

Antiviral agents in hepatitis B virus transfected cell lines: inhibitory and cytotoxic effect related to time of treatment

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The antiviral and cytotoxic effects of ara-arabinoside monophosphate, 2',3'-dideoxy-cytidine, ganciclovir, 9-2(-phosphonylmethoxyethyl) adenine, 2',3'-dideoxy-3'-thiacytidine and recombinant interferon-alpha were studied using two human hepatitis B virus transfected hepatoma cell lines, HepG2 2.2.15 and HB 611. After 9 days of exposure, starting on day 3 after seeding, inhibition of extracellular HBV-DNA expressed as ID₅₀ was in the 0.1–1.0 μM range for 2',3'-dideoxy-3'-thiacytidine and 9-2(-phosphonylmethoxyethyl) adenine and >10 μM for dideoxy-cytidine, ara-arabinoside monophosphate and ganciclovir in both cell lines. At 2.500 U/ml recombinant interferon-alpha showed less than 20% inhibition in both cell lines. The HBV-DNA inhibitory effects of 2',3'-dideoxy-3'-thiacytidine and 9-2(-phosphonylmethoxyethyl) adenine were also investigated after 1 and 3 days of exposure. In that setting ID₅₀'s were 10 and 3.3 μM for 2',3'-dideoxy-3'-thiacytidine and >100 and 30 μM for 9-2(-phosphonylmethoxyethyl) adenine, respectively. No major inhibitory effect on hepatitis B surface antigen and hepatitis B e antigen secretion was observed for any agent in this study, except for 9-2(-phosphonylmethoxyethyl) adenine in HB 611 cells. Cytotoxicity

measured by inhibition of [³H-methyl] deoxythymidine incorporation and expressed as CD₅₀ on day 4 was in the 10–100 μM range for ara-arabinoside monophosphate; in the 100–1000 μM range for 9-2(-phosphonylmethoxyethyl) adenine, ganciclovir and dideoxy-cytidine; and >1000 μM for 2',3'-dideoxy-3'-thiacytidine. This CD₅₀ decreased considerably (7–100 fold) when measured on day 12 for dideoxy-cytidine, ganciclovir, 9-2(-phosphonylmethoxyethyl) adenine and 2',3'-dideoxy-3'-thiacytidine, but was similar for ara-arabinoside monophosphate. Since the order of antiviral HBV activity and cytotoxicity of nucleoside analogues was similar in the two transfected hepatoma cell lines, we conclude that 2',3'-dideoxy-3'-thiacytidine and 9-2(-phosphonylmethoxyethyl) adenine are very potent inhibitors of HBV-DNA, with a long-lasting effect. In view of the progressive toxicity with continuous administration, intermittent administration might be an alternative mode of therapy.

Key words: Antiviral agents; Cell culture; Cytotoxicity; Hepatitis B virus.

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RESEARCH on hepatitis B virus has been hampered by the absence of an *in vitro* cell culture system for hepatitis B infection. Infection of hepatitis B virus in primary cultures of human hepatocytes has been reported, but great variability has been observed in levels of HBV replication (1).

As an alternative, transfection of various hepatoma cell lines with hepatitis B virus DNA has been introduced. Stable transfection was established for the

HepG2 2.2.15 and the HB 611 cell lines. Both cell lines excrete hepatitis B surface antigen, hepatitis B e antigen and complete Dane particles (2,3). These two cell lines are now widely used as model systems for screening of antiviral agents with activity against HBV. In previous studies various experimental designs were described to assess the therapeutic effect of antiviral agents. Differences in time of start of exposure of cells to the agents, duration of exposure, cell system, time lapse between exposure and measurement of anti-HBV effect and different types of cytotoxicity tests may ultimately have influenced the results. Therefore, it is of interest to investigate the two commonly used HBV-producing cell lines, the HepG2 2.2.15 and the HB 611

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cells, simultaneously. For comparison of the feasibility of these cell lines, we have chosen antiviral agents with different inhibitory properties: ara-arabinoside monophosphate (Ara-AMP) (4) and ganciclovir (DHPG) as suspected DNA polymerase inhibitors (non-reactive in growth inhibition of retroviruses: J. Bazarini, personal communication); 9-2(-phosphonylmethoxyethyl) adenine (PMEA) as inhibitor of DNA polymerase as well as reverse transcriptase (5), and dideoxy-cytidine (ddC) and 2'-3'-dideoxy-3'-thiacytidine (Lamivudine) as reverse transcriptase inhibitors (6,7). Recombinant interferon- α (IFN- α) is a presumed inhibitor of the viral RNA and protein synthesis (8,9). It is very important to establish the effects of drug activity on cytotoxicity and the effect of cytotoxicity on the evaluation of the antiviral effect in the earliest phase of selection of new drugs. For this reason we investigated the time and duration of treatment in relation to antiviral effect and cytotoxicity.

Material and Methods

Antiviral agents

Ara-arabinoside monophosphate and 2'3'-dideoxy cytidine were purchased from Sigma Chemical Co. (St Louis, USA). Ganciclovir was purchased from Sarva Syntex Corporation (Palo Alto, USA). Recombinant interferon- α was a gift from the Schering Plough Corporation (Kenilworth, NY, USA). 9-2(-phosphonylmethoxyethyl) adenine was a gift from Dr. J. Balzarini (Rega Institute of the Catholic University, Leuven, Belgium). Lamivudine is the negative enantiomer of 2',3'-dideoxy-3'-thiacytidine and was a gift from Glaxo Research and Development (Greenford, UK).

Human hepatoma cell lines

The HepG2 2.2.15 hepatoblastoma cell line was kindly donated by Dr. G. Acs (Mount Sinai Medical Center, New York, NY, USA); the HB 611 cell line was a gift from Dr. K. Matsubara (Institute of Molecular and Cellular Biology, Osaka, Japan).

Cells were seeded onto 25 cm² tissue culture flasks (Costar) at a density of 4×10^4 cells/cm² in Dulbecco's modification of Eagle's medium (DMEM), supplemented with 2 mmol/l L-glutamine (Flow Laboratories), garamycine (40 μ g/ml), amphotericin B (2.5 μ g/ml), the neomycin analogue G418 (360 μ g/ml for HepG2 2.2.15 cells; 200 μ g/ml for HB 611 cells) and 10% fetal bovine serum (FBS, Hyclone Laboratories Inc). When cells had reached confluence (day 6), FBS was reduced to 2%. Cell cultures were maintained in 5% CO₂ atmosphere at 37°C.

All antiviral agents were investigated for their anti-HBV effects after continuous exposure of the cells to

these drugs for 9 days. Initially, tenfold dilution steps were used. Intermediate dilution steps were chosen for the final experiments to obtain drug levels closer to the expected 50% inhibitory concentration. In these experiments exposure of the cells to the drug started on day 3 by replacing the culture medium with culture medium supplemented with the antiviral agent. This was repeated on day 6 and 9.

In short-term exposure experiments, medium was also replaced on day 3 by culture medium containing the antiviral agent. Exposure of the cells to the drug ended after, respectively, 1 and 3 days. Medium without antiviral agent was changed on day 6 and 9.

Cell culture supernatants from continuous (9 days) and short-(1 day and 3 days)-term exposures were harvested on day 12 for HBV-DNA extraction.

Analysis of HBV-DNA

For analysis of extracellular HBV-DNA the cell culture supernatant was clarified by centrifugation at 1000 g for 10 min. Aliquots were stored at -20°C until further analysis. After concentration by polyethylene glycol (PEG, 10% w/v) precipitation, the pellets were resuspended in a buffer consisting of 10 mM Tris-HCl pH 8.2, 400 mM NaCl, 2 mM Na₂EDTA, 0.6 mg/ml proteinase K and 1% SDS and incubated overnight at 45°C. Total HBV-DNA was extracted with phenol, followed by extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1). The DNA was precipitated overnight in ethanol. After two washes with ethanol 70%, the DNA pellets were dissolved in TE (10 mM Tris-HCl pH 7.5, 2 mM Na₂EDTA) in 10% of the starting volume.

For dot blot analysis, extracted DNA was applied to a Hybond N+ membrane (Amersham Life Science Products) in the Convertible Filtration Manifold System (BRL, Life Technologies Inc.) as described previously (10). Three reference samples were included in any hybridization run for quality and sensitivity control.

Hybridization of HBV-DNA sequences was performed with a [³²P]dCTP labelled EcoRI fragment of pCP10 containing the full length 3.2 kb genome of HBV. All labelling reactions were carried out with the Prime-a-Gene Labelling System (Promega). HBV-DNA was visualized by autoradiography. The limit of detection was 0.1 pg HBV-DNA.

The intensity of the autoradiographic dot spots was quantitated by scanning in an LKB 222-020 Ultra Scan XL laser densitometer. The 50% inhibitory dose (ID₅₀) was defined as the drug concentration that reduced the level of HBV-DNA in the culture medium by 50%. The ID₅₀ values were obtained by plotting percentage inhibition compared with control (no drug) versus the drug

TABLE 1

Inhibitory effects of antiviral agents in HepG2 2.2.15 and HB 611 cells

Anti-viral agent	ID ₅₀ (μM) day 12		CD ₅₀ (μM)				TI ₅₀ (μM)			
			HB 611		HepG2 2.2.15		HB 611		HepG2 2.2.15	
	HB 611	HepG 2.2.15	day 4	day 12	day 4	day 12	day 4	day 12	day 4	day 12
	A	B	C	D	E	F	C/A	D/A	E/B	F/B
Lamivudine	0.04	0.02	2800	150	3000	450	>1000	>1000	>1000	>1000
PMEA	0.70	1.20	150	20	150	20	214	10	516	21
ddC	10	50	>500	40	>500	4	>50	4	>10	0.08
AraAMP	40	100	10	30	20	25	0.25	0.33	0.20	0.25
DHPG	>100 ¹	>100 ²	180	20	430	25	<1.80	<0.20	<4.30	<0.20
IFN-α U/ml	>2500 ³	>2500 ⁴	>2500	>2500	>2500	>2500	-	-	-	-

¹ At 100 μM; 44% Inhibition.

² At 100 μM; 20% Inhibition.

³ At 2500 U/ml; 10% Inhibition.

⁴ At 2500 U/ml; 17% Inhibition.

concentration. The ID₅₀ could be determined with a 15% coefficient of variation.

Cytotoxicity

The 50% cytotoxic dose (CD₅₀) was determined by inhibition of [³H-methyl]deoxythymidine ([³H-methyl] dThd) incorporation and measured after 24 h of incubation at respectively 3 and 12 days after seeding (11).

The Therapeutic Index (TI) is the ratio of CD₅₀ over ID₅₀.

Extracellular HBsAg and HBeAg analysis

HBsAg and HBeAg secretion in clarified tissue culture medium were analyzed on day 12. HBsAg and HBeAg were determined semi-quantitatively by end-point titration in radioimmunoassays (AUSRIA II and HBe rDNA, Abbott Laboratories, Chicago, Ill., USA).

Results

Anti-HBV effect

The inhibitory effect on the level of HBV-DNA in tissue culture medium from HepG2 2.2.15 and HB 611 cells was determined on day 12. The level of HBV-DNA in supernatants of HB 611 cells not incubated with drugs resulted in weak signals after hybridization. Therefore Dane particle concentration by polyethyleneglycol (PEG) preceded HBV-DNA extraction and dot blot hybridization. For comparison, PEG precipitation was also applied to tissue culture medium of HepG2 2.2.15 cells.

The order of effectiveness in HepG2 2.2.15 cells, based on 50% inhibition of HBV replication, was: Lamivudine, PMEA, ddC, Ara-AMP, DHPG and interferon-α. The same order was obtained for HB 611 cells (Table 1). The 50% inhibitory dose ranged from about

0.02 μM for Lamivudine to >100 μM for DHPG. For IFN-α only minimal inhibition was observed (10 and 17% at 2500 U/ml for HB 611 and HepG2 2.2.15, respectively).

Inhibition of extra-cellular HBsAg and HBeAg

HBsAg and HBeAg secretion from both HepG2 2.2.15 and HB 611 cell cultures were not inhibited for the antiviral agents under study except for PMEA, which inhibited HBsAg secretion in HB 611 cell cultures. A reproducible 50% inhibition was found at 30 μM.

Cytotoxicity

Table 1 also shows CD₅₀ results in the two hepatoma cell lines. On day 4 the order of CD₅₀ was similar for HepG2 2.2.15 and HB 611, with the highest level of CD₅₀ for Lamivudine and lowest for Ara-AMP.

Comparing day 4 with day 12, lower levels (7–100 fold) of CD₅₀ were observed on day 12 for Lamivudine, PMEA, ddC and DHPG, but not for Ara-AMP.

The most dramatic change was noticed for ddC in HepG2 2.2.15 cells. In contrast to the results on day 4, the level of CD₅₀ on day 12 suggests that the antiviral activity on day 12 may be strongly influenced by cytotoxicity. This change steeply affected the therapeutic index of the drug (Table 1).

Short treatment with PMEA and Lamivudine

Fig. 1 illustrates that for PMEA and Lamivudine the concentration ranges of inhibitory activity and cytotoxicity are clearly separated. These two agents were studied for post-exposure inhibitory properties after short-term exposure in HepG2 2.2.15 cells. HepG2 2.2.15 cells were chosen for their favourable growth characteristics and higher virus yields compared to HB

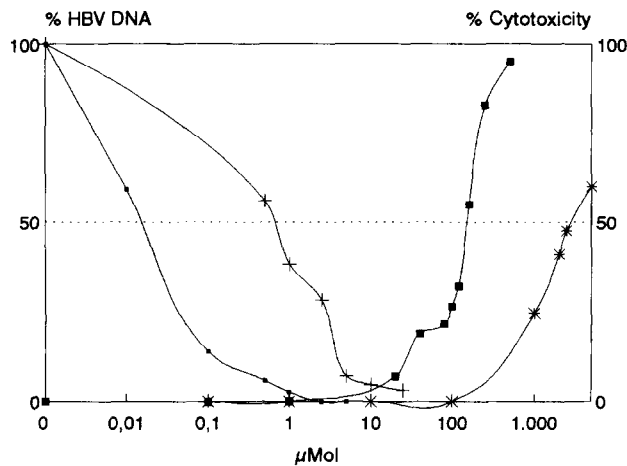


Fig. 1. Decrease of extracellular HBV-DNA in HepG2 2.2.15 cells (—■— Lamivudine; + PMEa) and increase of cytotoxicity (—*— Lamivudine; —■— PMEa) with increasing concentrations of antiviral agent. HBV-DNA was extracted from culture medium on day 12 after 9 days of exposure of the cells to the drug. Cytotoxicity was measured on day 4 by [³H-methyl] dThd incorporation and measured after 24 h of incubation.

611 cells. Extracellular HBV-DNA was evaluated on day 12.

After 3 days of exposure to PMEa and Lamivudine, the ID₅₀ was 30 μM and 3.3 μM, respectively, compared to 1.2 and 0.02 μM, after 9 days of exposure. In the 1-day exposure experiment PMEa and Lamivudine had ID₅₀'s of >100 and 10 μM, respectively.

Discussion

This is the first report on simultaneous investigation of antiviral agents in the two HBV-transfected hepatoma cell lines HepG2 2.2.15 and HB 611. The HBV inhibitory and cytotoxic effects of the agents under study were very similar for the two hepatoma cell lines after continuous exposure for 9 days. The order of effectiveness based on inhibition of extracellular HBV-DNA for both HepG2 2.2.15 and HB 611 cell cultures was Lamivudine, PMEa, ddC, Ara-AMP, DHPG and IFN-α.

For comparison, in the literature ID₅₀'s for Ara-AMP using HepG2 2.2.15 cells were reported as 36 μM for Ara-AMP (10), 0.05 μM (12, 13) and 0.006 μM (14) for Sddc and 1.6 μM (15) and 3.6 μM (16) for ddC.

PMEa (10 μM) inhibited HBV-DNA by 33% in HepG2 2.2.15 cells (10) and in HB 611 cells and ID₅₀ of 0.18 μM was reported (16). IFN-α inhibited HBV-DNA by 45% at a concentration of 10 000 IU/ml in

HepG2 2.2.15 cells (17) and by 50% at 400 IU/ml in HB 611 cells (15).

DHPG was never assayed in HepG2 2.2.15 and HB 611 cells.

In most studies on HBV inhibitory agents, cytotoxicity in cell culture systems was measured in lymphoblastoid or hepatoma cell cultures early after plating of the cells. At that time cellular DNA synthesis is expected to be highest.

Antiviral activity, measured as inhibition of the level of HBV-DNA under continuous exposure to the drug is, in fact, because of the need to change the culture medium every 3 days, determined at the end of the treatment period. Therefore, we included in our study cytotoxicity measurements on day 12 in addition to the usual measurement of cytotoxicity at the start of treatment (day 4). For both cell lines we observed an increase in cytotoxicity for Lamivudine, PMEa, ddC and DHPG after prolonged drug treatment. The increase in cytotoxicity as measured by [³H-methyl] dThd incorporation was confirmed by cell death in dye fixing experiments (results not shown).

The most dramatic change in CD₅₀ upon time of evaluation was measured for ddC in HepG2 2.2.15 cells. An unfavourable cytotoxic aspect of ddC was also apparent in inhibitory measurements of mitochondrial DNA (12,13). In clinical trials peripheral neuropathy was observed at all doses (18–20). Our experiments suggest that the elevated cytotoxicity of ddC on day 12 may result in an overestimation of the inhibitory effect on the level of HBV-DNA and a too favourable therapeutic index for this drug.

Reduction of HBsAg concomitant with HBV-DNA in the culture medium could not be explained by accumulation within a subcellular fraction, since with increasing drug concentration both intra-, and extracellular levels of HBV-DNA decreased quite similarly.

Compared to the other agents in our study, Lamivudine is the most vigorous antiviral agent, with low toxicity on day 4 as well as day 12. Our *in vitro* findings with Lamivudine in these two cell lines were confirmed in pilot studies with Lamivudine in chronic hepatitis B patients (21). Furthermore, the outcome of our short exposure experiments are of interest for intermittent administration of antiviral drugs to reduce or avoid delayed toxic effects in man.

In summary, with the limited number of agents in this study we conclude that HepG2 2.2.15 cells are more suitable than HB 611 because of higher virus yields and better culturing characteristics, with a similar inhibitory effect on Dane particle secretion.

The high yields of HBV from the HepG2 2.2.15 cells made it possible to use a 24-well tissue culture system

(results not shown) and to perform direct analysis of extra-cellular HBV-DNA in cell-culture medium without PEG concentration and DNA extraction or PCR capturing (22).

Measuring cytotoxicity at the end of the incubation period may have a further discriminatory effect on the calculated and often overestimated therapeutic index. The severe toxicity observed in the FIAU study stresses once more the need for sensitive laboratory methods to predict toxic effects in man. Cell culture systems like HBV-producing hepatoma cell lines may be helpful in exploring this area.

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