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Use of a standardized cell culture assay to assess activities of nucleoside analogs against hepatitis B virus replication

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Summary

A cell culture system for the evaluation of compounds which inhibit HBV replication (Korba and Milman, Antiviral Res. 15:217, 1991) has been developed into a standardized assay. Toxicity of test compounds was assessed by the uptake of neutral red dye under culture and treatment conditions which were identical to those used for the antiviral assays. A total of 667 separate cultures of 2.2.15 cells were evaluated for this study. In 86 untreated cell cultures, representing 15 experiments over a 24-month period, the levels of extracellular HBV virion DNA and intracellular HBV DNA forms were found to vary by less than 2.5-fold overall. Virion DNA in serum and intracellular viral DNA replication intermediates [RI] are the two most reliable and commonly followed markers of hepadnavirus replication in patients and experimental animals. In these assays, levels of extracellular HBV virion DNA and intracellular HBV RI were well correlated in 2.2.15 cells. Less correlation was observed between the levels of HBV virion DNA and the 3.2-kb episomal HBV genomes present in the cells. A threshold level of 22–37 intracellular replicating HBV genomes appeared to be required before virions were detected in the culture medium. The activities of several 2′-substituted and 3′-substituted deoxynucleoside analogs against HBV replication were compared using this standardized assay. Dideoxycytosine [ddC] and dideoxyguanosine [ddG] were the most selective 2′,3′-dideoxynucleosides against HBV in 2.2.15 cells. Substitution of fluorine at the 2′ position abolished the antiviral activity of ddC, but enhanced the selective antiviral activities of dideoxythymidine and dideoxyuracil. Several 2′-fluorinated pyrimidine arabinosyl furanosides,

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reported to be potent (but toxic) inhibitors of hepadnaviruses in vivo, demonstrated relatively low selective antiviral activities in 2.2.15 cells. The current data base allows for validation of any given set of test evaluations through statistical analysis of both the positive and the negative treatment controls present in each experiment; thus, relevant comparisons of the selectivity of anti-HBV activities for different compounds examined in future experiments can be made.

Hepatitis B virus; Standardized assay; Cell culture; Nucleoside analog; Antiviral agent

Introduction

Hepatitis B virus (HBV) infection is a major worldwide health problem. Infection by HBV causes both acute and chronic liver disease and has been epidemiologically linked to primary hepatocellular carcinoma (HCC) (Beasley and Hwang, 1984; Popper et al., 1987). To date, no effective antiviral treatments against chronic HBV infection have been developed.

Until recently, the development of effective therapeutic agents against HBV has been hampered by the lack of suitable culture systems which can be used in a predictive manner for antiviral therapies in vivo. Relevant and predictive animal models of HBV-induced infection and disease, including HCC, such as the woodchuck hepatitis virus (WHV) and its natural host, the Eastern woodchuck, have been developed (see Gerin et al., 1989, for a review). Analysis of the antiviral effect of therapeutic agents on hepadnaviruses is currently tedious. Prolonged treatment schedules are required to induce substantial reductions in viral replication either in cell culture, WHV-infected animals or HBV-chronic carriers (Korba and Milman, 1991; Korba et al., 1991; Hoofnagle, 1991). The most reliable markers for hepadnaviral replication are extracellular levels of virion DNA and intracellular viral DNA replication intermediates, both of which are traditionally analyzed in a quantitative manner by blot hybridization techniques.

The time-consuming nature of the treatment regimens and analysis of viral replication markers prevents the rapid and simultaneous examination of large panels of potential antiviral agents. Therefore, it is essential that standardized in vitro screening systems be developed to permit relevant and statistically significant comparisons of the antiviral effects of compounds examined in separate experiments. This report details the parameters and limitations of a previously described cell culture assay (Korba and Milman, 1991) developed to examine the antiviral effects of compounds against HBV in a human hepatoblastoma cell line, 2.2.15 (Sells et al., 1989), using data compiled over a 24-month period. The comparative effects of a panel of nucleoside analogs, representing a variety of structural families, examined during this time period,

on HBV replication, using this culture system, is then described.

Materials and Methods

Cell culture and antiviral assays

Details of the design of the antiviral procedure and the growth conditions for 2.2.15 cells (Sells et al., 1989) have been previously described (Korba and Milman, 1991). Briefly, confluent cultures in 24-well tissue culture dishes were treated with 10 consecutive daily doses of antiviral compounds in RPMI1640 medium with 2% fetal bovine serum. Culture medium was removed daily and assayed for extracellular (virion) HBV DNA at day 0 (before drug additions) and after 3, 6, and 10 days of treatment. Intracellular HBV DNA forms were analyzed at the end of the treatment period (day 10). Stock cultures of 2.2.15 cells were routinely passaged only 3–4 times after recovery from cryopreservation.

DNA extraction and blot hybridization

HBV DNA was extracted from culture media and analyzed by a slot blot hybridization technique as previously described (Korba and Milman, 1991). Before culture media samples were aliquoted for HBV DNA analysis, each sample was centrifuged for 1 min at maximum speed (14000 × *g*) in a microcentrifuge. Cellular DNA was prepared and analyzed using a Southern blot hybridization technique which has been described (Korba et al., 1989; Korba and Milman, 1991).

The probe used for the hybridization analyses was a ^{32}P-labelled, gel-purified, 3.2-Kb *Eco RI* HBV DNA fragment from plasmid AM12 (Korba et al., 1986). HBV DNA concentrations were determined by comparisons to HBV standards present on every nitrocellulose filter using an AMBIS beta scanner (AMBIS systems, San Diego, CA) as previously described (Korba et al., 1989; Korba and Milman, 1991). EC_{90} values (90% effective concentration: drug concentration which induces a 90% decrease in the levels of HBV DNA replication intermediates in treated versus untreated control cells) were calculated by linear regression analysis. For all regression analyses, experimental data corresponding to the widest range of test compound concentrations which produced regression coefficients (r) of 0.80 or greater were used for the EC_{90} (and CC_{50}, see below) calculations. For each compound, data from multiple experiments were combined into a single group which was then used for the regression analysis.

Toxicity measurements

Toxicity was determined by the inhibition of the uptake of neutral red dye (Finter, 1969). Cells were grown to confluence in 96-well flat-bottomed tissue culture plates and treated with compounds (in 0.2 ml culture medium/well) as described above. Four concentrations of each compound were assayed, each in

triplicate cultures, in 3–10-fold steps. Twenty-four hours following the final addition of compound, culture medium was removed and 0.2 ml of DPBS containing 0.01% neutral red dye (Sigma, Inc.) was added to each culture well. The cells were then returned to the tissue culture incubator for 2 h. The dye was removed, the cells were washed once with DPBS (0.2 ml/well), and then 0.2 ml of 50% EtOH/1% glacial acetic acid was added to each well. Following 30 min of gentle mixing on a rotary platform at room temperature, absorbance at 510 nanometers [A_{510}] were determined using an Immunoreader NJ-2000 plate reader (Intermed, Japan). On each 96-well plate, wells containing no cells were used to correct for possible light-scattering effects. Untreated control cultures were also maintained on each 96-well plate. CC_{50} values (50% cytotoxic concentration: drug concentration which induces a 50% inhibition of dye uptake versus control cultures) were calculated by linear regression analysis, as described above.

Test compounds

Test compounds were diluted into culture media each day from 100- to 1000-fold concentrated stock solutions. Stock solutions were stored in daily aliquots at −20°C. The sources of the compounds used were as follows: AraA (Sigma Inc.); 2′,3′-dideoxynucleosides (Calbiochem, Inc.); 3′-fluoro and 3′-azido-dideoxynucleosides (Rega Institute, Leuven, Belgium); FEAU, FMAU (Memorial Sloan Kettering Cancer Center, New York, NY); FIAC, FIAU (O'Classen Biochemicals, San Rafael, CA; 2′-CDG (Southern Research Institute, Birmingham, AL); ddDAPR and EHNA (Burroughs Wellcome Co., Research Triangle Park, NC).

Results

Levels of HBV DNA replication intermediates

A total of 667 separate cultures of 2.2.15 cells, representing 6 different passages, were examined in 15 experiments over a 24-month period. These included 86 untreated control cultures and cultures treated with 63 different compounds. The levels of intracellular HBV DNA replication intermediates [HBV RI] and intracellular 3.2-Kb HBV episomal genomes [MONOMER] in the untreated cultures in the 15 experiments varied overall by only 1.8-fold and 2.0-fold, respectively (Table 1). HBV DNA concentrations in the different cultures were analyzed using a one-tailed *T*-test with corrections for small numbers to assign Fisher's probability values to the observed differences in HBV DNA values. In a routine assay, where the HBV DNA values in untreated cultures fell within the overall distribution represented by the data base collected from these 86 untreated cultures (see Table 1), depressions of 3.0-fold (for HBV RI) or 3.6-fold (for HBV virion DNA) from the average HBV DNA levels observed in untreated cells were found to be statistically significant ($P < 0.05$).

TABLE 1

HBV DNA replication in 2.2.15 cells in separate experiments

Experiment date	Treatment*	Intracellular HBV DNA[@] [pg/μg cell DNA (± S.D.)]			HBV DNA in culture medium [pg/ml ± (S.D.)][#]			
		Integrated	Monomers	Rep.Int.	day 0	day 3	day 6	day 10
5-89	Untreated cells (10)	1.3 ± 0.2	2.3 ± 0.4	73 ± 8	57 ± 11	60 ± 16	58 ± 9	56 ± 13
7-89	Untreated cells (4)	1.2 ± 0.1	2.0 ± 0.2	68 ± 8	58 ± 12	52 ± 8	54 ± 15	60 ± 17
9-89	Untreated cells (6)	1.2 ± 0.1	2.3 ± 0.2	66 ± 9	56 ± 12	57 ± 11	58 ± 11	62 ± 14
10-89	Untreated cells (8)	1.1 ± 0.2	2.0 ± 0.5	78 ± 12	73 ± 15	78 ± 16	93 ± 16	92 ± 11
12-89	Untreated cells (4)	1.3 ± 0.1	2.5 ± 0.2	87 ± 7	53 ± 3	75 ± 15	44 ± 2	48 ± 3
1-90	Untreated cells (8)	1.1 ± 0.2	2.2 ± 0.4	74 ± 17	70 ± 20	71 ± 13	75 ± 20	83 ± 17
2-90	Untreated cells (4)	1.2 ± 0.1	2.3 ± 0.3	67 ± 10	56 ± 7	69 ± 18	71 ± 18	59 ± 18
3-90	Untreated cells (4)	1.0 ± 0.2	2.4 ± 0.4	81 ± 13	75 ± 14	85 ± 11	90 ± 8	80 ± 6
5-90	Untreated cells (8)	1.3 ± 0.3	2.6 ± 0.4	84 ± 17	71 ± 16	77 ± 8	79 ± 20	83 ± 20
7-90	Untreated cells (4)	1.1 ± 0.2	2.5 ± 0.5	82 ± 11	60 ± 11	81 ± 12	52 ± 5	80 ± 9
9-90	Untreated cells (4)	1.1 ± 0.1	2.4 ± 0.5	71 ± 5	47 ± 1	90 ± 20	69 ± 2	74 ± 13
11-90	Untreated cells (6)	0.9 ± 0.1	2.3 ± 0.4	81 ± 4	81 ± 15	75 ± 17	65 ± 16	79 ± 18
2-91	Untreated cells (8)	1.1 ± 0.1	2.1 ± 0.2	73 ± 15	57 ± 12	77 ± 13	81 ± 12	78 ± 10
3-91	Untreated cells (4)	1.2 ± 0.1	2.6 ± 0.4	70 ± 10	80 ± 7	70 ± 13	72 ± 11	73 ± 17
4-91	Untreated cells (4)	1.0 ± 0.1	2.7 ± 0.4	77 ± 15	73 ± 13	87 ± 15	92 ± 19	95 ± 20
Totals for all untreated cultures (86)		1.1 ± 0.3	2.3 ± 0.5	75 ± 12	64 ± 16	73 ± 15	71 ± 14	74 ± 16

[@]Analysis of intracellular HBV DNA was 24 h following the 10th day of treatment. INTEGRATED, integrated HBV DNA; MONOMERS, 3.2-Kb episomal HBV genomes; REP.INT., HBV DNA replication intermediates.

*Numbers in parentheses indicate the total number of cultures used to calculate the values presented.

[#]Values presented are the mean values calculated from multiple analyses and multiple cultures. Standard deviations (S.D.) were calculated and are also presented. Values for REP.INT. and culture medium DNA have been rounded to two significant figures.

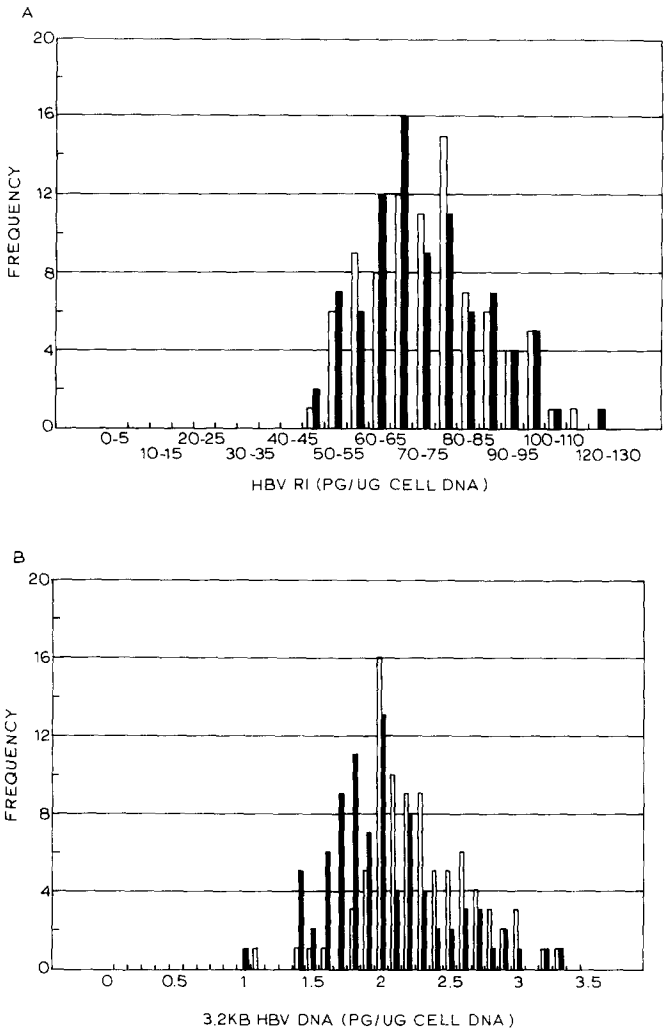

Fig. 1. Frequency distributions of episomal intracellular HBV genomic forms in untreated 2.2.15 cell cultures. Open bars display originally measured levels of HBV DNA and solid bars display normalized HBV DNA levels (see text for discussion). Panel A: HBV replication intermediates [HBV RI]. HBV DNA levels were grouped into 5-pg intervals for this presentation. Panel B: 3.2-Kb monomeric HBV genomes.

The levels of episomal intracellular HBV DNA in the 86 untreated cultures resolved into patterns which approximated normal distributions (Fig. 1). These data indicated that no subpopulation of cells producing unusually high or low levels of HBV DNA were inadvertently selected. The distributions of HBV RI or MONOMER levels within each of the 15 individual experiments were not different in their overall pattern from the combined distributions for all 86 cultures (data not shown).

HBV RI and MONOMER levels in each separate culture were normalized

for technique-related variations in the measured levels of HBV DNA from the Southern blot analyses. This was accomplished by dividing the observed HBV RI or MONOMER concentrations in each gel lane by the levels of integrated HBV DNA in the same gel lane. This normalization was possible since the levels of stably integrated HBV DNA in 2.2.15 cells can be expected to remain constant on a per cell basis in these assays (these viral DNA sequences are not involved in HBV replication (Summers and Mason, 1981; Seeger et al., 1989)). This correction allows for a direct comparison of HBV DNA levels between different cultures and assays since it removes variations caused by the blot transfer and DNA hybridization techniques. The normalization of HBV RI and MONOMER levels, while lowering the mean values 10–20%, did not change the overall shape of the frequency distribution for either class of intracellular HBV DNA (Fig. 1).

Levels of HBV virion DNA in culture media

The levels of HBV virion DNA released from 2.2.15 cells into the culture media were relatively constant (variations of: 2.1-fold (day 0), 2.5-fold (day 3), 2.6-fold (day 6), and 2.6-fold (day 10)) over the 10-day assay period between each of the 15 experiments (Table 1). HBV virion DNA levels were similarly consistent (2.7-fold variance overall) between the 15 different experiments (Table 1).

As observed for intracellular HBV DNA, the levels of HBV virion DNA in the untreated cultures resolved into patterns which approximated normal distributions (Fig. 2). This was observed not only for the combined distribution of virion DNA levels present in samples from all 4 days (Fig. 2, Panel A), but also for the separate analyses of each of the 4 individual days assayed (Fig. 2, panels B–E). The levels of HBV virion DNA in the day-10 media samples for the untreated cultures were representative of the day-0, day-3 and day-6 HBV virion levels in these cultures with respect to both average DNA concentration (Table 1) and distribution of DNA levels (Fig. 2).

HBV virion DNA levels observed in the day-10 samples from the 86 untreated cell cultures were also representative of the day-0 HBV virion DNA levels for all 667 separate cultures (untreated and treated combined) used for these studies (Fig. 3): average HBV virion DNA levels, 74 $\pm$ 16 pg/ml (day-10, untreated) versus 76 $\pm$ 14 (day-0, all cultures combined). For all of the treated cultures, the day-0 media samples contain HBV virion DNA released by these cultures prior to the addition of test compounds.

Levels of HBV virion DNA were correlated with levels of HBV RI

Viral DNA replication intermediates and virion DNA are the two classes of viral DNA forms routinely used to determine current levels of hepadnaviral replication both in vitro and in vivo (Korba and Milman, 1991; Korba et al., 1991; Thomas, 1987; Suzuki et al., 1988; Lee et al., 1989; Ueda et al., 1989). The levels of HBV virion DNA and either the levels of HBV RI or levels of MONOMER forms in untreated 2.2.15 cells, where HBV replication occur at a

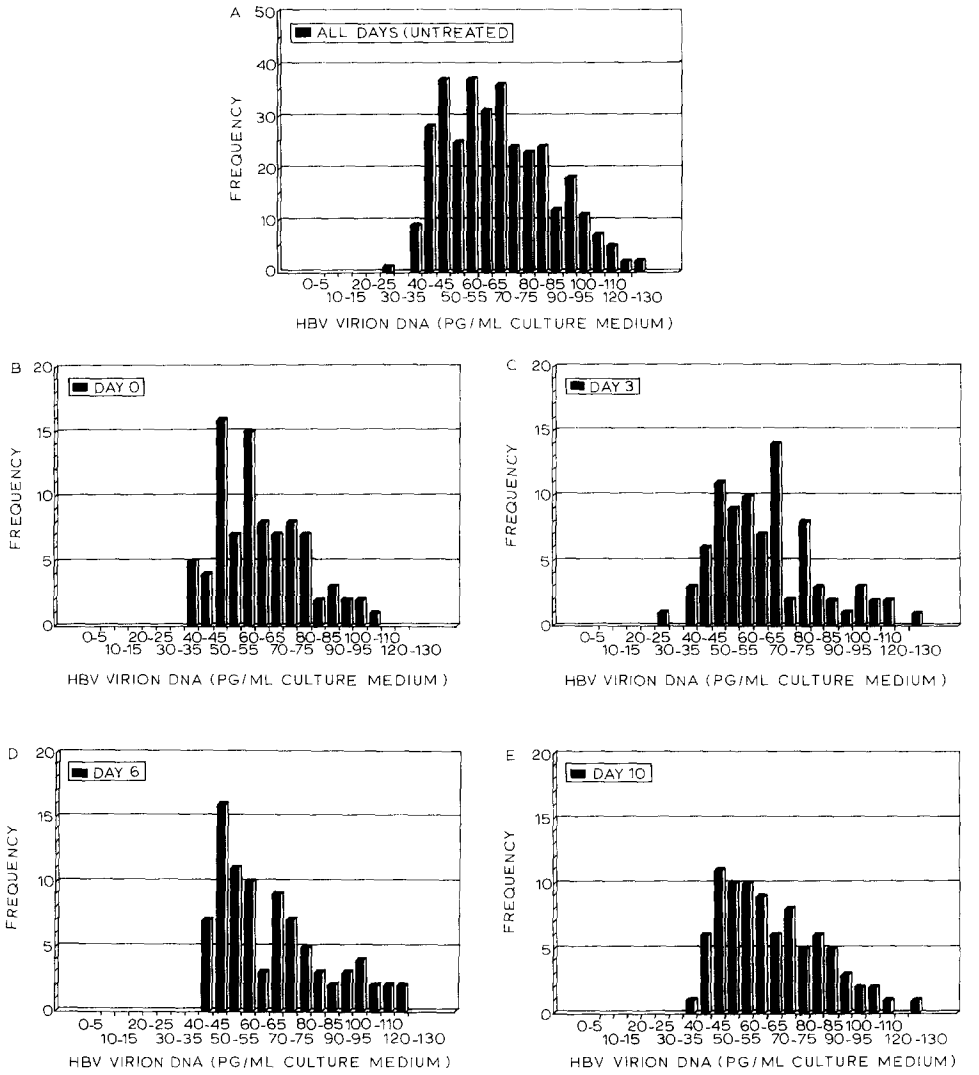

Fig. 2. Frequency distributions of extracellular HBV virion DNA in medium from untreated 2.2.15 cell cultures. HBV DNA levels were grouped into 5 pg intervals. Panel A: HBV virion DNA levels from all four days analyzed in these assays. Panels B–E: HBV virion DNA levels from each individual day of analysis; day 0 (B), day 3 (C), day 6 (D), day 10 (E).

maximal rate (Sells et al., 1989), were only weakly correlated over the limited range of HBV DNA concentrations observed in these cultures (Fig. 4A and 4B). The regression coefficients (r) for these analyses were virtually identical when either the raw or the normalized HBV RI values were used for this analysis (r-values of 0.40 and 0.42, respectively).

A strong correlation between HBV RI and virion HBV DNA levels was

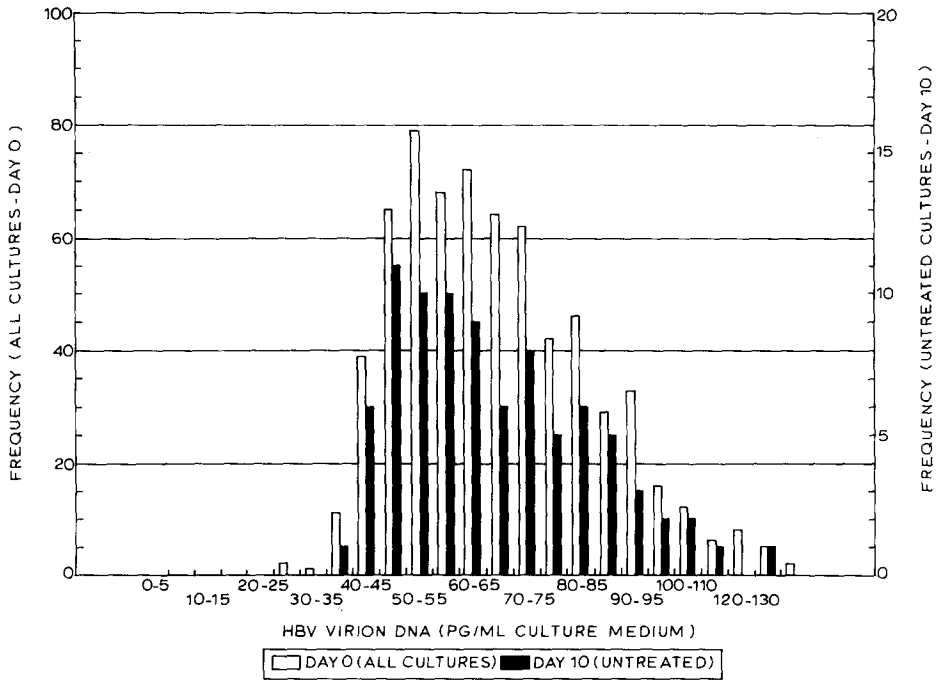

Fig. 3. Frequency distributions of extracellular HBV virion DNA in culture medium. The distribution of HBV virion DNA levels in day 10 medium from 86 untreated cultures [solid bars] is compared to the distribution of HBV virion DNA levels in day 0 (pretreatment) medium from 667 cultures (treated and untreated combined) [open bars]. HBV DNA levels were grouped into 5pg intervals.

observed when this analysis was performed using a combination of all untreated and treated cultures (r-value of 0.81, Fig. 4C). The intercept for this analysis was equivalent to approximately 22 to 37 HBV genomic copies per cell (Fig. 4C). These calculations were based on an equivalence of approximately 3.3×10^5 HBV genomes/pg HBV DNA and a yield of 1 μg DNA from 1×10^6 cells (Korba et al., 1989; Sells et al., 1989). Essentially no correlation was observed between the MONOMER form of HBV DNA and HBV virion DNA levels for the combined population of untreated and treated cultures (r-values of 0.12, Fig. 4D). Depending upon the specific antiviral agent used, MONOMER levels may or may not decline in conjunction with HBV virion levels (Fig. 4D). Similar analyses revealed a weak correlation between the levels of MONOMER and HBV RI; r-values were 0.34 for untreated cultures and 0.43 for untreated and treated cultures combined (data not shown).

Effect of nucleoside analogs on HBV DNA replication

A comparison of the anti-HBV activities for selected sets of chemical families of nucleoside analogs are displayed in Table 2. The standard deviations listed in Table 2 for the EC_{90} and CC_{50} values were calculated using the coefficients of

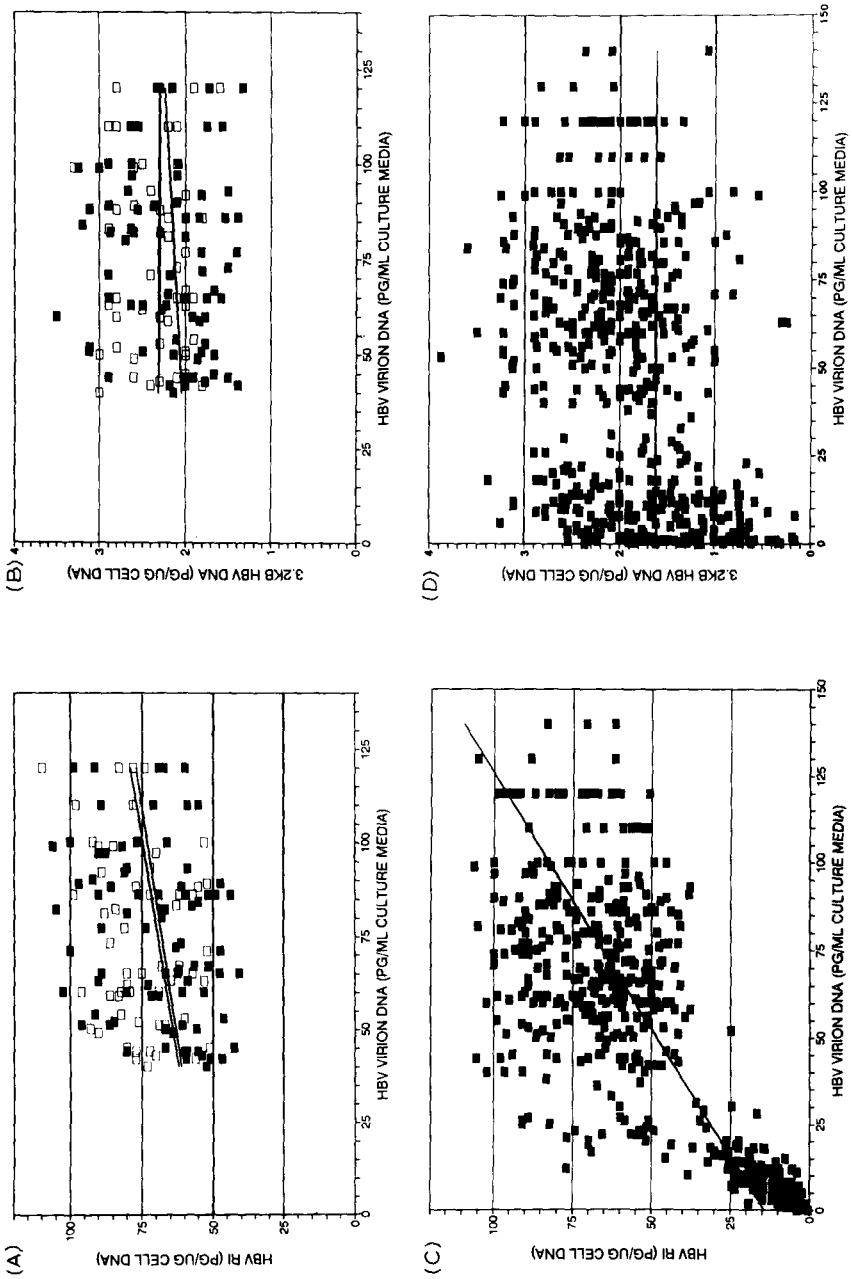

Fig. 4. Relationship of day-10 extracellular HBV virion DNA and episomal intracellular HBV genomic forms. Panels A and B: untreated cultures, HBV RI (A), 3.2-Kb monomer HBV genomes (B). Lines represent plots of linear regression analyses. Open symbols and upper lines in both panels correspond to analyses using the originally measured HBV DNA levels. Solid symbols and upper lines correspond to analyses using normalized HBV DNA levels. Panels C and D: all cultures (treated and untreated combined), HBV RI (A), 3.2-Kb monomer HBV genomes (B). Lines represent plots of linear regression analyses. Only normalized HBV DNA levels were used for the analyses presented in these two panels.

variance produced by each regression analysis. A selectivity index [SI] was calculated for each compound and is expressed as the ratio of CC_{50} to EC_{90} (Table 2).

Of the 2′,3′-dideoxynucleosides, ddG and ddC were the most effective and selective agents against HBV replication in 2.2.15 cells (Table 2). AraA and Ara-AMP, by comparison, demonstrated a lesser degree of specific activity against HBV replication (Table 2). The addition of the deaminase inhibitor, EHNA (Schaeffer et al., 1970), improved the SI of AraA, primarily by limiting toxicity (Table 2). EHNA, when used in the culture assay, did not effect HBV DNA replication: HBV RI levels were 80 $\pm$ 14 pg/μg cell DNA at Day 10 (data not shown).

Pyrimidine arabinofuranosyls with substitutions of fluorine or iodine at the 2′ position of the carbohydrate ring (FEAU, FMAU, FIAU, FIAC) have been shown to be effective against HSV replication in vitro and woodchuck hepatitis B virus in vivo (Chou et al., 1987; Fourel et al., 1990). These compounds were effective inhibitors of HBV replication in 2.2.15 cells and, except for FEAU, demonstrated approximately the same level of selectivity as AraA (Table 2).

A group of 3′-fluoro-2′,3′-dideoxynucleosides was recently shown to substantially reduce the toxicity associated with the parent analogs but retained the anti-HIV activity in vitro (Van Aerschot et al., 1990). Several of these compounds, when used in this assay system, demonstrated reduced toxicity relative to the parent compounds, but produced variable effects on their ability to inhibit HBV replication (Table 2). The substitution of fluorine at the 3′ position of the carbohydrate ring of ddC (FddC) almost completely abolished its anti-HBV activity (Table 2). While a fluorine substitution at the same position of ddT or ddU (FddT, FddU) increased the anti-HBV activity of the two parent compounds, the substitution of an azido group at this position on ddU (AzddU) did not improve its anti-HBV activity (Table 2). A chlorine substitution at the 5′ position of the base of either ddC, FddC, or FddU abolished the antiviral activity of these nucleoside analogs (Table 2).

The SI of 2′,6′-diaminopurine 2′,3′-dideoxyriboside [ddDAPR], previously shown to be effective against the duck hepatitis B virus both in vivo and in vitro (Suzuki et al., 1988; Lee et al., 1989) and HBV replication in this culture system (Korba and Milman, 1991), was similar to that of ara-AMP (Table 2). The SI of the carbocyclic analog of deoxyguanosine [2′-CDG], previously shown to be effective against HBV replication in 2.2.15 cells (Price et al., 1990; Korba and Milman, 1991), was the highest yet observed in this assay (Table 2). While the CC_{50} of 2′-CDG was at least 10-fold lower than the other nucleoside analogs used in this study, 2′-CDG exhibited an especially potent activity against HBV replication (Table 2).

The topoisomerase inhibitor, nalidixic acid [NalA] has been previously shown to be an effective agent against duck hepatitis B virus replication both in vitro and in vivo (Civitico et al., 1990). In this assay system, NalA inhibited HBV DNA replication, but the SI of NalA was found to be less than that of AraA (Table 2).

TABLE 2

Antiviral activity of test compounds

See text for description of assay parameters. Values presented (± standard deviations [S.D.]) were calculated by linear regression analysis as described in the Materials and Methods section using data combined from multiple experiments. S.D. were calculated using the standard error of regression generated from the linear regression analyses. Abbreviations: 2′,3′-dideoxynucleosides of adenosine (ddA), inosine (ddI), guanosine (ddG), cytidine (ddC), thymidine (ddT), or uracil (ddU); AraA, adenine-9-B-D-arabinofuranoside; EHNA, erythro-9(2-hydroxynonyl)adenine; (FMAU) 2′-fluoro-1-B-D-arabino-furanourosyl-5-methyluracil; (FEAU) 2′-fluoro-1-B-D-arabinofuranourosyl-5-ethyluracil; (FIAU) 2′-fluoro-1-B-D-arabinofuranosyl-5-iodouracil; (FIAC) 2′-fluoro-1-B-D-arabinofuranosyl-5-iodocytidine; (FddCyt) 3′-fluoro-ddC; (ddClCyt) 5-chloro-ddC; (FddClCyt) 3′-fluoro-ddClCyt; (AzddClCyt) 3′azido-ddClCyt; (FddThy) 3′-fluoro-ddT; (FddUrd) 3′-fluoro-ddU; (FddClUrd) 5-chloro-FddUrd; (AzddUrd) 3′-azido-ddU; (AzddClUrd) 5-chloro-FddUrd; ddDAPR, 2′,6′-diaminopurine 2′,3′-dideoxy-riboside; 2′-CDG, carbocyclic analog of deoxyguanosine.

Compound	EC_{90} (μM)	CC_{50} (μM)	SI (CC_{50}/EC_{90})
ddA	96 ± 12	154 ± 14	1.6
ddI	127 ± 15	233 ± 25	1.8
ddG	9 ± 2	197 ± 18	22
ddC	6 ± 1	168 ± 19	28
ddT	134 ± 14	82 ± 7	0.6
ddU	92 ± 8	53 ± 6	0.6
AraA	23 ± 3	191 ± 22	8.3
AraA + EHNA	33 ± 5	563 ± 15	17
Ara-AMP	61 ± 6	293 ± 28	4.8
FMAU	38 ± 7	205 ± 32	5.4
FEAU	87 ± 9	228 ± 37	2.6
FIAU	24 ± 4	212 ± 28	8.8
FIAC	34 ± 7	207 ± 25	6.1
FddCyt	57 ± 5	336 ± 20	5.9
ddClCyt	>100	396 ± 32	<1.0[@]
FddClCyt	>100	338 ± 26	<1.0
AzddClCyt	>100	357 ± 37	<1.0
FddThy	40 ± 4	361 ± 24	9.0
FddUrd	36 ± 7	338 ± 29	9.4
FddClUrd	>100	362 ± 30	<1.0
AzddUrd	>100	338 ± 39	<1.0
AzddClUrd	>100	332 ± 27	<1.0
ddDAPR	42 ± 7	230 ± 19	5.5
2′-CDG	0.1 ± 0.03	12 ± 2	120
Nalidixic acid	57 ± 6	196 ± 20	3.4

[#]A 'greater than' symbol indicates that no antiviral effect was observed at the concentration indicated, which was the highest concentration used in the culture assays for the indicated compound.

[@]N.D., Since no statistically significant depression in HBV DNA levels was observed at the highest drug concentration tested, calculations of the selectivity index assume that, based upon linear regression analysis of HBV DNA levels and drug concentrations, the EC_{90} value was greater than the CC_{50} value.

Discussion

This report describes the continued development of a standardized cell culture assay for the evaluation of compounds directed against HBV replication. This assay system permits comparisons of the potency of different compounds against HBV replication in 2.2.15 cells in a statistically relevant manner. It is especially important for the activities of new classes of compounds to be directly compared to compounds for which both the in vitro and in vivo antihepadnaviral activities are known. Toxicity measurements, which use standard assay criteria, provide critical information on the specificity of the observed antihepadnaviral activities. The current data base allows for validation of any given set of test evaluations through statistical analysis of both the positive and the negative treatment controls included in each experiment.

The overall levels of HBV DNA replication and virus production in untreated 2.2.15 cell cultures were relatively constant among the different cell cultures examined during the two-year study period, indicating that no apparent selection of cell subpopulations occurred. This was further supported by the relatively normal distribution of HBV RI, MONOMER, and virion DNA levels in the untreated cultures and the consistent effect on HBV replication in the different cultures to model antiviral compounds. These observations may be a consequence of the limited number of passages of 2.2.15 cells used (6 total). The variation in HBV DNA levels observed in the 2.2.15 cell line is a consequence of the natural biology of the virus. Similar or greater variation in hepadnavirus DNA levels is observed in vivo (Lee et al., 1989; Korba et al., 1989, 1991; Suzuki et al., 1988; Korba et al., 1991).

It appears that a pool of intracellular replicating HBV genomes accumulates in 2.2.15 cells before virions are released. Linear regression analysis showed that approximately 30 copies of replicating HBV genomes are present, on average, in 2.2.15 cells in the absence of any released virions (Y intercept in Fig. 4C). This observation is consistent with the overall scheme of HBV replication (Summers and Mason, 1982). A similar accumulation of intracellular HBV DNA in 2.2.15 cell cultures was previously observed immediately prior to the rapid increase in virion release following the onset of confluence (Sells et al., 1988). This pool of intracellular HBV genomes may represent an additional target for future antiviral agents.

In these studies, toxicity was determined under culture conditions which were identical to those used in the antiviral assays. While measurements of toxicity in actively dividing 2.2.15 cell cultures may provide a more sensitive assessment of toxicity (Matthes et al., 1990; Yokota et al., 1991), such measurements may not be relevant to the effects and metabolism of compounds in confluent cultures, the condition under which the antiviral assessments are actually made.

The use of confluent cultures in the assay provides for a cell population in a relatively homogenous state of the cell growth cycle and for a consistent cell

density in each separate culture and in each different experiment. In contrast, cultures of actively dividing cells represent a cell population with a continuously changing proportion of actively growing and non-dividing cells. The homogeneity of cell cycle growth states in confluent cultures eliminates potential variabilities in metabolism of the antiviral compounds which may occur between actively dividing and non-dividing cells.

HBV replication and virus production in 2.2.15 cells are maximal and most stable in confluent cultures (Sells et al., 1989). This feature of 2.2.15 cells makes use of the actively dividing cultures for antiviral analysis (Price et al., 1989; Matthes et al., 1990; Lampertico et al., 1991) especially difficult since only slight inhibitions of cell growth rates (which may be at the limit of detection by standard methods) could inhibit the onset of confluence and the accompanying 10-fold or greater rise in HBV RI levels and HBV virion production. For example, over a 10-day assay period, an increase in doubling time of 24 to 27 h (a 12.5% increase), could theoretically reduce the number of cells in a growing culture by 50%. Several sets of control cultures representing different stages of confluence would thereby be required in order to discriminate true antiviral effects in such a background.

Relationships between the relative potency of specific compounds against HBV replication in cell culture and their antiviral effects in vivo need to be continually developed. Currently, there are few compounds which have been tested against HBV in patients or against hepadnaviruses in animal models and in cell culture. For those compounds which have been tested, good correlations exist between observed antiviral effects in vivo and in vitro (Suzuki et al., 1989; Price et al., 1989; Ueda et al., 1989; Korba and Milman, 1991; Lee et al., 1991; Lampertico et al., 1991;). However, the general relationships between relative potencies of antiviral compounds in vitro and in vivo for HBV may not always hold for specific compounds. For example, the 2′-fluorinated nucleosides examined in these culture studies have similar potencies to AraA or Ara-AMP in culture. But, FEAU, FMAU and FIAU demonstrate effective antiviral activities at 10-fold lower concentrations than ara-AMP in vivo (Chou et al., 1987; Fourel et al., 1990; Korba et al., 1991). These differences may be due to factors such as differential rates of metabolism or relatively poor intracellular uptake by 2.2.15 cells. These types of differences will, however, most likely represent a minor proportion of antiviral compounds as more parallel studies are conducted.

The cell culture assay system described in this and in a previous report (Korba and Milman, 1991) represents a standardized and accurate model system for determining the anti-HBV activity of candidate compounds for in vivo analyses. In addition, because of the current level of knowledge concerning the life cycle of HBV (Tiollais et al., 1985; Summers and Mason, 1981; Seeger et al., 1988), the 2.2.15 cell system is also amenable to studies on the mechanisms of action of these agents. The combination of parallel studies in both cell culture and in experimental animal models should accelerate the development of safe and effective antiviral strategies directed against HBV infection.

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