8
(0.02 μmol/l)	4	6 ± 4.6	14 ± 7.4	4 ± 4.0	8 ± 5.6	0
	6[a]	2 ± 2.8	18 ± 8.4	2 ± 2.8	2 ± 2.8	2 ± 2.8
	8[a]	4 ± 4.0	2 ± 2.8	0	0	0
Zn-Ac-ACV	2[a]	14 ± 7.6	20 ± 8.8[d]	0	4 ± 4.0	0
(0.02 μmol/l)	4[a]	4 ± 4.0	20 ± 8.8	4 ± 4.0	8 ± 5.6	4 ± 4.0
	6[a]	6 ± 4.6	18 ± 8.4	0	6 ± 4.6	6 ± 4.6
	8[a]	2 ± 2.8	8 ± 5.6	0	4 ± 4.0	0

[d]Statistically significant differences from the control. For the rest of the legend see Table 2.

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