THE SELECTIVE INHIBITION OF VIRAL DNA SYNTHESIS BY CHEMOTHERAPEUTIC AGENTS: AN INDICATOR OF CLINICAL USEFULNESS?*

John C. Drach and Charles Shipman, Jr.

Dental Research Institute
The University of Michigan
Ann Arbor, Michigan 48109

INTRODUCTION

The fundamental reasons and test systems available for the empirical screening of antiviral substances was discussed by Herrmann in his review published some 15 years ago.[1] Today there are a number of *in vitro* procedures available to investigators interested in antiviral chemotherapy. Herrmann and coworkers described an agar diffusion method for the detection and bioassay of antiviral substances.[2] Plastic panels frequently are used for determining the cytotoxicity and antiviral efficacy of candidate compounds.[3,4] A mathematical treatment of data derived from panel procedures yields a "virus rating" (VR). The VR is a measure of the inhibition of the virus cytopathogenic effect by a drug at levels nontoxic or partially toxic for the host cell. In an attempt to measure the selective inhibition of viral nucleic acid synthesis compared with host nucleic acid synthesis, Miller and associates[5] developed a procedure utilizing the uptake of radioisotopically labeled uridine or thymidine as a criterion for antiviral activity. Although each of the above procedures has been successfully employed in antiviral screening studies, they have not been able to uniformly predict the clinical usefulness of putative antiviral drugs.

In previous studies on the biochemical action of the antiviral drug 9-β-D-arabinofuranosyladenine (ara-A),[6,7] we noted that herpes simplex virus (HSV) DNA synthesis was inhibited to a greater degree by the drug than was host DNA synthesis. It is possible that the ability of ara-A to preferentially inhibit viral DNA synthesis may be related to the drug's favorable therapeutic index in experimental animals[8] and man.[9] Therefore, we have developed an evaluation procedure to quantitate the selective activity of ara-A and have applied it to the evaluation of other potentially useful compounds. The procedure can be used advantageously with human herpesviruses and involves the mathematical determination of a value that we have termed the "selective index" (SI). The SI is derived from a measurement of the effects of a drug on host and viral DNA synthesis by comparing 50% inhibitory (I_{50}) concentrations. A drug will have a positive SI if it inhibits viral DNA synthesis to a greater extent than host DNA synthesis. Conversely, a drug that inhibits host DNA synthesis more than viral DNA synthesis will have a negative SI.

MATERIALS AND METHODS

Materials

The following drugs were obtained through the courtesy of the corresponding investigators: 9-β-D-arabinofuranosyladenine (ara-A, Vira-A®), Dr. H. E.

Machamer, Parke, Davis and Co., Detroit, Mich.; 1-β-D-arabinofuranosylcytosine (ara-C, Cytosar[R]), Dr. H. E. Renis, The Upjohn Co., Kalamazoo, Mich.; coformycin, Dr. H. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan; stallimycin (formerly Distamycin A), Dr. J. A. Page, Adria Laboratories, Inc., Wilmington, Del.; phosphonoacetic acid (PAA), Dr. R. G. Duff, Abbott Laboratories, North Chicago, Ill.; and ribavirin (Virazole[R]), Dr. R. W. Sidwell, ICN Nucleic Acid Research Institute, Irvine, Calif. The following materials were purchased from the indicated sources: 9-β-D-arabinofuranosylhypoxanthine (ara-H), Pfanstiehl Laboratories, Inc., Waukegan, Ill.; 4-(2-hydroxyethyl)-1-piperazineëthanesulfonic acid (HEPES) and pronase, Calbiochem, La Jolla, Calif.; sarkosyl, Geigy Industrial Chemicals, Chicago, Ill.; [methyl-³H]-thymidine (dThd), New England Nuclear, Boston, Mass.; and CsCl, Kawecki Berylco Industries, Inc., Boyertown, Pa.

Cell Culture Techniques

The source of cells and HSV Type 1, the routine growth and passage of BHK 21/4 cells and KB cells, the propagation and titration of HSV, and the techniques used for the enumeration of cells, the determination of cell viability, and the detection of mycoplasma contamination have been described previously.[6]

Protocol for Studies in Monolayer Cultures

KB cells were planted in plastic tissue culture flasks (75 cm² surface area) (Falcon Plastics, Oxnard, Calif.) 18 to 24 hr prior to infection with HSV. At the onset of experiments, logarithmically growing, replicate monolayer cultures were 60% to 70% confluent and contained 4 to 6 × 10⁶ cells/flask. The medium was decanted and the cultures were infected with 10 plaque-forming units (PFU) of HSV per cell. The HSV was contained in 1.0 ml of minimal essential medium with Hanks salts [MEM(H)] supplemented with 10% unheated calf serum. Mock-infected monolayer cultures received 1.0 ml of medium alone. After a 1-hr adsorption period at 37°, the cell sheet was rinsed twice with 5 ml of MEM(H) to remove unadsorbed virus and 10.0 ml of medium containing [³H]thymidine (dThd, 5 μM, 2 or 3 μCi/ml), 2X arginine, and 10% extensively heated calf serum[8] was added. Drugs were added to the medium at the selected concentrations immediately prior to use. Following a 12- or 16-hr† incubation at 37°, 0.5 ml of 0.25 mM ethylenediaminetetraacetate (EDTA) was added to uninfected monolayer cultures and after several minutes at 37° flasks were shaken to suspend the cells. Infected monolayers were shaken to suspend the cells and 1.0 ml aliquots were removed and stored at −76° for subsequent virus assay. EDTA (0.45 ml) was added to the remaining medium to bring the final concentration to that in the uninfected cultures. Aliquots (100 μl) of the suspensions were spotted in triplicate on filter paper circles, which were processed for the determination of incorporated label as described previously.[6] Data were tabulated on the basis of tritium incorporated into acid-insoluble material per 10⁵ cells. Inhibition of DNA synthesis was determined at the various drug levels as a percent of a control without drugs. Five-ml aliquots of infected cell suspensions were centrifuged at 600g for 5 min and the pellets stored at −76° for subsequent separation of viral and cellular DNA. Following isopycnic centrifugation in CsCl gradients[6] the amount of

†Previous work in our laboratory[6,7] established that the synthesis of viral DNA was complete and drug effects were maximal 10–12 hr postinfection.

label was proportioned between viral and cellular DNA on the basis of peak areas. Areas were determined directly or by integration after the peaks were fully separated using a Dupont 310 curve resolver set to fit Lorentzian distribution curves.

Statistical Analyses

Dose-response relationships were constructed by linearly regressing probit values of the percent inhibition of DNA synthesis or plaque formation against log drug concentrations. The 50% inhibitory (I_{50}) concentrations and the 95% confidence intervals were calculated from the regression lines using the methods described by Goldstein.[10] Identity of any two dose-response curves was tested by calculating p values for the equality of the regressions and slopes.[11] Unless otherwise noted, the slopes of all the pairs of dose-response curves compared in this study were similar (p > 0.350); consequently p values given in the text refer only to the comparison of regressions.

RESULTS

Inhibition of Total DNA Synthesis

Incorporation of labeled dThd into acid-insoluble material was determined in the presence and absence of drugs. Incorporation of label was taken as measure of the total amount of DNA synthesized by uninfected and HSV-infected monolayer cultures of KB cells. FIGURE 1 illustrates the inhibition of total DNA synthesis in uninfected and infected cells by ara-C and ara-A plus 3.5 μM coformycin.‡ Ara-C inhibited total DNA synthesis in uninfected cells more effectively than it inhibited DNA synthesis in HSV-infected cells. This was observed at all drug concentrations tested in three separate experiments. Construction of dose-response relationships using all values from these experiments (FIGURE 1) showed the degree of inhibition to be significantly different (p < 0.0001) in infected and uninfected cells. Interpolation of 50% inhibitory (I_{50}) concentrations from these lines (FIGURE 1, TABLE 1) indicated that DNA synthesis in uninfected cells was inhibited more than three times as effectively. In contrast, no consistent differences were observed in five experiments on the effects of ara-A plus coformycin on total DNA synthesis. Analysis of the overall dose-response relationships (FIGURE 1) confirmed that the drug effect was virtually the same (p = 0.41) in uninfected and HSV-infected cells. Fifty percent inhibitory concentrations also were virtually identical for uninfected and infected cells with a complete overlap of the corresponding 95% confidence intervals (FIGURE 1, TABLE 1). We previously have shown that ara-H also inhibited total DNA synthesis to the same extent (p = 0.69) in uninfected and HSV-infected KB cells.[7] Ara-A without an adenosine deaminase inhibitor, however, was two times more effective in inhibiting total DNA synthesis in HSV-infected cells (TABLE 1); the two dose-response curves were parallel but not coincident (p = 0.015).[7]

Several other drugs have been examined in the same manner. TABLE 1 also summarizes the effects of these drugs, expressed as I_{50} concentrations, on total DNA synthesis (TABLE 1, columns 1 & 2). PAA and stallimycin appeared to be somewhat more effective (45% and 78%, respectively) in inhibiting total DNA synthesis in HSV-infected cells compared with uninfected cells. However, the pairs of dose-

‡Coformycin at this concentration totally inhibited adenosine deaminase activity without affecting either viral replication or cellular and viral DNA synthesis.[7]
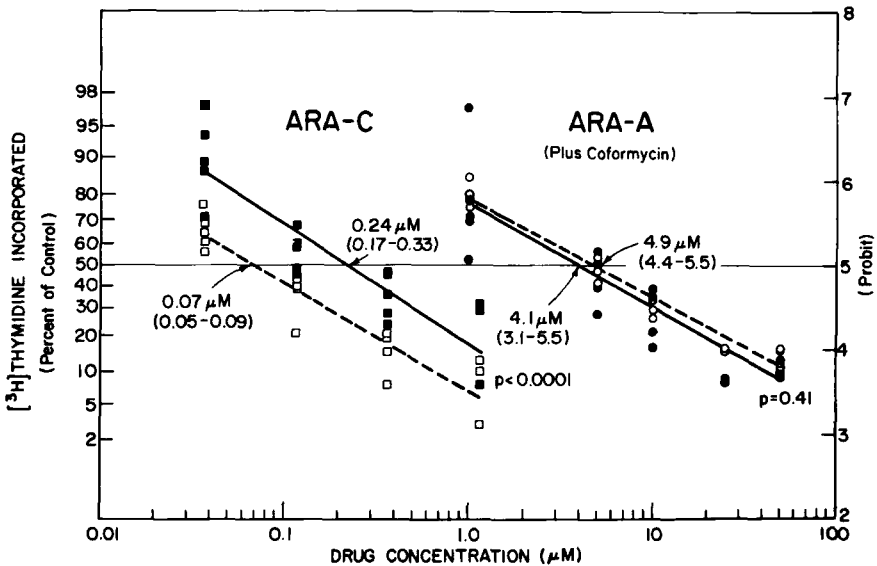
FIGURE 1. Dose-response relationships between drug concentration in culture medium and inhibition of total DNA synthesis. Mock-infected (open symbols, dashed lines) and HSV-infected (closed symbols, solid lines) monolayer cultures of KB cells were exposed to [³H]dThd and drugs for 12 hr. Amount of label incorporated into acid precipitable material was determined in triplicate on aliquots of harvested cells. In the absence of drugs approximately 20,000 or 40,000 cpm were incorporated per 10⁵ cells by mock-infected or HSV-infected cultures, respectively. I₅₀ concentrations (and corresponding 95% confidence intervals) were calculated from the regression lines. The p values refer to a test of the equality (coincidence) of the pairs of regression lines.

response curves used to calculate these concentrations were not significantly different (p = 0.25 and 0.13, respectively). Like ara-C, ribavirin was more potent (10-fold) in uninfected KB cells than in HSV-infected cells. The two dose-response curves were parallel but separate (p < 0.0001).

Inhibition of DNA Synthesis in HSV-Infected Cells

Lysates were prepared from all HSV-infected cultures and were subjected to isopycnic centrifugation in CsCl gradients to separate cellular and viral DNA. The effects of ara-A plus 3.5 μM coformycin (panel A) and ara-C (panel B) on the amount of [³H]dThd incorporated during 12 hr of labeling are shown in Figure 2. As is evident from the figure, viral DNA synthesis was inhibited to a significantly greater extent than was cellular DNA synthesis by ara-A plus 3.5 μM coformycin. In contrast, ara-C inhibited the synthesis of both DNA species to approximately the same extent. The data from five ara-A and three ara-C replicate experiments are summarized as dose-response curves in FIGURE 3. Ara-A plus 3.5 μM coformycin inhibited viral DNA synthesis three times more effectively than it inhibited cellular DNA synthesis based upon the difference in the dose-response curves (p < 0.0001) and the difference in the corresponding I₅₀ concentrations (TABLE 1, columns 3 & 4). In a previous report[7] we

TABLE 1

INHIBITION OF DNA SYNTHESIS BY ANTIVIRAL DRUGS*

Drug	I_{50} Concentration, in μM (and Corresponding 95% Confidence Interval†)			
	DNA Synthesis in Uninfected KB cells	Total DNA Synthesis in HSV-Infected KB Cells	Cellular DNA Synthesis in HSV-Infected KB Cells	Viral DNA Synthesis in HSV-Infected KB Cells
Ara-C	0.068	0.24	0.23	0.21
	(0.053–0.087)	(0.17–0.33)	(0.14–0.39)	(0.15–0.30)
Ara-A plus	4.9	4.1	8.0	2.6
3.5 μM coformycin‡	(4.4–5.4)	(3.1–5.5)	(6.1–10.5)	(2.0–3.3)
Ara-A‡	62	31	67	20
	(45–85)	(19–48)	(34–120)	(13–30)
Ara-H‡	309	215	> 750	126
	(165–578)	(130–357)	ND§	(88–182)
Ara-H	299	402	583	30
(MOI = 1)	(200–448)	(277–584)	(322–1050)	(23–38)
Ribavirin	1.9	184	> 1500	145
	(1.1–3.3)	(47–723)	ND§	(111–191)
Stallimycin¶	423	238	671	206
	(244–734)	(171–333)	(268–1680)	(130–327)
IMPY**	ND††	ND††	455	763
			(297–697)	(615–946)
PAA	1038	712	1398	485
	(337–2202)	(384–1323)	(389–5025)	(307–766)

*Performed in monolayer culture at a multiplicity of infection (MOI) = 10.

†That range of values which, at the 95% confidence level, contains the true value.

‡Taken in part from the data of Schwartz, Shipman, and Drach.[7]

§Not determined accurately owing to a poor fit ($r^2 \leq 0.3$) of data points to dose-response curve.

¶Formerly designated Distamycin A.

**Calculated from the data of Pelling and Shipman.[12]

††Not determined.

established that both ara-A (without coformycin) and ara-H also were three times more effective as inhibitors of viral DNA synthesis (TABLE 1). In comparison, ara-C inhibited neither cellular nor viral DNA synthesis preferentially (p = 0.39, FIGURE 3). Examination of individual gradients showed, however, that in all experiments ara-C concentrations ≤ 0.2 μM inhibited cellular DNA synthesis in slight preference to viral DNA synthesis, whereas at concentrations greater than this the opposite was true (FIGURE 2).

Similar experiments have been performed to evaluate the effects of PAA, ribavirin, stallimycin, and 2,3-dihydro-1H-imidazo[1,2-b]pyrazole (IMPY). The activity of the last compound against HSV has been reported by Pelling and Shipman.[12] The first three compounds all produced a greater inhibition of viral DNA synthesis than of cellular DNA synthesis. Stallimycin was approximately 3-fold more potent in this regard. However, variation in the degree of inhibition of cellular DNA synthesis observed in duplicate experiments resulted in some overlap of 95% confidence intervals (TABLE 1) and a degree of identity between the two dose-response

curves (p = 0.11, data not shown). More pronounced variations in the effects of PAA and ribavirin on cellular DNA synthesis (TABLE 1) make exact comparisons of the I_{50} concentrations difficult—even though cellular DNA synthesis appears to be much less sensitive to the two drugs than does viral DNA synthesis. IMPY affected the synthesis of the two DNA species in an opposite manner. This compound was 68% more potent in its inhibition of cellular DNA synthesis. The two dose-response curves used to calculate I_{50} concentrations were not coincident (p = 0.003, data not shown).

Calculation of the Selective Index

In the preceding sections we have compared drug effects on total DNA synthesis and on separated cellular and viral DNA synthesis. We believe, however, that the
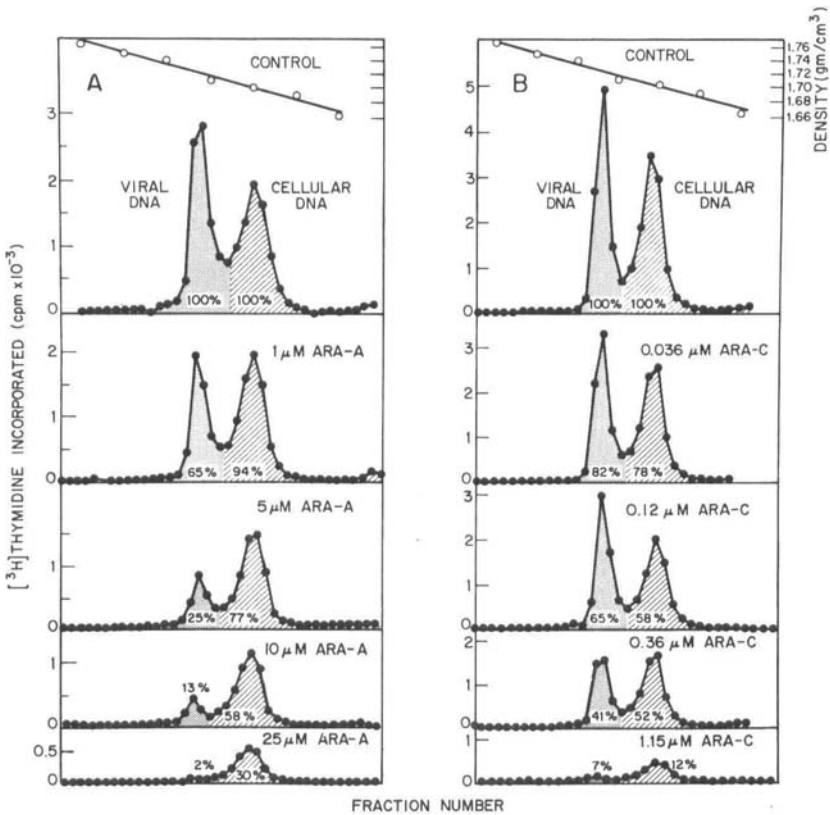

FIGURE 2. Separation of viral and cellular DNA from HSV-infected cells by isopycnic centrifugation in CsCl gradients. Amount of label incorporated into cellular and viral DNA was measured in the absence and presence of ara-A plus 3.5 μM coformycin (panel A) and ara-C (panel B). The amount is expressed as a percentage of the amount incorporated into the respective DNA species in cultures without drugs.
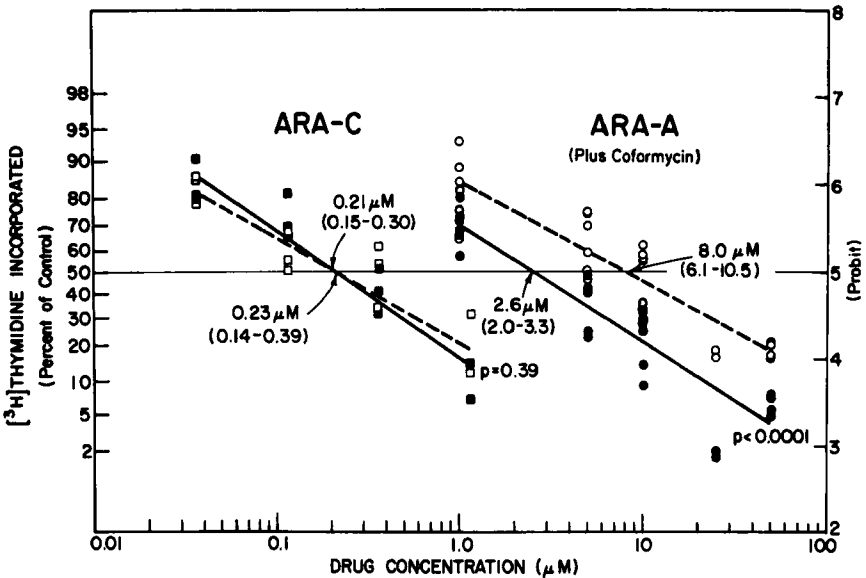
FIGURE 3. Dose-response relationships between drug concentration in culture medium and inhibition of DNA synthesis in HSV-infected cells. DNA was labeled in the experiments depicted in FIGURE 1 and separated as illustrated in FIGURE 2. The amount of [³H]dThd incorporated into cellular DNA (open symbols, dashed lines) and viral DNA (closed symbols, solid lines) is expressed as a percentage of the amount incorporated into the respective DNA species in the absence of drugs. I_{50} concentrations and p values were determined as described in the legend to FIGURE 1.

most valid comparison is between the effects of drugs on DNA synthesis in uninfected cells and the effects on viral DNA synthesis in HSV-infected cells. To aid in this comparison and to more clearly define the relationship, we have derived an index that quantifies the preferential inhibition of viral or uninfected cellular DNA synthesis. The I_{50} concentration of a drug for DNA synthesis in uninfected cells is divided by the I_{50} concentration for viral DNA synthesis. We have termed the logarithm of this ratio the "selective index" (SI). That is,

$$SI = \log_{10} \frac{I_{50} \text{ concentration for DNA synthesis in uninfected cells}}{I_{50} \text{ concentration for viral DNA synthesis in HSV-infected cells}}.$$

The SI is positive if viral DNA synthesis is inhibited preferentially and negative if uninfected cellular DNA synthesis is more strongly inhibited.

SI values were calculated for the drugs evaluated in this study from the data listed in TABLE 1 (columns 1 & 4). TABLE 2 ranks these drugs according to their SI. Ara-A plus coformycin, ara-A, ara-H, PAA, and stallimycin were positively selective whereas ara-C and ribavirin were negatively selective.§ The coincidence of the two dose-response regression lines (from which the component I_{50} values were calcu-

§To simplify descriptions, we have termed drugs that preferentially inhibit viral DNA synthesis as "positively selective" and drugs that preferentially inhibit host cell DNA synthesis as "negatively selective."

TABLE 2

SELECTIVE INDICES OF DRUGS EVALUATED IN UNINFECTED
AND HSV-INFECTED* MONOLAYER CULTURES OF KB CELLS

Selective Index	Drug
0.5	Ara-A
0.4	Ara-H
0.3	Stallimycin, PAA, ara-A + †
0.2	
0.1	
0.0	- - - - - - - - - - - - - - - - - -
−0.1	
−0.2	
−0.3	
−0.4	
−0.5	Ara-C
.	
.	
.	
−1.9	Ribavirin

*MOI (multiplicity of infection) = 10.
†Ara-A + = ara-A plus 3.5 μM coformycin.

lated) was tested by comparing p values for the equalities of the slopes and regressions of the two lines. The pairs of dose-response regression lines for ara-C, ara-A plus coformycin, ara-A, ara-H, and stallimycin were parallel (the slopes were similar, $p \gtrless 0.3$). With the exception of stallimycin, the regressions were different (the parallel lines were defined by separate regressions, $p \leq 0.05$). For example, the dashed line in FIGURE 1 for ara-C was parallel to, but separate from, the solid line in FIGURE 3. On the other hand, the pairs of dose-response lines for PAA and ribavirin were not parallel ($p < 0.005$) and therefore not coincident.

Effect of Cell Propagation Method on SI

In a previous communication[7] we reported that ara-A and ara-H were more selective in suspension cultures than in monolayer cultures. This was the result of a greater inhibition of viral DNA synthesis in HSV-infected cells grown in suspension culture. We speculated that the cause was a less efficient adsorption of HSV than had occurred in monolayer cultures. We now have tested this speculation by evaluating ara-H in monolayer cultures infected with HSV at a multiplicity of infection (MOI) of 1 rather than 10. The selectivity of ara-H was increased to 1.0 in monolayer cultures infected at a MOI of 1 (TABLE 3). This was the result of an increase in the drug sensitivity of viral DNA synthesis and not of a change in the drug sensitivity of DNA synthesized in uninfected KB cells (TABLE 1).

Inhibition of HSV Replication

The effect of drugs on HSV replication in KB cells was measured by assaying in BHK-21/4 cells the number of plaque-forming units of newly synthesized virus. Dose response relationships were constructed for each drug (data not shown) and com-

TABLE 3

SELECTIVE INDICES OF DRUGS EVALUATED
UNDER VARIOUS CULTURE CONDITIONS

Method of Cell Propagation	Drug	MOI	Selective Index
Suspension	Ara-A+*	3	0.7†
	Ara-H	3	0.6†
Synchronized Suspension	Ara-A	15	1.1‡
	Ara-H	15	1.1‡
Monolayer	Ara-A	10	0.5
	Ara-A+*	10	0.3
	Ara-H	10	0.4
	Ara-H	1	1.0

*Ara-A + = ara-A plus 3.5 µM coformycin.
†Calculated from the data of Schwartz et al.[7]
‡Calculated from the data of Shipman et al.[6]

pared with the corresponding relationships for inhibition of viral DNA synthesis. TABLE 4 illustrates that there was close correspondence between the I_{50} concentrations for viral DNA synthesis and the I_{50} concentrations for HSV replication. With the exception of stallimycin, the pairs of dose-response lines used to calculate the respective I_{50} concentrations were nearly coincident ($p > 0.25$). Stallimycin appeared to inhibit HSV replication to a slightly greater extent than it inhibited viral DNA synthesis.

TABLE 4

INHIBITION OF HSV REPLICATION BY ANTIVIRAL DRUGS

Drug	I_{50} Concentration, in µM (and Corresponding 95% Confidence Interval)		pt
	Viral DNA Synthesis*	HSV Replication	
Ara-C	0.21 (0.15–0.30)	0.19 (0.13–0.26)	0.30
Ara-A plus 3.5 µM coformycin‡	2.6 (2.0–3.3)	2.1 (1.0–4.6)	0.27
Ribavirin	145 (111–191)	123 (50–302)	0.64
Stallimycin	206 (130–327)	142 (103–194)	0.01
PAA	485 (307–766)	577 (293–1137)	0.83

*Presented also in TABLE 1.
†Dose-response regression lines for inhibition of viral DNA synthesis were compared to the corresponding lines for inhibition of HSV replication by calculating p values for the equality of the slopes and regressions of the two lines. The slopes of each pair of lines for the listed drugs were virtually the same ($p \geq 0.5$), consequently the p value in the table refers only to a comparison of regressions.
‡Calculated from the data of Schwartz et al.[7]

DISCUSSION

The biochemical method described herein has been used to evaluate the specificity of drug action on host cell and viral DNA synthesis. The method is proposed not as a primary screening technique for antiviral drugs but rather as an additional, more specific test of drugs found active in the more routine *in vitro* tests.[1-4] Together, information derived from both procedures could be valuable in predicting drug efficacy and toxicity *in vivo*. That is, positively selective drugs that are active against DNA viruses might be expected to cause less cytotoxic manifestations *in vivo* (e.g., depression of immune mechanisms, bone marrow, etc.) than drugs that are negatively selective. Although we have utilized only HSV Type 1 in the current study, the method should be applicable to any herpesvirus whose DNA can be separated from host cell DNA by isopycnic centrifugation. This would include HSV Type 2, cytomegalovirus, Epstein-Barr virus, and possibly varicella-zoster virus.[13]

Other biochemical methods have been utilized for the evaluation of antiviral agents. Miller *et al.*[5] developed a method to detect compounds that were selective inhibitors of viral RNA synthesis. Calculation of I_{50} concentrations permitted a direct comparison of drug effects on RNA synthesis in uninfected cells and viral RNA synthesis in infected cells. (Actinomycin D was used to block host RNA synthesis in infected cells.) The method was extended to DNA viruses by digesting unincapsidated DNA with DNAse. De Clercq and Shugar[14] also used a comparison of I_{50} concentrations to calculate "antiviral indices." Inhibition of dThd incorporation or growth of uninfected cells was compared to inhibition of vaccinia-virus-induced cytopathogenicity.

In the present study we have concluded that the best measure of a drug's selectivity should be based upon a comparison of drug effects as described in our definition of the SI, namely, a comparison of the inhibition of DNA synthesis in uninfected, logarithmically growing cells to inhibition of viral DNA synthesis. This appears to best approximate the *in vivo* situation where a drug will exert its antiviral effect based on inhibition of viral DNA synthesis in the infected cell and will cause toxic effects on uninfected proliferating cells at sites that may be remote to the viral infection. We rejected the more simple comparison of drug effects on total DNA synthesis in uninfected and HSV-infected cells as the sole measure of selectivity because host cell DNA synthesis contributes to the latter measurement. The exact nature of cellular DNA in HSV-infected cells and the control of its synthesis is somewhat unclear, and, as judged from the variability of inhibition (see confidence intervals, TABLE 1, column 3), drug effects are more difficult to quantitate. That is not to say, however, that effects on host cell DNA synthesis in infected cells should be ignored. As is evident in FIGURE 2, the selectivity expressed by ara-A plus coformycin is most readily discerned by comparison with cellular DNA synthesis within the infected cells.

Our choice of the 50% inhibitory concentration as the basis for comparing drug effects may complicate the interpretation of selectivity in some instances. If dose-response curves are parallel the I_{50} concentration is simple to determine and will yield the same SI as would the 10% or 90% inhibitory concentrations. However, if dose-response curves are not parallel, as was the case with ribavirin and PAA, then the choice of any inhibitory concentration as the basis of comparing the two curves may be improper. In the latter case comparisons based on the slopes of the lines might be more valid. Fortunately, in the case of PAA and ribavirin the interpretation was the same on either basis of comparison.

The additional possibility that we did not measure drug action on DNA synthesis must be considered. In this and our prior articles[6,7] the incorporation of [3H]dThd

into acid-precipitable material has been equated with DNA synthesis. We have not ruled out the possibility, however, that the noted selective effects were the result of differential changes in the specific activity of [^{3}H]dThd nucleotides, which might accompany drug treatment or HSV-infection. In fact, it has been known for a number of years that HSV-infection increases dThd nucleotide pools in mammalian cells.[15,16] However, for this increase to influence the reported selectivities (both positive and negative) pool sizes in uninfected and HSV-infected cells also would have to change independently with changes in drug concentration. Although we have no data for most of the drugs studied, we have observed that 120 μM ara-A did not alter the labeling of dThd nucleotides in B-mix K-44/6 cells (unpublished observation). Moreover, to attribute the preferential inhibition of viral or cellular DNA synthesis within the infected cell to drug-related pool size changes requires that viral and cellular DNA be synthesized from separate dThd nucleotide pools in the nuclei of HSV-infected cells. Also these pools would have to change independently with drug concentration changes. In addition, the close correspondence between inhibition of viral DNA synthesis and inhibition of HSV replication (TABLE 4) argues that, for the drugs tested, inhibition of [^{3}H]dThd incorporation into viral DNA is a fair measure of inhibition of viral DNA synthesis. Nonetheless, experimental determination of nucleotide pool sizes or specific activity of [^{3}H]dThd incorporated into DNA will be required to totally resolve the issue.

If drug-induced pool size changes do alter the SI, the alteration most likely would be with those drugs whose selectivity arose from differential inhibition of total DNA synthesis—such as ara-C (FIGURE 1) or ribavirin. In the case of ara-C, however, it is interesting that our results are in accord with those of Kaplan and Ben-Porat[17,18] who also observed a preferential inhibition of DNA synthesis in uninfected rabbit kidney cells compared to HSV- or PRV-(pseudorabies virus)-infected cells. These investigators concluded that ara-C was not a specific antiviral agent. The accord between the two sets of data is particularly noteworthy because our experiments were performed with HSV, which increases dThd nucleotide pool size,[15,16] and theirs with PRV, which does not.[19] In contrast, Sidwell and Huffman[4] concluded that ara-C possessed a high degree of antiherpes activity having a VR greater than that of ara-A (1.3 compared with 1.1). Our results with ribavirin also differ with those of Huffman and coworkers.[20] The antiviral activity of the drug appears lower in our KB cells (I_{50} = 123 μM, TABLE 4) than in theirs [minimum inhibitory concentration (MIC) = 4.1 μM], but direct comparisons between I_{50} values and MIC values may be misleading. Of more concern is the potent inhibition of DNA synthesis we observed with ribavirin in uninfected KB cells (I_{50} = 1.9 μM) compared with a relative lack of cytotoxicity measured microscopically by Huffman et al.[20] Although drug-induced changes in dThd nucleotide pool sizes or use of logarithmically growing cultures of KB cells (compared with Sidwell and associates' use of stationary cultures[4,20]) may account for the noted differences, we have no data to support either possibility. On the other hand, the observation that ribavirin exhibited negative selectivity when examined in the usual manner (TABLE 1, columns 1 & 4) but showed positive selectivity against viral DNA synthesis within HSV-infected cells (TABLE 1, columns 3 & 4) may be related to the known cell line specificity of this drug.[20,21]

The selective inhibition of viral DNA synthesis compared with cellular DNA synthesis has been noted with other inhibitors. Becker and Weinberg[22] have reported that a concentration of 96.5 μM stallimycin completely inhibited the synthesis of viral DNA but only partially reduced the amount of cellular DNA. The effects of this drug were not as potent in the current study (TABLE 1, column 4). Furthermore, we observed a progressive decrease in the bouyant density of viral and cellular DNA synthesized in the presence of increasing drug concentrations. This indicates that

even though cellular DNA was synthesized, it may not have been able to function normally. Phosphonoacetic acid similarly has been shown to selectively inhibit the synthesis of HSV DNA.[23] Although we did not observe the nearly total selectivity against viral DNA synthesis (TABLE 1, columns 3 & 4) noted previously[23] the two sets of data probably can be reconciled by Overby and associates' use of confluent cultures.[23] In fact, our results agree in general with those of Overby et al.[23] and Huang[24] who found that concentrations of PAA required to totally block viral DNA synthesis also partially inhibited DNA synthesis in logarithmically growing, uninfected cells. This observation is consistent with DNA polymerase β being insensitive to the drug, whereas DNA polymerase α has equal[25] or lesser[26] sensitivity than does HSV DNA polymerase. Schildkraut et al.[27] have reported the selective inhibition of HSV replication by 5-bromodeoxycytidine and 5-iododeoxycytidine. The selective action appears to be dependent upon a virus-induced enzyme that catalyzed the conversion of the halogenated analogues of deoxycytidine to the corresponding nucleotides. Zinc ions also have been found to inhibit the replication of HSV.[28] Inhibition appears to be a consequence of the selective blockage of viral DNA synthesis through the inhibition of HSV DNA polymerase. Prusoff and colleagues[29] recently reported on the selective inhibition of HSV by 5'-amino-2',5'-dideoxy-5-iodouridine (AIU). Again, the high selectivity of AIU for HSV-infected cells probably is related to the specificity of a HSV-induced enzyme. In contrast, a selective inhibition of *cellular* DNA synthesis in HSV-infected and Epstein-Barr virus-infected cells has been reported with cordycepin[30] and with hydroxyurea.[31]

SUMMARY

A cell culture system has been utilized to measure the effects of drugs on DNA synthesis in uninfected and HSV-(herpes simplex virus)-infected KB cells. DNA from HSV-infected cells was separated into viral and cellular components by isopycnic centrifugation in CsCl gradients. The amount of [³H]thymidine incorporated into acid-insoluble material was measured in the absence and presence of drugs. Dose-response relationships were established by linearly regressing the probit value of the percent inhibition of DNA synthesis against the logarithm of drug concentration. Fifty percent inhibitory (I_{50}) concentrations were interpolated from the corresponding regression lines for inhibition of the following: (i) DNA synthesis is uninfected KB cells, (ii) total DNA synthesis in HSV-infected KB cells, (iii) cellular DNA synthesis in HSV-infected cells, and (iv) viral DNA synthesis in HSV-infected cells.

We have derived an index (SI, selective index) that quantifies the preferential inhibition of viral or uninfected cellular DNA synthesis. This index can be expressed as

$$SI = \log_{10} \frac{I_{50} \text{ concentration for DNA synthesis in uninfected cells}}{I_{50} \text{ concentration for viral DNA synthesis in HSV-infected cells}}.$$

The SI is positive if viral DNA synthesis is inhibited preferentially and negative if uninfected cellular DNA synthesis is more strongly inhibited.

A positive SI value of 0.5 was obtained for the clinically useful antiviral drug arabinosyladenine (ara-A) and a value of 0.4 for its metabolite, arabinosylhypoxanthine (ara-H). Although the adenosine deaminase inhibitor coformycin greatly increased the potency of ara-A, the inhibitor did not increase the selectivity of the drug (SI = 0.3). Stallimycin (distimycin A) (SI = 0.3) and phosphonoacetic acid (SI = 0.3) were similarly effective in preferentially inhibiting the

synthesis of HSV DNA. In contrast, arabinosylcytosine (ara-C) and ribavirin inhibited DNA synthesis in uninfected cells to a greater degree than viral DNA synthesis (SI = -0.5 and -1.9, respectively).

An analysis of the advantages and limitations of this experimental procedure is made and the suggestion is offered that the *in vitro* determination of a drug's selective index may be a valid predictor of clinical usefulness.

REFERENCES

1. HERRMANN, E. C., JR. 1961. The detection, assay and evaluation of antiviral drugs. Progr. Med. Virol. **3**: 158–192.
2. HERRMANN, E. C., JR., J. GABLIKS, C. ENGLE & P. L. PERLMAN. 1960. Agar diffusion method for detection and bioassay of antiviral antibiotics. Proc. Soc. Exp. Biol. Med. **103**: 625–628.
3. EHRLICH, J., B. J. SLOAN, F. A. MILLER & H. E. MACHAMER. 1965. Searching for antiviral materials from microbial fermentations. Ann. N.Y. Acad. Sci. **130**: 5–16.
4. SIDWELL, R. W. & J. H. HUFFMAN. 1971. Use of disposable micro tissue culture plates for antiviral and interferon studies. Appl. Microbiol. **22**: 797–801.
5. MILLER, P. A., H. L. LINDSAY, M. CORMIER, B. R. MAYBERRY & P. W. TROWN. 1973. Rapid semi-automated procedures for assaying antiviral activity. Ann. N.Y. Acad. Sci. **173**: 151–159.
6. SHIPMAN, C. JR., S. H. SMITH, R. H. CARLSON & J. C. DRACH. 1976. Antiviral activity of arabinosyladenine and arabinosylhypoxanthine in herpes simplex virus-infected KB cells. I. Selective inhibition of viral DNA synthesis in synchronized suspension cultures. Antimicrob. Agents Chemother. **9**: 120–127.
7. SCHWARTZ, P. M., C. SHIPMAN, JR. & J. C. DRACH. Antiviral activity of arabinosyladenine and arabinosylhypoxanthine in herpes simplex virus-infected KB cells. II. Selective inhibition of viral DNA synthesis in the presence of an adenosine deaminase inhibitor. Antimicrob. Agents Chemother. **10**: 64–74.
8. KURTZ, S. M. 1975. Toxicology of adenine arabinoside. *In* Adenine Arabinoside: An Antiviral Agent. D. Pavan-Langston, R. A. Buchanan & C. A. Alford, Jr., Eds.: 145–157. Raven Press. New York, N.Y.
9. PAVAN-LANGSTON, D., R. A. BUCHANAN & C. A. ALFORD, JR., Eds. 1975. *In* Adenine Arabinoside: An Antiviral Agent. (*Vide* Parenteral and ophthalmic studies: 197–416). Raven Press. New York, N.Y.
10. GOLDSTEIN, A. 1964. Analysis of a single curve with graded responses. *In* Biostatistics: An Introductory Text: 156–161. The Macmillan Company. New York, N.Y.
11. FOX, D. J. & K. E. GUIRE. 1973. MIDAS: Michigan Interactive Data Analysis System: 59–60. The Statistical Research Laboratory of The University of Michigan, Ann Arbor, Mich.
12. PELLING, J. C. & C. SHIPMAN, JR. 1976. Antiviral activity of 2,3-dihydro-*1H*-imidazo[1,2-*b*]pyrazole (IMPY) in herpes simplex virus type 1-infected mammalian cells. Biochem. Pharmacol. **25**: 2377–2382.
13. GOODHEART, C. R. & G. PLUMMER. 1975. The densities of herpesviral DNAs. *In* Progress in Medical Virology. E. Berger and J. Melnick Eds. Vol. 19: 324–352. Hafner Publishing Co., New York, N.Y.
14. DE CLERCQ, E. & D. SHUGAR. 1975. Antiviral activity of 5-ethyl pyrimidine deoxynucleosides. Biochem. Pharmacol. **24**: 1073–1078.

15. Newton, A., P. P. Dendy, C. L. Smith & P. Wildy. 1962. A pool size problem associated with the use of tritiated thymidine. Nature **194**: 886- 887.

16. Cheng, Y. C., B. Goz & W. H. Prusoff. 1975. Deoxyribonucleotide metabolism in herpes simplex virus infected HeLa cells. Biochim. Biophys. Acta **390**: 253-263.

17. Kaplan, A. S., M. Brown & T. Ben-Porat. 1968. Effect of 1-β-D-arabinofuranosylcytosine on DNA synthesis. I. In normal rabbit kidney cell cultures. Mol. Pharmacol. **4**: 131-138.

18. Ben-Porat, T., M. Brown & A. S. Kaplan. 1968. Effect of 1-β-D-arabinofuranosylcytosine on DNA synthesis. II. In rabbit kidney cells infected with herpes viruses. Mol. Pharmacol. **4**: 139-146.

19. Kaplan, A. S., & T. Ben-Porat. 1970. Nucleoside analogues as chemotherapeutic antiviral agents. Ann. N.Y. Acad. Sci. **173**: 346-361.

20. Huffman, J. H., R. W. Sidwell, G. P. Khare, J. T. Witkowski, L. B. Allen & R. K. Robins. 1973. In vitro effect of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole, ICN 1229) on deoxyribonucleic acid and ribonucleic acid viruses. Antimicrob. Agents Chemother. **3**: 235-241.

21. Marks, M. I. 1974. Variables influencing the in vitro susceptibilities of herpes simplex viruses to antiviral drugs. Antimicrob. Agents Chemother. **6**: 34-38.

22. Becker, Y. & A. Weinberg. 1972. Distamycin A inhibition of Epstein-Barr virus replication in arginine-deprived Burkitt lymphoblasts. Israel J. Med. Sci. **8**: 75-78.

23. Overby, L. R., E. E. Robishaw, J. B. Schleicher, A. Ruether, N. L. Shipkowitz & J. C.-H. Mao. 1974. Inhibition of herpes simplex virus replication by phosphonoacetic acid. Antimicrob. Agents Chemother. **6**: 360-365.

24. Huang, E.-S. 1975. Human cytomegalovirus. IV. Specific inhibition of virus-induced DNA polymerase activity and viral DNA replication by phosphonoacetic acid. J. Virol. **16**: 1560-1565.

25. Bolden, A., J. Aucker, & A. Weissbach. 1975. Synthesis of herpes simplex virus, vaccinia virus, and adenovirus DNA in isolated HeLa cell nuclei. I. Effect of viral-specific antisera and phosphonoacetic acid. J. Virol. **16**: 1584-1592.

26. Leinbach, S. S., J. M. Reno, L. F. Lee, A. F. Isbell & J. A. Boezi. 1976. Mechanism of phosphonoacetate inhibition of herpesvirus-induced DNA polymerase. Biochemistry **15**: 426-430.

27. Schildkraut, I., G. M. Cooper & S. Greer. 1975. Selective inhibition of the replication of herpes simplex virus by 5-halogenated analogues of deoxycytidine. Mol. Pharmacol. **11**: 153-158.

28. Shlomai, J., Y. Asher, Y. J. Gordon, U. Olshevsky & Y. Becker. 1975. Effect of zinc ions on the synthesis of herpes simplex virus DNA in infected BSC-1 cells. Virology **66**: 330-335.

29. Cheng, Y. C., B. Goz, J. P. Neenan, D. C. Ward & W. H. Prusoff. 1975. Selective inhibition of herpes simplex virus by 5'-amino-2',5'-dideoxy-5-iodouridine. J. Virol. **15**: 1284-1285.

30. Becker, Y., & U. Olshevsky. 1973. Inhibition of herpes simplex virus replication by cordycepin. Israel. J. Med. Sci. **9**: 1581-1585.

31. Hampar, B., J. G. Derge, L. M. Martos, M. A. Tagamets, & M. A. Burroughs. 1972. Sequence of spontaneous Epstein-Barr virus activation and selective DNA synthesis in activated cells in the presence of hydroxyurea. Proc. Nat. Acad. Sci. U.S.A. **69**: 2589-2593.