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ANTIVIRAL ACTIVITY OF 2,3-DIHYDRO-1H-IMIDAZO[1,2-b]PYRAZOLE IN HERPES SIMPLEX VIRUS TYPE 1-INFECTED MAMMALIAN CELLS

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Abstract—2,3-Dihydro-1H-imidazo[1,2-b]pyrazole (IMPY), a known inhibitor of DNA synthesis, has been shown to be a useful drug for the synchronization of mammalian cells in culture. Recent studies in our laboratory indicate that IMPY may possess significant antiviral activity against herpes simplex virus (HSV) type 1. IMPY, at a concentration of 80 μg/ml or 0.73 mM, reduced syncytia formation approximately 80 per cent. A 50 per cent inhibitory dose was calculated for each drug in order to compare potency in syncytia reduction of IMPY with that of arabinosyladenine (ara-A) and arabinosylhypoxanthine (ara-H). Our results indicated that the antiviral potencies of the three drugs were ranked in the order ara-A > ara-H > IMPY, the 50 per cent inhibitory doses being 22, 195 and 309 μM respectively. Utilizing the microplate procedure of Sidwell and Huffman [*Appl. Microbiol.* **22,** 797 (1971)], inhibition of viral cytopathic effect was rated against drug cytotoxicity and a virus rating (VR) established. A virus rating of 0.68 was calculated for IMPY. In comparison, VR values of 0.84 and 0.66 were obtained for ara-A and ara-H respectively. In contrast to the syncytia reduction studies, IMPY appeared to possess antiviral activity equivalent to that of ara-H according to the criteria of the virus rating assay. A technique was developed for evaluating the degree of selectivity (°*S*) of a drug with respect to its differential effect on viral and cellular DNA synthesis. IMPY was found to possess a negative selectivity at all concentrations studied, reflecting the fact that it inhibited cellular DNA synthesis more than viral DNA synthesis. In contrast, ara-A and ara-H both expressed positive degrees of selectivity in that they inhibited viral DNA synthesis more extensively than cellular DNA synthesis.

The antiviral and antitumor activities of many agents depend, at least in part, on their ability to inhibit DNA synthesis. Studies in L cells have shown that 2,3-dihydro-1H-imidazo[1,2-b]pyrazole (IMPY) reversibly inhibited DNA synthesis without significantly affecting RNA or protein synthesis [1]. Although the exact mode of action of this drug is unknown, it was postulated that IMPY may be interfering with deoxyguanosine triphosphate synthesis. The inhibition of DNA synthesis seen with IMPY was prevented and reversed by deoxyguanosine and potentiated by deoxycytidine. More recently, it has been demonstrated that both monolayer and suspension cultures of HeLa cells could be synchronized at the point of entry into the synthetic (S) phase of the cell cycle by IMPY [2]; addition of deoxynucleosides, however, failed to reverse or prevent the inhibition of DNA synthesis by IMPY. It was suggested that this limitation may be due to the poor capacity of HeLa cells to phosphorylate and utilize deoxyguanosine.

The first indication that IMPY possessed antiviral activity appeared during studies with the nucleoside antibiotic 9-β-D-arabinofuranosyladenine (ara-A), a known antiviral compound [3]. IMPY was used in our laboratory as an agent for synchronizing suspension cultures of KB cells. After synchronization was achieved, the cells were infected with herpes simplex virus type 1 (HSV-1) and treated with ara-A. The intracellular concentration of IMPY remaining after washing appeared to exert antiviral activity against HSV-1 even in infected cell cultures not treated with ara-A.

In this paper, we describe studies of IMPY and its action in HSV-infected mammalian cells. Our studies of syncytia reduction and microplate assays indicate that IMPY possesses a significant antiviral action against HSV-1. However, biochemical studies involving the separation and quantitation of viral and cellular DNA from HSV-infected KB cells revealed that IMPY inhibited cellular DNA synthesis to a slightly greater extent than the synthesis of viral DNA. This lack of selectivity is in direct contrast to the selective inhibition of viral DNA synthesis seen with ara-A and its deaminated catabolite arabinosylhypoxanthine (ara-H) [4,5].

MATERIALS AND METHODS

Chemicals. IMPY was generously provided by Dr. Herbert L. Ennis, Roche Institute of Molecular Biology (Nutley, N.J.), and Drs. P. Schmidt and K. Scheibli, Ciba-Geigy Ltd. (Basel, Switzerland). Ara-A

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was the gift of Dr. Harold E. Machamer, Parke-Davis, & Co. (Detroit, Mich.). Ara-H was purchased from Pfanstiehl Laboratories, Inc. (Waukegan, Ill.). [^{3}H-methyl]thymidine was obtained from New England Nuclear Corp. (Boston, Mass.). HEPES [4-(2-hydroxyethyl)-1-piperazineëthane-sulfonic acid] and pronase were purchased from CalBiochem (La Jolla, Calif.). Cesium chloride (technical grade) was obtained from Kawecki Berylco Industries, Inc. (Revere, Pa.). Sarkosyl was purchased from Geigy Industrial Chemicals (Chicago, Ill.), and thymidine from P-L Biochemicals, Inc. (Milwaukee, Wisc.).

Sera. Donor calf serum, free of mycoplasma and bacteriophage, was purchased from Flow Laboratories, Inc. (Rockville, Md.). Fetal bovine serum was obtained from K. C. Biologicals, Inc. (Lenexa, Kan.). In experiments involving the use of ara-A, extensively heat-treated (12 hr at 56°) calf serum was used to prevent the serum-associated deamination of ara-A to ara-H [6].

Cell culture. Monolayer cultures of KB cells were grown at 37° in 32-oz glass prescription bottles or 75 cm^2 plastic flasks (Falcon Plastics, Oxnard, Calif.) in Eagle minimum essential medium with Hanks salts [MEM(H)] and 10% calf serum.

Suspension cultures of KB cells were grown at 37° in 500-ml spinner flasks (Bellco Glass Co., Inc., Vineland, N.J.) with 300–400 ml of minimal essential medium containing spinner salts [MEM(S)] ($NaHCO_3$ omitted) and buffered at pH 7.0 with 0.01 M HEPES. The medium was supplemented with 10% calf serum.

BHK-21/4 and BHK-21/13 cells were grown in plastic flasks in MEM with Earle Salts [MEM(E)], supplemented with 10% calf serum and 5% tryptose phosphate broth. The sodium bicarbonate concentration was reduced to 1.85 g/liter. Incubation was performed at 37° in a humidified atmosphere of 97% air and 3% CO_2. Virus assays and syncytia reduction assays were carried out with BHK-21/4 cells in Ham Nutrient Mixture F-12 supplemented with 5% fetal bovine serum.

Both cell lines were passaged every 2–3 days according to conventional procedures using 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid in a HEPES buffered salt solution at pH 7.4 [7].

Antibiotics were not used in any cell culture media in order to facilitate the detection of bacterial or mycoplasmal contamination of the cell lines. All cell lines were regularly screened (tests performed by Microbiological Associates, Bethesda, Md.) and were found to be free of mycoplasma.

Virus. Herpes simplex virus strain HF was the gift of Dr. Gary H. Cohen, Philadelphia, Pa. High titer virus stock was prepared according to a previously published method [4]. Briefly, monolayer cultures of KB cells were grown in MEM(H) with 10% calf serum and 2X arginine. The cultures were infected with virus at an input multiplicity of 5–10 plaque forming units (PFU)/cell. The cells were harvested after 24 hr and washed by centrifugation in fresh medium (5 min at 300 *g*). The cell pellet was resuspended and sonicated for 60 sec, followed by centrifugation at 500 *g* for 10 min.

Titration of virus. Monolayer cultures of BHK-21/4 cells were prepared by planting 1 to 1.5 × 10^6 cells in 60 × 15 mm Contur dishes (Lux Scientific Corp., Thousand Oaks, Calif.) using modified MEM(E) medium supplemented with 10% calf serum. After incubating the dishes at 37° in a humidified 97% air–3% CO_2 atmosphere overnight, the medium was aspirated and 0.2 ml of the appropriate viral dilution was added. The dishes were incubated in a CO_2 atmosphere for 1 hr to permit viral adsorption.

After infection, a syncytial assay [4] was performed in order to determine the titer of the virus sample being studied. In a syncytial assay, excess virus was aspirated and 5 ml of F-12 medium containing 10% fetal calf serum was added to each dish and incubated at 37° in a CO_2 atmosphere for 20–24 hr. After this interval, syncytia were counted with an inverted microscope (60× magnification) and the titer of the virus sample was calculated.

Syncytia reduction studies. Syncytia reduction assays were performed on monolayers of HSV-infected BHK-21/4 cells planted in 60 × 15 mm Integrid tissue culture dishes (Falcon Plastics) according to the procedure described above. After a 1-hr viral adsorption period, F-12 medium containing 0, 10, 20, 40, 80 or 160 μg/ml of IMPY was added and allowed to incubate for 24 hr, after which time syncytia could be counted with the aid of an inverted microscope. A linear regression plotting program was used to determine the best linear relationship between per cent syncytia inhibition and drug concentration.

Microtest assay. The micro tissue culture plate procedures developed by Sidwell and Huffman [8] were used in our laboratory as a means of evaluating the antiviral activity of IMPY and other drugs. Inhibition of viral cytopathic effect (CPE) was rated against drug cytotoxicity and a virus rating (VR) established.

Disposable plastic microplates (Microtest II tissue culture plates, Falcon Plastics) containing circular wells were planted with BHK-21/13 cells in modified MEM(E). A Cornwall syringe was used to deliver 0.2 ml of a cell suspension/well such that each well received approximately 3.6 × 10^4 cells. After a 24-hr incubation at 37° in a CO_2-enriched atmosphere, the medium was removed and each well was infected with 0.1 ml virus suspended in MEM(E) at a concentration of 10 tissue culture infective dose 50 per cent ($TCID_{50}$)/well.

Several min after addition of the virus suspension, 0.1 ml medium or medium containing various concentrations of the drug to be tested was added to each well, bringing the total volume/well to 0.2 ml. Included in each plate were toxicity controls (containing drug and medium only), virus controls (containing virus and medium only), and cell controls (containing medium only). The plates were sealed with a plastic lid (Falcon Plastics), incubated for 3 days, and examined microscopically for CPE and drug cytotoxicity every 24 hr using an inverted phase microscope.

The condition of the cell layer in each well was graded on a scale of 0 (normal cells, no cytotoxicity or CPE) to 4 (virtually complete destruction of cell layer). From these data, a VR was computed for each drug tested.

Preparation and labeling of DNA. To study the effect of IMPY on DNA replication, tritium-labeled cellular and viral DNA were obtained from HSV-infected monolayers of KB cells by incubating each

monolayer in MEM(H) medium containing [^{3}H-methyl]thymidine (2 μCi/ml final concentration) for 12 hr at 37°. The medium in each flask also contained a specific concentration of IMPY (0, 10, 20, 40, 80 and 160 μg/ml), and cold thymidine (5 μM concentration) to prevent the premature depletion of label. At the end of 12 hr the flasks were shaken vigorously to suspend the cells and a 5-ml sample was removed from each flask. The cells were pelleted by centrifugation at 2900 g for 15 min and the pellets were stored at $-76°$ for subsequent analysis. After resuspending each pellet in 0.5 ml TES [30 mM Tris-(hydroxymethyl)aminomethane (Tris), 5 mM EDTA, 50 mM NaCl, pH 8.0], an equal volume of a solution containing 2 mg/ml of pronase (autodigested for 15 min at 37°) and 1% sarkosyl in TES was added and incubated for 15 min at 37° or until the mixture cleared. The preparations were rapidly pipetted three or four times to partially shear the DNA present; shearing of DNA was necessary to afford good separation of viral and cellular DNA in the CsCl density gradient.

Ten μl of each DNA preparation was spotted on 2.5-cm filter paper circles (Schleicher & Schuell, Inc., Keene, N.H.) which were processed as described below. The radioactivity contained on each filter paper circle was counted in a Packard liquid scintillation spectrometer model 3320 (Packard Instrument Co., Downers Grove, Ill.) to determine what volume of the DNA preparation should be applied to each CsCl gradient in order to achieve satisfactory separation. In general, 20 or 30 μl of the DNA preparation was the volume added to each gradient.

Isopycnic density gradient centrifugation. CsCl density gradient ultracentrifugation was used to separate viral DNA from cellular DNA. The DNA preparation (20–30 μl) was added to 9.9 g CsCl in 8.0 ml TES. After centrifugation at 15° using a Ti-50 rotor in a Beckman model L3-50 preparative ultracentrifuge, eight drop fractions were withdrawn from the bottom of the tube using a Buchler polystaltic pump and an LKB Ultrorac fraction collector (LKB Products, Bromma, Sweden). Fifty-μl samples were spotted on filter paper circles and processed and counted as described below. About forty fractions were collected from each tube and every fifth fraction was used to measure refractive index and determine buoyant density.

Liquid scintillation spectrometry. In order to determine the amount of [^{3}H]thymidine incorporated into DNA, 50 μl of each CsCl fraction was spotted in triplicate on filter paper circles and processed as follows. The filter papers were dried rapidly under an infrared lamp and washed three times with ice-cold 5% trichloroacetic acid, twice with 95% ethanol, and once with diethyl ether. The dried circles of filter paper were placed in vials containing 12 ml of 0.26% PPO (2,5-diphenyloxazole) in toluene for counting in a liquid scintillation spectrometer.

RESULTS

Inhibition of HSV-induced syncytia formation by IMPY, ara-A and ara-H. Table 1 compares and contrasts the inhibitory effects of IMPY, ara-A and ara-H on the ability of the HF strain of herpex simplex virus type 1 to induce syncytia in monolayers of BHK-21/4 cells. IMPY, at a concentration of 80 μg/ml or 0.73 mM, reduced syncytia formation approximately 80 per cent. In order to compare IMPY, ara-A and ara-H in potency of syncytia reduction, a 50 per cent inhibitory dose was calculated for each drug. Our results indicated that the antiviral potencies of the three drugs were ranked in the order ara-A > ara-H > IMPY, the 50 per cent inhibitory doses being 22, 195 and 309 μM respectively.

Determination of virus rating. Utilizing the microplate procedure of Sidwell and Huffman [8] a virus rating (VR) was calculated for IMPY, ara-A and ara-H. A summary of the results is listed in Table 2. In contrast to the syncytia reduction studies, the three drugs were rated in the order ara-A > IMPY = ara-H, on the basis of the VR that each received (0.84, 0.68 and 0.66 respectively). Thus, IMPY appeared to possess antiviral activity equivalent to that of ara-H according to the criteria set forth in the studies of Sidwell and Huffman [8]. In addition, IMPY showed less cytotoxicity than ara-A and approximately the same cytotoxicity as ara-H on a per mole basis.

Inhibition of cellular and viral DNA synthesis in HSV-infected KB cells. DNA synthesized in HSV-infected monolayer cultures of KB cells during a 12-hr exposure to [^{3}H]thymidine was separated into viral and cellular components in CsCl density gradients. Each species of DNA was separated on the basis of its buoyant density, owing to the different G + C ratios of HSV DNA and KB cell DNA. Typical profiles of radioactivity in fractions from CsCl

Table 1. Effect of IMPY, ara-A and ara-H on syncytia formation in HSV-infected BHK-21/4 cells*

	Per cent reduction of HSV-induced syncytia forming units when compared to control cultures (no drug) Concentration of drug (μg/ml)								
Drug	0.1	1	2	5	10	20	40	80	160
IMPY					14 ± 21†	50 ± 23	55 ± 4	80 ± 4	78 ± 15
Ara-A‡	20	26	29	40	71	97	99	>99	100
Ara-H‡					4	2	35	65	85

* Two hundred syncytium forming units (SFU) of herpes simplex virus were added to nearly confluent monolayers of BHK-21/4 cells in 60 × 15 Integrid plastic dishes. Adsorption was allowed to occur at 37° for 1 hr, after which time 5 ml of Ham's Nutrient Mixture F-12 medium supplemented with 5% fetal bovine serum and the appropriate drug concentration was added. Syncytia were counted after a 20-hr incubation.

† All values are ± the standard error of the mean.

‡ Data were obtained from previous studies in our laboratory [4].

Table 2. Microtest screen of IMPY, ara-A and ara-H on BHK-21/13 cells infected with HSV-1 (10 $TCID_{50}$/well)*

Drug	Concentrations used in test wells† (μg/ml)	Virus rating at 72 hr
IMPY	0, 3.2, 10, 32, 100 and 320	0.68 ± 0.01‡
Ara-A	0, 3.2, 10, 32, 100 and 320	0.84 ± 0.01
Ara-H	0, 10, 32, 100, 320 and 1000	0.66 ± 0.02

* Monolayers of BHK-21/13 cells, grown in micro tissue culture plates, were infected with HSV-1 (10 $TCID_{50}$/well) and incubated with the appropriate drug concentration at 37° in a humidified CO_2 atmosphere for 72 hr. The virus rating for each drug was calculated according to the methods of Sidwell and Huffman [8].

† In all cases, concentrations were selected which ranged from completely inactive to totally cytotoxic.

‡ All values are ± the standard error of the mean.

density gradients are shown in Fig. 1. Presumably because a technical grade of CsCl was used to prepare the gradients, buoyant densities slightly less than the expected values were obtained.

Viral and cellular DNA synthesis in drug-treated cultures was compared to the viral and cellular DNA synthesized in the virus-infected cultures without drug. The radioactivity associated with viral and cellular DNA was determined by summing the cpm in the fractions which composed each peak in Fig. 1. The ratio of viral DNA to cellular DNA was calculated and used to proportion the total DNA synthesized in the infected cells into the viral and cellular components. Data from drug-treated cultures were normalized to data from the control culture. The per cent reduction of viral and cellular DNA synthesized at each drug level was then computed with respect to the control cultures containing no drug (Fig. 2). This figure illustrates that, in infected monolayer cultures, IMPY inhibited cellular DNA synthesis to a greater extent than viral DNA synthesis at all drug levels studied.

The results shown in Fig. 2 were used to determine the degree of selectivity (°S) shown by IMPY and that of ara-A and ara-H in reducing viral and cellular DNA synthesis. The equation below allows the calculation of a value which expresses the positive or negative selectivity of a drug in its action on DNA synthesis in virus-infected cell cultures:

$$^{\circ}S_n = \log_{10}\left[\frac{C/C'}{V/V'}\right],$$

where C is equal to the total number of cpm/cell contained within the CsCl gradient peak representing cellular DNA synthesized when drug is present at concentration n. C' is equal to the total number of cpm/cell contained within the CsCl gradient peak representing cellular DNA synthesized when no drug was present. V is the total number of cpm/cell contained in the CsCl gradient peak representing viral DNA synthesized when drug was present at concentration n. V' is equal to the total number of cpm/cell contained within the CsCl gradient peak representing viral DNA synthesized in the control cultures when no drug was present.

The results of a selectivity analysis of IMPY, ara-A and ara-H are illustrated in Fig. 3. Positive values of °S indicate that the drug inhibits viral DNA syn-

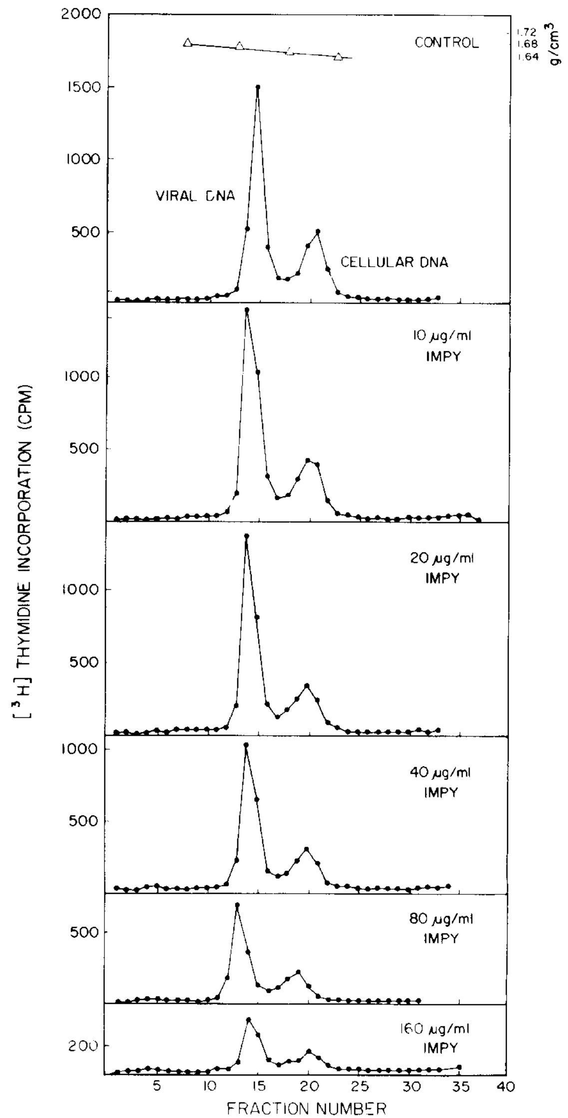

Fig. 1. Separation by isopycnic centrifugation in CsCl density gradients of viral and cellular DNA synthesized in HSV-infected monolayer cultures of KB cells. Location of viral and cellular DNA in the gradients was monitored by [^{3}H]thymidine incorporation. Cultures contained no drug or 10, 20, 40, 80 or 160 μg/ml of IMPY.

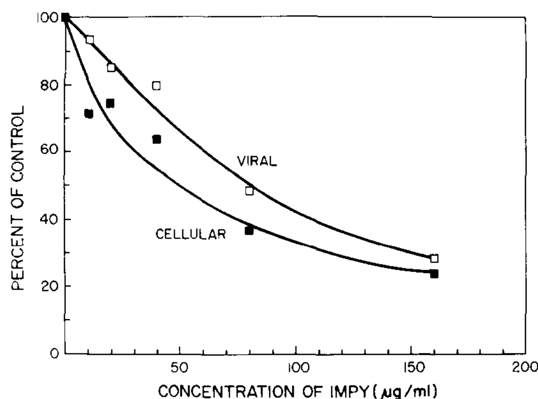

Fig. 2. Effect of IMPY on the reduction of viral and cellular DNA synthesis in monolayer cultures of KB cells. The total cpm contained within each peak of viral or cellular DNA was computed from Fig. 1 and corrected for variation in cell number.

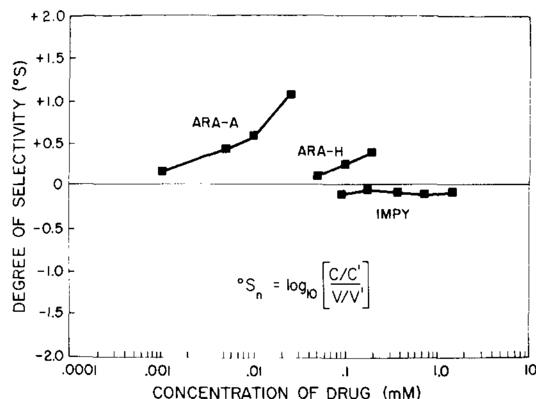

Fig. 3. Degree of selectivity (°*S*) at drug concentration *n* of IMPY, ara-A and ara-H in HSV-infected monolayer cultures of KB cells. *C* is equal to the total number of cpm/cell contained within the CsCl gradient peak representing cellular DNA synthesized when drug is present at concentration *n*. *C'* is equal to the total number of cpm/cell contained within the CsCl gradient peak representing cellular DNA synthesized when no drug was present. *V* is the total number of cpm/cell contained in the CsCl gradient peak representing viral DNA synthesized when drug was present at concentration *n*. *V'* is equal to the total number of cpm/cell contained within the CsCl gradient peak representing viral DNA synthesized when no drug was present. A positive value of °*S* indicates that the inhibition of viral DNA synthesis is greater than cellular DNA synthesis at the dose under consideration. When a drug concentration results in cellular DNA synthesis being inhibited more extensively than viral DNA synthesis, a negative value of °*S* results.

thesis to a greater extent than cellular DNA synthesis at the concentration indicated, whereas negative °*S* values reflect more extensive inhibition of cellular than viral DNA synthesis. IMPY was found to possess negative selectivity at all concentrations studied. In contrast, ara-A and ara-H both express positive selectivity.

DISCUSSION

The antiviral activity of IMPY was assessed in mammalian cell cultures infected with herpes simplex virus type 1. Our results indicated that IMPY exerted an antiviral action on HSV-1 in syncytia reduction studies and microplate tissue culture assays but further biochemical studies demonstrated that the inhibitory effects of IMPY were not directed solely toward viral infection. Determination of the drug's effect on viral and cellular DNA synthesis indicated that IMPY reduced cellular DNA synthesis to a greater extent than viral DNA synthesis at all drug levels studied.

We have attempted to evaluated the degree of selectivity (°*S*) of a drug with respect to its differential effect on viral and cellular DNA synthesis. A drug was assigned a positive °*S* if it acted to reduce viral DNA synthesis to a greater extent than cellular DNA synthesis. Conversely, a drug which inhibited cellular DNA synthesis more than viral DNA synthesis was designated as being negatively selective. Although this index of selectivity has proved useful in our studies comparing the antiviral activities of various drugs, our definition of °*S* possesses several limitations which should be considered.

The primary limitation of degree of selectivity as we have defined it resides in the fact that each value of °*S* is dose dependent. As a result, as the denominator of the equation approaches zero, °*S* becomes artificially high, and this exaggeration must be taken into account when interpreting the data.

Our definition of °*S* evaluates viral and cellular DNA synthesis in virus-infected cell cultures only. This application does not consider the general inhibitory effect of a viral infection on the cellular DNA synthetic process. Depending on the multiplicity of viral infection, the initiation of viral DNA synthesis with its inherent domination of the cellular DNA synthetic machinery may affect the total amount of cellular DNA produced. Thus, by not incorporating the total amount of cellular DNA synthesized in the uninfected cell into our concept of degree of selectivity, we may be introducing an error into our evaluation. Further selectivity studies of various drugs have been performed in our laboratory involving both virus-infected and uninfected cell cultures. A modified, dose-independent definition of °*S* has been formulated and will be presented at a future time [9].

On the basis of the criteria discussed above, our results indicated that IMPY possessed a negative value of °*S* in HSV-infected mammalian cells at all drug levels studied. The reason for this negatively selective action is unknown at the present time. IMPY may act by exerting a differential inhibition of a cellular enzyme as opposed to its viral-induced enzyme counterpart in the infected cell.

In contrast to IMPY, ara-A and ara-H were found to exhibit different degrees of positive selectivity, reflecting the fact that both drugs inhibit viral DNA synthesis more than cellular DNA synthesis. Previous studies in our laboratory suggest that the selective inhibition of viral DNA synthesis by ara-A and ara-H may be due to the preferential inhibition of a step in nucleotide metabolism catalyzed by a virus-specified enzyme [4,5].

The results presented in this paper do not offer any definitive explanation for the mechanism of action of IMPY. Previously, Ennis *et al.* [1] suggested that IMPY inhibited DNA synthesis by interfering with deoxyguanosine triphosphate synthesis. They observed that the inhibition of DNA synthesis seen with IMPY was prevented and reversed by deoxyguanosine and potentiated by deoxycytidine in L cells [1]. In contrast to these studies, Beer *et al.* [2] demonstrated that addition of deoxynucleosides failed to reverse or prevent the inhibition of DNA synthesis by IMPY in HeLa cells. Further experimentation with regard to the effect of IMPY on cellular and viral metabolism and enzyme action is warranted.

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