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A cell culture assay for compounds which inhibit hepatitis B virus replication

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Introduction

Infection by hepatitis B virus (HBV) is a major worldwide health problem with over 200 million individuals chronically-infected with HBV (Beasley and Hwang, 1984). In addition to causing both acute and chronic liver disease, HBV infection is epidemiologically linked to the formation of primary hepatocellular carcinoma (HCC) (Beasley and Hwang, 1984; Popper, et al., 1987). Several types of treatment regimens have been reported for individuals with chronic HBV infection, including interferons and nucleoside analogs (see Tabor, 1987 and Thomas, 1987 for reviews). However, these treatments have moderate to serious side effects, are only transiently effective in suppressing HBV, or are effective for only a small percentage of the general population of HBV-infected individuals.

A major obstacle in the development of new therapeutic agents for HBV is the lack of a suitable in vitro culture system which accurately models chronic HBV infection in man. Relevant animal models of HBV infection and disease, including HCC, have been developed (e.g. the woodchuck hepatitis virus (WHV) and its natural host, the Eastern woodchuck (Gerin, 1984; Popper et al., 1986)). Several potentially suitable HBV culture systems have been developed by transfection of human liver cell lines with cloned HBV DNA (Sureau et al., 1986; Sells et al., 1987; Tsurimoto et al., 1987). The various HBV genomic forms present in chronically infected cells represent well characterized stages of viral replication (Sum-

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mers and Mason, 1982; Seeger et al., 1986), making it possible to assess the biological effects of potential antiviral agents by examining specific changes in HBV DNA genomic forms and relative concentrations. We describe here the incorporation of the 2.2.15 cell line (Sells et al., 1987) into a practical screening system for the identification of compounds which inhibit HBV replication.

Materials and Methods

Cell culture conditions and overall experimental design

The 2.2.15 cell line was maintained in RPMI1640 culture media containing 5% fetal bovine serum (FBS), 2 mM glutamine and 50 μg/ml gentamicin sulfate. Cells were routinely checked for resistance to G418 (Sells et al., 1987) (Gibco, Inc., Grand Island, NY) and mycoplasma contamination (Mycotrim TC system, Hanna Biologics, Inc., Alameda, CA). For the anti-HBV assay, cells were seeded into 24-well tissue culture plates at approximately $1 \times 10^4/cm^2$ and grown to confluence (approximately 7 days). Cells were maintained at confluence for 2 to 3 days prior to the initiation of the treatment protocols to allow for HBV DNA levels to stabilize (Sells et al., 1987). The culture medium was changed twenty-four hours prior to exposure to test compounds. During the 10 day treatment period, the culture medium was removed and test compounds were added to the cultures in fresh culture medium at 24-h intervals. Immediately prior to the first dose of test compound (Day 0), and after 3, 6 and 10 days of treatment, culture medium was collected and stored at −70°C for HBV DNA analysis. Cells were lysed at the end of the treatment period and analyzed for intracellular HBV DNA.

DNA and RNA extraction

For the analysis of extracellular HBV DNA, 0.2 ml samples of culture medium were incubated for 20 min at 25°C in 1 M NaOH/10× SSC (1× SSC is 0.15 M NaCl/0.015 M Sodium Citrate, pH 7.2) and then directly applied to nitrocellulose membranes presoaked in 20 × SSC (Schleicher and Schuell, Keene, NH) using a slot blot apparatus (Schleicher and Schuell). Samples were neutralized by washing twice with 0.5 ml of 1 M Tris, pH 7.2/2 M NaCl and once with 0.5 ml of 20 × SSC. Filters were then rinsed in 2 × SSC and baked at 80°C for 1 h under vacuum.

Cells were lysed for the analysis of intracellular HBV DNA with 0.5 ml/well of lysis buffer (4 M guanidine isothiocyanate – 7% 2-mercaptoethanol – 2% sarkocyl), and dialyzed for 1 h against 6 l of 50 mM Tris, pH 8.0 – 1 mM Na_2EDTA using a microdialysis apparatus (Life Technologies, Gaithersburg, MD). Lysates were then digested with proteinase K, extracted with phenol and chloroform, precipitated with ethanol, and resuspended in 10 mM Tris, pH 8.0 – 1.0 mM EDTA as previously described (Korba et al., 1989). Cultures maintained in 10-cm diameter dishes were lysed in 6 ml lysis buffer and cellular RNA and DNA were prepared as previously described (Korba et al., 1989).

Gel electrophoresis

Samples of cellular DNA (10 μg/lane) were digested with *Hin*dIII, electrophoresed in 1% agarose gels, and transferred to nitrocellulose membranes (Schleicher and Schuell) as previously descrjbed (Korba et al., 1989). Samples of unfractionated cellular RNA (30 μg/lane) were denatured, electrophoresed in 6% formaldehyde/$NaPO_4$ (pH 6.5) 1% agarose gels, and transferred to nitrocellulose membranes (mRNC, Schleicher and Schuell) as previously described (Korba et al., 1989).

Hybridization analysis of HBV DNA

A purified, 3.2 Kb *Eco*RI HBV DNA fragment (Galibert et al., 1979) was labelled with [^{32}P]dCTP by nick translation and used as the hybridization probe (specific activity, 3–5 × 10^8 DPM/μg). The hybridization and post-washing conditions have been described (Korba et al., 1989). HBV nucleic acid levels in the samples were measured using an Ambis beta scanner (Ambis Systems, Inc., San Diego, CA). The relative amounts of ^{32}P signal hybridized to the samples were compared to the signal hybridizing to known amounts of HBV DNA standards applied to each nitrocellulose filter (gel or slot blot). Standard curves, generated by multiple analyses, were used to correlate relative cpm measurements with HBV DNA quantities.

Toxicity testing

Toxicity was approximated by the analysis of cell growth rates in the presence of the various test compounds. Briefly, cells were seeded at 1000 cells/cm^2 and compounds were added 24 h later for three consecutive daily intervals. Cell growth rates were then determined by standard procedures as previously described (Korba et al., 1990).

Results

Presentation of HBV DNA patterns in 2.2.15 cells and application to antiviral testing

The 2.2.15 cell line was obtained by transfection of the Hep G2 human hepatoblastoma cell line with a plasmid containing multiple tandem copies of the HBV genome (subtype ayw) (Sells et al., 1988). A typical pattern of intracellular HBV DNA in 2.2.15 cells is displayed in Fig. 1 (lanes 1 and 2).

The quantity of HBV DNA in episomal monomers, replication intermediates, and integrated HBV DNA were quantitatively compared in order to evaluate viral replication status. The relative amounts of cellular DNA in each lane were normalized to the amounts of integrated HBV DNA because the per cell levels of this

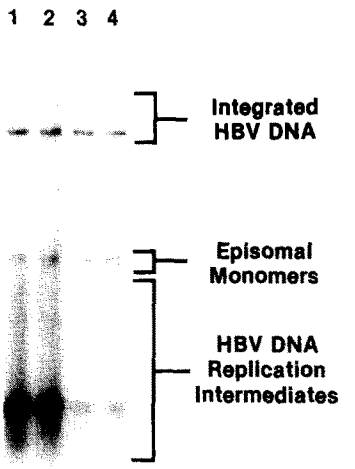

Fig. 1. Examples of intracellular HBV DNA patterns used for the analysis of antiviral activity against HBV. Cellular DNA (10 μg/lane) was digested with *Hin*dIII [no recognition sequence in HBV (Galibert et al., 1979)], and examined for HBV DNA sequences by Southern blot hybridization analysis. Lanes 1 and 2, DNA from untreated (control) cultures; Lanes 3 and 4, DNA from cultures treated for 10 days with 50 μg/ml (100 μM) ara-AMP. Exposure to film was for 20 h with a 5× Dupont lightning-plus enhancing screen.

class of HBV DNA would be expected to remain constant. The quantities of HBV DNA replication intermediates and episomal monomers were used as an indicator of the relative rate of HBV replication. Intracellular HBV DNA patterns indicative of a decline in HBV replication show a specific loss of replication intermediates without a change in the level of integrated HBV DNA (Fig. 1, lanes 3 and 4).

Response of HBV in 2.2.15 cells to a model antiviral compound

The nucleoside analog, ara-AMP (adenine arabinoside monophosphate) demonstrates how an active anti-HBV compound affects viral replication in 2.2.15 cells. Ara-AMP inhibits HBV replication in vivo (Alexander and Williams, 1986) and, unlike the parent compound ara-A, ara-AMP is soluble in aqueous solutions and is nontoxic at high concentrations to confluent cultures of 2.2.15 cells (ID_{50} approximately 500 μg/ml, unpublished observations).

Ara-AMP had a dose-dependent inhibitory effect on HBV replication and extracellular virus production in 2.2.15 cells (Table 1). Extracellular production of HBV was reduced over 10-fold (compared to the day 0 values) following 3 days of treatment with 300 μg/ml ara-AMP and HBV DNA levels in the culture medium became undetectable (>1000-fold depression) by the sixth day of treatment (Table 1). Within 4 days after the last dose of ara-AMP was administered, HBV production returned to near pretreatment levels (day 4P, Table 1).

The effect of ara-AMP on intracellular HBV DNA in 2.2.15 cells paralleled the effect of ara-AMP on HBV DNA in the culture medium. After 10 days of treatment

TABLE 1

Effect of ara-AMP on the production of extracellular HBV particles

Ara-AMP (μg/ml)	HBV DNA levels in culture media (pg/ml)				
	Day 0	Day 3	Day 6	Day 10	Day 4P
0	38	65	39	85	60
	72	45	50	35	42
30	75	38	65	50	45
	33	20	26	22	21
100	90	50	4	0.3	8
	34	12	3	1	13
300	60	5	0*	0	18
	65	1	0	0	12

HBV DNA levels were quantitated in 0.2 ml samples of culture medium as described in the Materials and Methods section. Cells were grown on 10-cm culture dishes. Ara-AMP (Sigma, Inc., St. Louis, MO) was present in the cultures up to day 10; day 4P represents 4 days of culture following withdrawal of ara-AMP. A value of 1.0 pg HBV DNA/ml corresponds to approximately 3×10^5 HBV virions/ml. For each treatment, two separate sets of data are presented. These correspond to the analysis of duplicate cultures.

*Undetectable level of HBV DNA, sensitivity cutoff was 0.1 pg HBV DNA/ml (approximately 3×10^4 virions/ml).

with 300 μg/ml ara-AMP the quantities of HBV DNA replication intermediates were reduced more than 300-fold (day 10, Table 2, Fig. 2A). Within 4 days following the termination of drug treatment, the levels of HBV DNA replication intermediates had returned to near pretreatment levels (day 4P, Table 2, Fig. 2A).

Ara-AMP did not affect the levels of HBV or b-actin RNA transcripts in 2.2.15 cells (Table 2). No changes in the pattern of HBV RNA transcripts (Tiollais et al., 1985; Sells et al., 1988) were observed during the treatment with ara-AMP and no change in the level of HBV surface antigen released by the 2.2.15 cells was observed (data not shown).

Reduction of culture size for the antiviral assay

The results described above were obtained using cells grown on 10-cm diameter culture dishes. This culture design is cumbersome for the screening of large numbers of compounds in vitro. Experiments conducted with 2.2.15 cell cultures maintained in 24-well tissue culture plates produced results which were equivalent to those obtained from larger cultures (Table 3, Fig. 2B).

Anti-HBV activity of other compounds

Table 4 lists the results of the examination of additional compounds for anti-HBV activity using the multiwell culture assay. The antiviral activities of all of

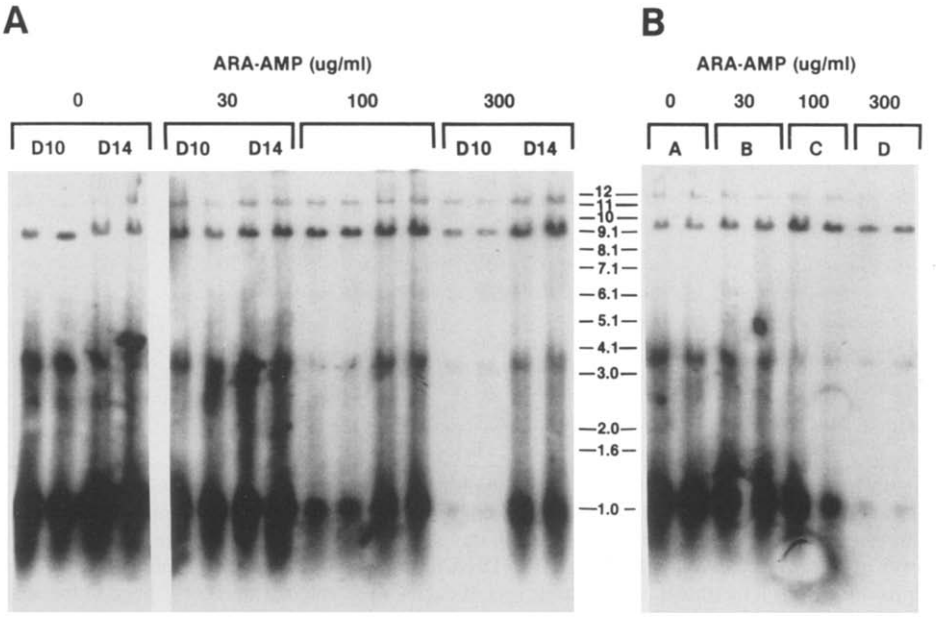

Fig. 2. Analysis of intracellular HBV DNA following treatment with ara-AMP. DNA size markers are from co-electrophoresis of the '1 Kb ladder' size markers (Life Technologies, Gaithersburg, MD). For each lane, 10 μg of *Hin*dIII-digested cellular DNA was used. Nitrocellulose filters were exposed to film for 3 days (with a 5× Dupont enhancing screen). For each treatment condition and time point, cultures maintained in duplicate dishes or wells were analyzed in separate lanes. Panel A: DNA extracted from cells grown in 10-cm culture dishes; ara-AMP was present in these cultures from day 0 to day 10 (D10); day 14 (D14) represents DNA from cultures 4 days following the removal of ara-AMP from the culture medium following 10 days of ara-AMP treatment. Panel B: DNA extracted from cells grown in 24-well culture plates and lysed after 10 days of treatment with ara-AMP.

these compounds was dose-dependent. Unless otherwise noted, no significant toxicity was observed for these compounds at the concentrations listed in Table 4.

Ara-A (adenine-9-B-D-arabino furanoside), the parent compound for ara-AMP, was found to be an effective anti-HBV agent in 2.2.15 cells (Table 4). However, ara-A was toxic at concentrations greater than 100 μg/ml (data not shown).

The nucleoside analog, ddG (2′,3′-dideoxyguanosine), had significant activity against HBV replication in the 2.2.15 cell culture assay (Table 4). Another dideoxyguanosine analog, dDAPR (2′,6′-diaminopurine 2′,3′-dideoxyriboside), was also effective in inhibiting HBV replication in 2.2.15 cells (Table 4). The carbocyclic analog of deoxyguanosine, 2′-CDG, was effective at inhibiting HBV replication in 2.2.15 cells at 100-fold lower concentrations than ddG or dDAPR (Table 4). The dideoxynucleoside ddA (2′,3′-dideoxyadenosine) displayed only a weak anti-HBV activity, while ddC (2′,3′-dideoxycytosine) was as effective as ddG in inhibiting HBV DNA replication in 2.2.15 cells (Table 4).

Several compounds known to be effective against other viruses were also used in this cell culture assay. AZT (3′azido-3′-deoxythymidine), did not inhibit HBV rep-

TABLE 2

Effect of ara-AMP on intracellular HBV DNA replication and RNA expression

Ara-AMP (μg/ml)	Day 10 of treatment					4 Days post-treatment				
	HBV DNA levels[a]			HBV[b] DNA	Actin[b] RNA	HBV DNA levels			HBV RNA	Actin RNA
	Integ	Mono	RI			Integ	Mono	RI		
0	1.3	3.9	62	11	0.3	1.3	3.5	68	12	0.5
	1.1	2.6	46	14	0.4	1.0	5.3	60	13	0.3
30	1.5	2.0	39	11	0.5	1.3	5.3	56	9	0.5
	1.3	1.7	27	18	0.4	1.3	5.3	56	13	0.3
100	1.5	0.2	9	16	0.4	1.3	2.3	35	17	0.3
	1.4	0.1	6	10	0.2	1.4	2.0	33	8	0.5
300	0.6	0.1	0.1	18	0.4	1.7	1.4	23	17	0.2
	0.5	0.1	0.1	11	0.3	1.7	1.3	27	8	0.3

See text and legend to Table 1 for experimental details. In this assay, a level of1.0 pg HBV DNA/μg cell DNA corresponds to approximately 3 HBV genomic copies/cell.

Integ., integrated HBV DNA; Mono., episomal, monomeric HBV genomes; RI, HBV DNA replication intermediates (see Fig. 1 for examples).

[a]HBV DNA levels are presented as pg HBV DNA/μg cellular DNA.

[b]HBV RNA and actin (cellular b-actin) RNA values are presented as pg RNA/μg infractionated cellular RNA. The human b-actin probe was obtained from Oncor, Inc., Gaithersburg, MD.

TABLE 3

Effect of ara-AMP on HBV production in multiwell plates

Ara-AMP (μg/ml)	Intracellular HBV DNA[a] (pg HBV DNA/μg cell DNA			Extracellular HBV DNA (pg HBV DNA/ml culture media)			
	Integ	Mono	RI	Day 0	Day 3	Day 6	Day 10
0	1.4	3.0	88	50	50	46	40
	1.2	2.0	79	55	100	42	45
30	1.1	1.4	36	60	65	100	103
	1.4	1.0	39	55	45	110	75
100	1.4	0.3	6	100	75	11	1
	1.7	0.1	3	45	50	16	0*
300	1.3	0.1	0.2	100	25	0	0
	1.1	0.1	0.3	95	30	0	0

Cells were grown in 24-well culture plates and treated for 10 days with ara-AMP as described in the Materials and Methods section.

[a]Cells were lysed for the analysis of intracellular HBV DNA following the tenth day of treatment.

*Undetectable level of HBV DNA, sensitivity cutoff was 0.1 pg HBV DNA/ml.

TABLE 4

Effect of nucleoside analogs on HBV production in 2.2.15 cell cultures

Compound[b]	Intracellular HBV DNA[a] (pg HBV DNA/μg cell DNA)			Extracellular HBV DNA (pg HBV DNA/ml culture media)			
	Integ	Mono	RI	Day 0	Day 3	Day 6	Day 10
Untreated	1.4	3.0	93	50	50	46	40
cells	1.2	2.0	80	55	100	42	45
Ara-A	1.1	0.8	0.4	77	60	10	1
(100 μM)	1.2	0.6	0.2	62	50	8	0*
AZT	1.3	3.0	75	51	55	30	66
(100 μM)	1.5	2.3	51	65	60	50	70
2′3′DDA	1.1	0.7	16	80	78	71	6
(100 μM)	1.1	0.8	12	66	72	59	8
2′3′DDC	1.8	0.2	0.1	59	45	9	0
(100 μM)	1.6	0.2	0.1	88	51	5	0
2′3′DDC	1.0	1.9	11	99	95	31	1
(10 μM)	0.8	1.2	21	88	51	45	1
2′3′DDG	1.6	0.2	0.1	49	35	8	0
(100 μM)	1.7	0.1	0.1	58	41	2	0
2′3′DDG	1.6	1.9	12	86	56	33	1
(10 μM)	1.5	1.2	10	75	60	25	0.4
2′6′DDAPR	1.9	1.0	1	83	90	2	0
(100 μM)	1.9	1.0	1	98	75	9	1
2′6′DDAPR	1.9	2.1	16	100	90	32	6
(10 μM)	1.6	1.3	20	77	75	49	13
2′-CDG	1.4	1.8	0.4	89	41	8	0
(1 μM)	1.5	2.0	1	90	55	17	0.1
2′-CDG	1.3	2.1	12	120	88	55	11
(0.1 μM)	1.2	1.4	18	95	75	77	5
Acyclovir	1.5	1.0	23	19	20	18	2
(50 μM)	1.9	1.3	27	20	27	12	2
PMEA	1.4	0.9	21	46	38	16	12
(10 μM)	1.2	0.9	25	63	90	29	14
HPMPC	1.4	2.4	36	96	70	24	12
(10 μM)	1.9	1.9	33	41	19	11	11

Cells were grown in 24-well culture plates and treated for 10 days as described in the Materials and Methods section.

[a]Cells were lysed for the analysis of intracellular HBV DNA following the tenth day of treatment.

[b]Ara-A, adenine-9-B-D-arabino furanoside (Sigma, Inc.); 2′3′DDG, 2′,3′-dideoxy-guanosine (Sigma, Inc.); 2′3′DDA, 2′,3′-dideoxyadenosine (Sigma, Inc.); 2′3′DDC, 2′,3′-dideoxycytosine (Sigma, Inc.); 2′6′DDAPR, 2′,6′-diaminopurine 2′,3′-dideoxy-riboside; 2′-CDG, carbocyclic analog of deoxyguanosine; HPMPC, S-1-[(3-hydroxy-2-phosphonylmethoxy)propyl; PMEA, 9-(2-phosphonyl methoxyethyl)adenine.

*Undetectable level of HBV DNA, sensitivity cutoff was 0.1 pg/ml.

lication in 2.2.15 cells (Table 4). The acyclic nucleoside acyclovir (acycloguanosine) reduced the levels of extracellular HBV DNA by the tenth day of treatment but had little effect on intracellular HBV replication in 2.2.15 cells (Table 4). Two other acyclic nucleosides, HPMPC, (S-1-[(3-hydroxy-2-phosphonyl-methoxy)propyl]cytosine) and PMEA (9-[2-phosphonyl methoxy-ethyl]-adenine), caused a minor suppression of HBV replication in 2.2.15 cells (Table 4). However, concentrations greater than 10 μM of either of these compounds were toxic to the 2.2.15 cell cultures (data not shown). HPMPC is an effective agent against members of the herpes virus family (Bronson et al., 1989). PMEA is a potent inhibitor of retroviruses (Balzarini et al., 1989).

Discussion

HBV is a circular, double-stranded DNA virus, 3.2 Kb in size (Tiollais et al., 1985). The presence of a discrete, intracellular DNA band migrating as a 3.2 Kb linear or open circular molecule indicates that HBV has successfully entered a cell, uncoated, and completed replication of the plus strand (Summers and Mason, 1982; Seeger et al., 1986). A heterogeneous population of single and partially double-stranded viral DNA molecules represents HBV DNA replication intermediates which are a consequence of the unique replication cycle of hepadnaviruses (Summers and Mason, 1982; Seeger et al., 1986). These viral DNA molecules disappear upon the termination of viral DNA replication. Although HBV can integrate into the genome of the host cell (Shafritz and Rogler, 1984; Tiollais et al., 1985), integration is not an obligatory or essential step in the HBV replication pathway (Summers and Mason, 1982; Seeger et al., 1986).

The 2.2.15 cell line accurately models all essential virologic features of chronic HBV infection (Acs et al., 1987; Sells et al., 1987, 1988) including (1) a stable pattern of integrated HBV DNA, (2) the presence of high levels of replicating virus (episomal open circular and superhelical monomers, and single-stranded DNA replication intermediates), (3) the appropriate sizes and patterns of viral-specific RNA transcripts and proteins, and (4) the release of high titres (10^6 to 10^7/ml) of infectious HBV virions. This cell line can be used for the primary evaluation of compounds for activity against HBV.

The inhibitory activities of several test compounds against HBV DNA replication in this culture assay system accurately model the inhibitory activities of these compounds against hepadnaviruses when administered to chronic viral carriers. As observed in vivo (Alexander and Williams, 1986; Tabor, 1987; Thomas, 1987), the inhibition of HBV DNA replication in 2.2.15 cells by ara-AMP was effective only while the compound was present and no inhibition of HBV surface antigen production was observed. Both ddG and dDAPR effectively inhibit hepadnavirus replication in 2.2.15 cells and in ducks chronically infected with the duck hepatitis B virus (DHBV) (Suzuki et al., 1988; Lee et al., 1989). Acyclovir is effective in the treatment of herpes simplex virus infections but has little activity against HBV in chronically infected patients (Alexander et al., 1987; Tabor, 1987; Tho-

mas, 1987) or in the 2.2.15 cell culture system. AZT is a potent inhibitor of the human immunodeficiency virus (HIV), but has not been reported to affect HBV replication when administered to patients infected with both HIV and HBV, exhibits no inhibitory activity against HBV DNA polymerase in in vitro assays (Nordenfelt et al., 1987), and was not inhibitory to HBV replication in 2.2.15 cells. The carbocyclic analog of deoxyguanosine, 2′-CDG, was effective at inhibiting HBV replication in 2.2.15 cells in this study and in a previous report (Price et al., 1989).

The compounds used in this study produced consistent and reproducible effects on HBV replication in 2.2.15 cells. Since HBV replication was reduced more than 10-fold by the more effective compounds, the inherent variation in the levels of HBV DNA replication intermediates or virions produced by 2.2.15 cell cultures did not interfere with the interpretation of the assay. Parallel cultures seeded in the same multiwell plates (from an identical cell passage) or cultures in different experiments can display 3-fold differences in the levels of intracellular and extracellular HBV DNA. This variability may be a consequence of the inherent biology of HBV, differences in specific host factors, or differences in specific virus-host interactions in the separate cultures. Similar variations (3 to 10-fold) in hepadnaviral DNA levels are also observed in vivo (Korba et al., 1989; Lee et al., 1989).

The inhibition of HBV replication by ara-AMP in 2.2.15 cells was not the result of general cell toxicity since the levels of HBV-specific and b-actin RNA transcripts did not change during the course of the study. The recovery of HBV DNA replication during the post-treatment period cannot be accounted for by the growth of new cells since actively dividing 2.2.15 cells do not support the high levels of HBV replication observed in confluent cultures (Sells et al., 1988). The frequent replacement of culture medium for the confluent cultures during the treatment period limits the accumulation of potentially toxic by-products of the test compounds in the culture medium and permits the analysis of extracellular HBV particle production during a discrete and consistent time interval for a constant number of cells.

Few correlations can be made regarding basic nucleoside structure and the inhibition of HBV replication with currently available information. Dideoxypurine nucleoside analogs appear to be more effective than the dideoxypyrimidine derivatives in the inhibition of DHBV either in chronically infected ducks or primary hepatocyte cultures (Suzuki et al., 1988; Lee et al., 1989). However, in this and a previous report (Ueda et al., 1989), both ddC and ddG were highly active against HBV in cell culture while ddA displayed little anti-HBV activity. Ara-AMP is a potent inhibitor of both DHBV and HBV (Alexander and Williams, 1986; Tabor, 1987; Thomas, 1987; Lee et al., 1989). Acyclic nucleosides (acyclovir, HPMPC, PMEA) show little activity against HBV, although early clinical trials with the acyclic nucleoside, ribavirin (1-B-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) showed antiviral activity in HBV-infected patients (Patki and Gupta, 1982).

Promising anti-HBV compounds identified by cell culture systems will be candidates for further testing in relevant animal models of HBV infection in man, such as WHV and DHBV. The availability of an in vitro testing/screening system for anti-HBV compounds should stimulate renewed interest in the development of antiviral therapies for chronic HBV infections.

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