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Antiviral action of 5-amino-2-(2-dimethyl-aminoethyl)benzo-[de]-isoquinolin-1,3-dione

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Summary

A newly synthesized imide derivative of 3-nitro-1,8-naphthalic acid, 5-amino-2-(2-dimethylaminoethyl)benzo-[de]-isoquinolin-1,3-dione (designated M-FA-142), was tested on chick embryo cells against herpes simplex virus type 1 (HSV-1) and vaccinia virus (VV), and on Vero cells against African swine fever virus (ASFV). At a concentration of 4 μg/ml the drug inhibited VV replication by about one order of magnitude, and that of HSV-1 by about three orders of magnitude. A minor effect was shown against ASFV. Virus inhibition was found to depend on the amount of drug and multiplicity of infection. No virucidal effect was observed on the viruses tested, except for a slight effect on HSV-1. Inhibition of virus growth could be reversed when the drug was removed from the cell culture medium. Serial passages of HSV-1 and VV in the presence of the drug caused the appearance of drug-resistant viruses.

imide derivatives; 3-nitro-1,8-naphthalic acid; antiviral activity; herpes simplex virus; vaccinia virus; African swine fever virus

Introduction

Braña et al. [1] have synthesized a new series of imide derivatives of 3-nitro-1,8-naphthalic acid having different basic side chains linked to the imide nitrogen. The drugs in question were synthesized as part of a programme aimed at combining the known antitumour activity of compounds containing a β-nitronaphthalene moiety, as occurs naturally in aristolochic acid [5,8], with other structural entities suspected to be potentially efficacious, such as a glutaramide ring and a positively-charged tertiary amine side-chain. Two compounds, 5-nitro-2-[2-(1-pyrrolidine)ethyl]benzo-[de]-isoquinolin-1,3-dione (M-12210) and 5-nitro-2-(2-dimethylaminoethyl)benzo-[de]-isoquinolin-1,3-dione (M-4212), were synthesized in this way (the latter denoted

mitonafide by the World Health Organization) and have been proved active against experimental tumours [2] and shown to be inhibitors of two DNA viruses [6].

Another new compound [3] of this series, 5-amino-2-(2-dimethylaminoethyl)-benzo-[de]-isoquinolin-1,3-dione, designated M-FA-142, has previously been studied for its antiviral action against herpes simplex type 2 (HSV-2) and adenovirus type 5. Four μg/ml of this drug inhibited by 5 logs the HSV-2 titer, while no inhibition was observed with adenovirus type 5 [7]. In view of the great inhibition found with this drug against HSV-2, it seemed interesting to study its inhibitory action against herpes simplex type 1 (HSV-1), as well as against two more DNA viruses, vaccinia (VV) and African swine fever virus (ASFV). The structural formula of the compound is shown in Fig. 1.

Materials and Methods

Drugs

The 5-amino-2-(2-dimethylaminoethyl)benzo-[de]-isoquinolin-1,3-dione, (M-FA-142), was provided by MADE Laboratories, Madrid, Spain. It was dissolved in sterile, double-distilled water and sterilized by filtration; final dilutions were made in the cell culture media. 5-Iodo-2'-deoxyuridine (IUdR), purchased from Sigma Chemical Co. (St. Louis, MO), was run in parallel as a known active drug.

Cell culture

Primary chick embryo (CE) cells were cultured using procedures previously established [6], and African green monkey cells (Vero) were maintained in Dulbecco's modified Eagle medium with glutamine (Flow Laboratories, Inc., Rockville, MD, U.S.A.), 10% calf serum, and either 0.85% sodium bicarbonate for flask and test tube cultures, or 3.7% sodium bicarbonate for cultures in Petri dishes incubated in a 5% CO_2 atmosphere. The maintenance medium was supplemented with 2% calf serum. No antibiotics were added. Vero cells were kindly provided by Dr. R. Nájera, National Center of Microbiology, Virology and Immunology, Majadahonda, Madrid, Spain.

Viruses

Herpes simplex type 1 (HSV-1) strain HFEM was kindly supplied by Dr. F.P. Gallardo of the National Center of Microbiology, Virology and Immunology, Madrid, Spain. The vaccinia virus (VV) was obtained from vaccinal, germ-free calf lymph

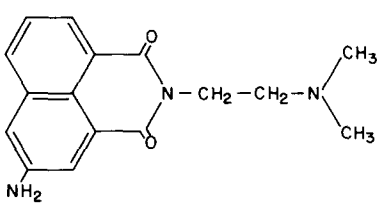

Fig. 1. Chemical structure of 5-amino-2-(2-dimethylaminoethyl)benzo-[de]-isoquinolin-1,3-dione.

by filtration, and maintained in our laboratory by serial passages in chick embryo cells. African swine fever virus (ASFV), adapted to growth in Vero cells, was obtained from Dr. E. Viñuela, Molecular Biology Center, Madrid, Spain.

Drug cytotoxicity

The effect of the drug was assessed in three different ways: (1) Study of the cytotoxic effect on chick embryo and Vero cells: Dilutions of 20, 15, 10, 8, 4, 2 and 1 μg/ml drug in maintenance medium were added to monolayers of chick embryo cells (four tubes per dilution). For six consecutive days the monolayers were examined microscopically for cytotoxic effects and compared with the cultures incubated in the absence of drug. (2) Effect of the drug on the attachment of cells to the glass surface: chick embryo and Vero cells were seeded in glass tubes with growth medium containing the above-mentioned concentrations of the drug (four tubes per dilution) and were observed daily for three days while keeping four tubes without drug as a control. (3) Effect of the drug on cell growth: suspensions of chick embryo and Vero cells were made as above in growth medium with and without drug, but with one fourth the normal number of cells. Every day for three consecutive days four cultures were trypsinized and the cells harvested and counted. Viability was determined by the trypan blue exclusion method.

The most sensitive method for detecting drug toxicity was the third, and we have employed the inhibitory effect of the drug on cell growth to define the 'maximum tolerated concentration' (MTC) as the highest dose of drug causing absolutely no discernible cytotoxic effect and no inhibition of cell growth.

Yield reduction test

Confluent monolayers of primary CE or Vero cells were infected with HSV-1, VV, or ASFV at a multiplicity of infection (m.o.i.) of approximately 0.1. After 120 min of adsorption at 37°C for ASFV, and 90 min for HSV-1 and VV, the cell sheet was washed with phosphate-buffered saline; then the maintenance medium with 4 μg/ml of the drug at the maximum tolerated concentration (MTC) was added. At appropriate times (24, 48, 72, 96 and 120 h, in the case of HSV-1; 24, 48, 72 and 96 h for VV; and 24, 48 and 72 h for ASFV) sets of four cultures were harvested and kept at –70°C, and the total yield of virus was obtained by freeze-thawing the cells followed by sonication. Cell debris was removed by low-speed centrifugation and the supernatant was titrated in CE cells by the plaque assay method (HSV-1 and VV) as previously described [9]. ASFV was titrated in Vero cells by plaque assay in a 5% CO_2 atmosphere. A known active drug, IUdR, was run in parallel. The virus titers are expressed in plaque-forming units (PFU) per ml.

Results

Drug cytotoxicity

No microscopic cytotoxic effects or inhibition of cell fixation was apparent at a drug concentration of 8 μg/ml for CE or Vero cells, but at this concentration of drug, proliferation of CE and Vero cells was partially inhibited. However, at a dose of 4

μg/ml no toxic effects were found and CE or Vero cell growth was not inhibited. We considered a concentration of 4 μg/ml as the highest dose without any discernible toxic effects on cell growth and without inhibition of cell growth, and we have defined this dose as the 'maximum tolerated concentration' (MTC).

Virucidal activity assays on HSV-1, VV and ASFV

The direct virus-inactivating effect of M-FA-142 was studied with HSV-1 and VV in CE cells, and with ASFV in Vero cells. The MTC dose of the drug (4 μg/ml) mixed with the appropriate amount of each virus and a control for each virus without the drug were kept at 4°C for 180 min with periodical shaking. After this time the different solutions of drug-plus-virus and drug-free virus were titrated by plaque assay in cell cultures. The compound tested did not exert virucidal effects against VV or ASFV, and only a slight virucidal effect for HSV-1. The results are shown in Table 1.

Inhibition of HSV-1, VV and ASFV multiplication by M-FA-142

Primary CE and Vero cell cultures were infected at a m.o.i. of 0.1 with each virus and then treated with M-FA-142 (4 μg/ml). The total virus yield was determined every 24 h after cell infection during 5 days for HSV-1, 4 days for VV and 3 days for ASFV. The growth curves, compared with the controls (virus-infected but untreated, and virus-infected and treated with IUdR), are plotted in Figs. 2, 3 and 4. The results of a representative assay for HSV-1 are shown in Fig. 2; an average reduction of 3 $\log_{10}$ in virus yield was achieved by M-FA-142. Fig. 3 shows the inhibition of VV multiplication by M-FA-142 (4 μg/ml); the reduction in VV yield was about 1 $\log_{10}$. Finally, treatment of ASFV-infected Vero cells with M-FA-142 gave a ca. 3-fold reduction of virus yield (Fig. 4). The results obtained with IUdR for HSV-1 and VV indicate a higher activity compared with M-FA-142 when using MTC doses for both compounds. No further experiments were made with ASFV because this virus did not appear sufficiently sensitive to the compound.

Influence of different multiplicities of infection (m.o.i.) on the inhibition of HSV-1 and VV replication by M-FA-142

The inhibition of HSV-1 and VV by M-FA-142 (4 μg/ml), when the viruses were inoculated at different m.o.i. ranging from 0.0001 to 1 PFU/cell, is illustrated in Fig. 5. Inhibition was measured by determining the amount of virus produced by cells

TABLE 1

Virucidal activity of M-FA-142 against herpes simplex type 1 (HSV-1), vaccinia (VV) and African swine fever (ASFV) viruses

Viruses	Virus titer after 3 h contact (PFU/ml)	
	Virus-plus-drug	Virus control
HSV-1	1.7×10^7	6.8×10^7
VV	1.2×10^5	1.2×10^5
ASFV	6.6×10^4	4.9×10^4

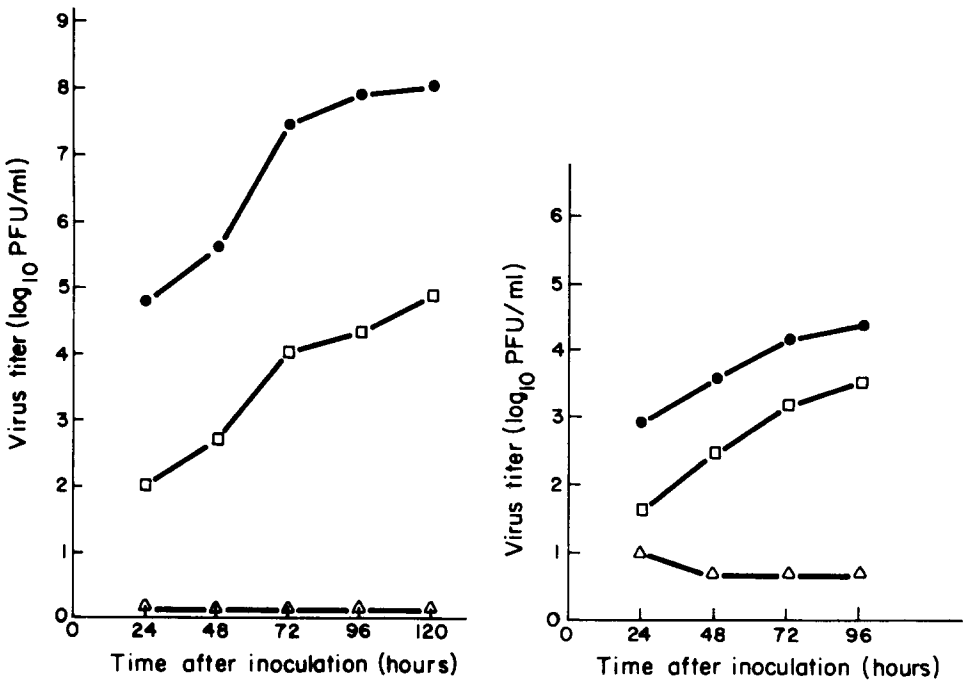

Fig. 2. Inhibition kinetics of HSV-1 replication in chick embryo monolayers using M-FA-142 (4 μg/ml) (□), no drug (●), or IUdR (100 μg/ml) (△).

Fig. 3. Inhibition kinetics of VV replication in chick embryo monolayers using M-FA-142 (4 μg/ml) (□), no drug (●), or IUdR (100 μg/ml) (△).

untreated and drug-treated for 72 h after virus inoculation. The upper panel of Fig. 5 represents drug inhibition of HSV-1 and the lower panel of the figure indicates the corresponding drug inhibition of VV. In both cases, a linear response was obtained. As compared to the controls, virus yield for infected cells treated with the drug was inhibited by the following factors: with HSV-1 by 3.0 $\log_{10}$ at a m.o.i. of 1 up to 4.0 $\log_{10}$ at a m.o.i. of 0.0001; with VV by 1.0 $\log_{10}$ at a m.o.i. of 1 up to more than 4.0 $\log_{10}$ at a m.o.i. of 0.0001.

Inhibition of HSV-1 and VV replication in the presence of different drug concentrations

CE cells infected with HSV-1 or VV were exposed to M-FA-142 concentrations ranging from 0.1 to 4 μg/ml. All samples were harvested at the same time (72 h) and titrated by plaque assay. In Fig. 6, residual virus titers are plotted against the concentration of M-FA-142. Virus yield reduction by M-FA-142 was closely dose-dependent. The minimal inhibitory concentration of the drug, defined as the concentration causing 1.0 $\log_{10}$ reduction, was found to be approximately 1 μg/ml for HSV-1 and 2 μg/ml for VV.

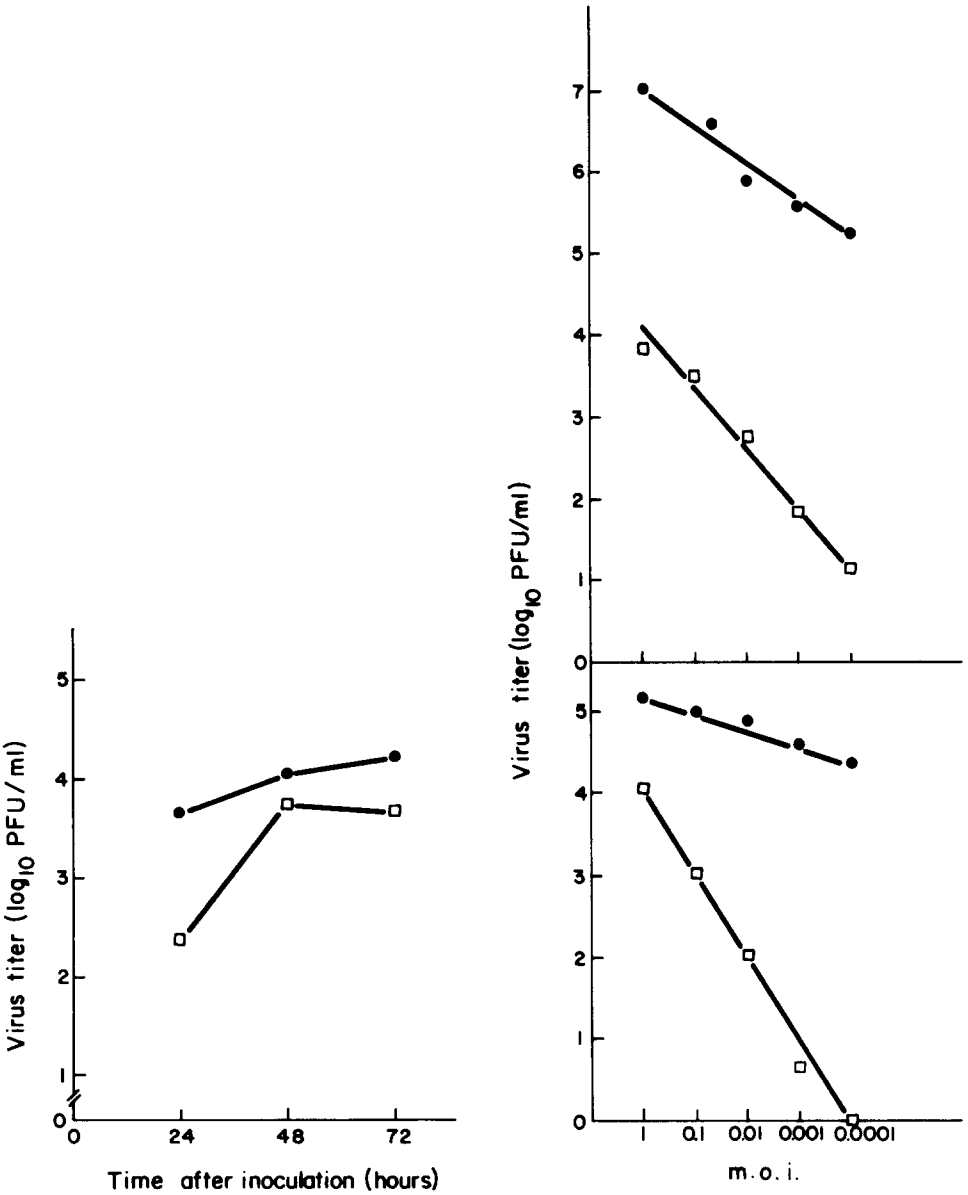

Fig. 4. Inhibition kinetics of ASFV replication in Vero monolayers using M-FA-142 (4 μg/ml) (□), or no drug (●).

Fig. 5. Influence of different multiplicities of infection (1, 0.1, 0.001, and 0.0001) on the inhibition of HSV-1 and VV replication in chick embryo cells by 4 μg/ml of M-FA-142. Virus titers were determined at 72 h for M-FA-142 (□) and control (●). The upper panel presents the inhibition of HSV-1 yields, while the lower panel presents the inhibition of VV yields.

Reversibility of the antiviral activity of M-FA-142 upon drug removal

In order to determine a possible reversal of drug inhibition, CE monolayers were infected with HSV-1 or VV (at a m.o.i. of 0.1) and exposed to M-FA-142 (4 μg/ml) in maintenance medium. At 24, 28 and 72 h after infection with HSV-1 or VV, the drug was removed and medium without drug was added. As shown in Table 2, all cultures exposed to the drug after infection were protected against viral c.p.e. However, the final virus yields (measured at 96 h) did not differ significantly from the control virus yield, suggesting a reversal of the antiviral effects of the drug upon its removal from the virus-infected cell cultures.

Development of drug resistance

Eight successive passages of HSV-1 and VV were made in four flasks either in the absence or presence of 4 μg/ml M-FA-142. Cultures were harvested and virus titrated by plaque assay at the time when the controls without drug showed complete c.p.e. Each flask with chick embryo cells was inoculated with 0.5 ml of a 10^{-1} virus dilution of the previous passage. During the first and second passages there was a decrease in the virus titer for HSV-1; afterwards, the virus titer gradually increased suggesting that the virus had become resistant to the drug. With VV a marked decrease in virus titer was seen only at the third passage, then the virus titer went up again as noted for HSV-1. By the eighth passage the virus titer was similar in the culture with or without drug (Fig. 7).

TABLE 2

Effect of M-FA-142 on herpes simplex virus type 1 (HSV-1) and vaccinia virus (VV) yields after removal of the drug

Virus	Time of drug removal (h)	c.p.e. at the time of drug removal		Final virus titer (PFU/ml) in treated cultures (96 h)	Final virus titer (PFU/ml) in untreated controls (96 h)
		With drug	Without drug		
HSV-1	24	0	0	2.5×10^7	
	48	0	+	2.0×10^7	5.7×10^7
	72	0	++	9.5×10^6	
VV	24	0	+	1.4×10^5	
	48	0	++	8.1×10^4	4.9×10^5
	72	0	+++	2.0×10^4	

The cytopathic effect (c.p.e.) was rated as follows: 0, no c.p.e.; +, 25%; ++, 50%; +++, 75% c.p.e.

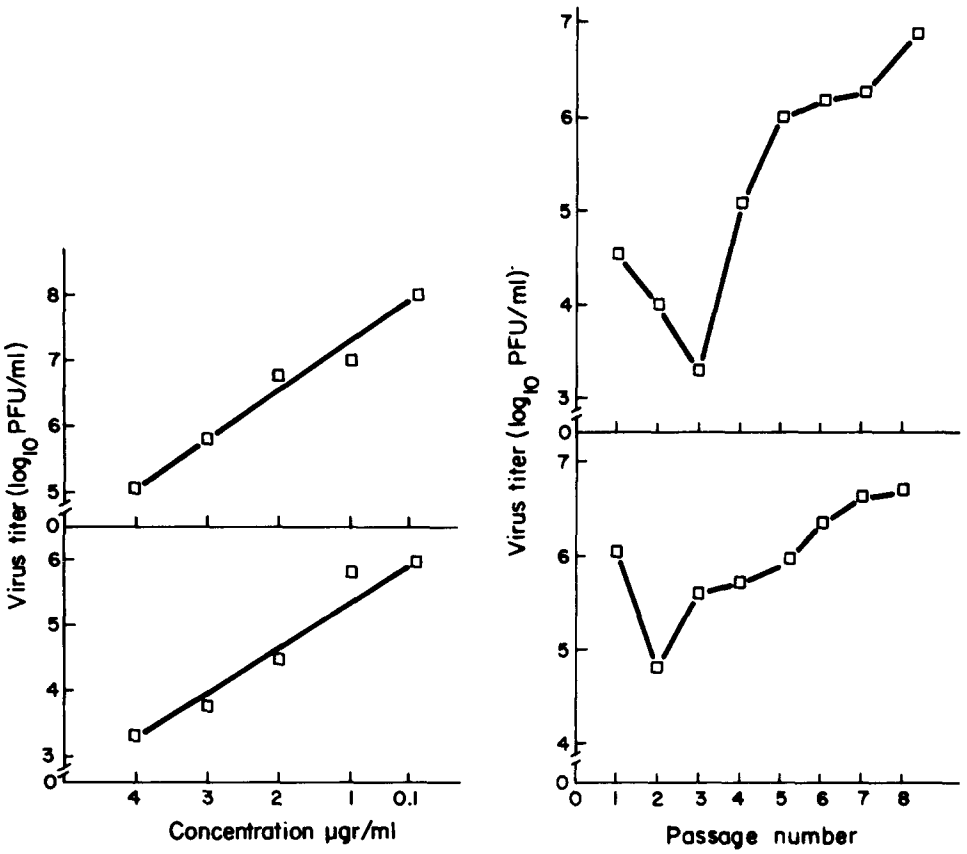

Fig. 6. Effect of varying M-FA-142 concentrations (4, 3, 2, 1 and 0.1 μg/ml) on the replication of HSV-1 or VV at 72 h in chick embryo cells. Control virus titer: HSV-1, 9.0×10^7 PFU/ml; VV, 8.5×10^5 PFU/ml. The upper panel presents the inhibition of HSV-1 yields, while the lower panel presents the inhibition of VV yields.

Fig. 7. Effect of consecutive passages of HSV-1 and VV in the presence of M-FA-142 (4 μg/ml). Control virus titer: HSV-1, 1.2×10^8 PFU/ml; VV, 1.0×10^7 PFU/ml. Upper panel: VV; lower panel: HSV-1.

Discussion

Two new antitumor drugs, imide derivatives of 3-nitro-1,8-naphthalic acid [1,2], have been shown to bind to double helical DNA by intercalation [10]. At an ionic strength of 0.01 M, their intrinsic association constant is about 1.45×10^5 M^{-1}, and each bound ligand molecule occludes about 3.4 nucleotides of the DNA lattice. They remove and reverse the supercoiling of closed circular duplex bacteriophage PM2 DNA with apparent unwinding angles of 11–12° per bound drug molecule, as compared to an assumed unwinding angle of 26° for ethidium. They increase the viscosity of sonicated rod-like DNA fragments, each bound molecule producing a calculated increment in length of 0.22–0.25 nm [10]. These drugs have been evaluated for

antiviral activity against double-stranded DNA viruses (herpes simplex and vaccinia) and an RNA virus (Sindbis). Experiments performed previously with these compounds showed significant antiviral activity against the DNA viruses, but not against the RNA virus, in vitro [6].

A new derivative, 5-amino-2-(2-dimethylaminoethyl)benzo-[de]-isoquinolin-1,3-dione (M-FA-142), has now been evaluated for its activity against double-stranded DNA viruses (herpes simplex, vaccinia and African swine fever virus). The activities were assessed using the MTC of the drug and, under these conditions, M-FA-142 brought about a significant reduction in HSV-1 titer; VV was also inhibited, although to a somewhat lesser extent, whereas a slight inhibitory action was found against ASFV. All three imide derivatives that have been studied are endowed with high cytotoxicity, but their cytotoxic and biochemical effects are reversible [2]. Vero and chick embryo cells are apparently not affected by low concentrations (4 μg/ml) of M-FA-142. The fact that virus synthesis resumed after removal of the drug shows that there was no permanent damage to the virus-synthesizing capacity of the cells. On the other hand, the cytotoxic and biochemical effects of these drugs are reversible and, thus, presumably do not involve a permanent covalent binding of the drug (or their metabolites) to a cellular target site. They have also been shown to stabilize double-helical DNA against heat denaturation [2], an effect typical of drugs complexing with DNA.

At 4 μg/ml, M-FA-142 diminished the replication of all viruses tested. The effect was small (less than one order of magnitude) for ASFV but reached three orders of magnitude for HSV-1. VV yield was reduced by one order of magnitude. From its structural similarity with the imide derivatives of 3-nitro-1,8 naphthalic acid [1,2], one might expect M-FA-142 to produce similar effects on virus DNA synthesis and to have similar mechanisms of action. Because M-FA-142 was not found to have a virucidal effect on any of the viruses, the antiviral effect of the drug can be explained by the inhibition of virus multiplication.

The inhibition of HSV-1 and VV viruses was most evident at a low m.o.i. The antiviral effects of M-FA-142 were also dose-dependent; at increasing levels of the drug the virus yields decreased proportionally.

The reversibility of the action of the drug, by simply replacing the drug-containing maintenance medium with fresh drug-free medium, was demonstrated for HSV-1 and VV viruses.

Serial passages of HSV-1 and VV viruses in the presence of M-FA-142 resulted in an increased resistance to the drug, as compared to the drug susceptibility exhibited by the viruses during the first passages.

The drug M-FA-142 has been proven to be less active against HSV-1 and VV viruses than the compounds M-4212 and M-12210 studied previously [6]. The three drugs were less effective against HSV-1 and VV than IUdR, except for M-12210 which at a concentration of 1 μg/ml effected the same reduction in HSV-1 titer as did IUdR at 100 μg/ml.

M-FA-142 at 4 μg/ml is about 100 times less inhibitory to HSV-1 than to HSV-2 [7]. This is in marked contrast with other antiherpes agents such as (*E*)-5-(2-bromovinyl)-2'-deoxyuridine which inhibits HSV-1 at a 100–1000 fold lower concentration than HSV-2.

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