The intracellular activation of lamivudine (3TC) and determination of 2′-deoxycytidine-5′-triphosphate (dCTP) pools in the presence and absence of various drugs in HepG2 cells

Stephen Kewn,[1] Patrick G. Hoggard,[1] Sean D. Sales,[1] Mark A. Johnson[2] & David J. Back[1]

[1] *Department of Pharmacology and Therapeutics, New Medical Building, University of Liverpool, Ashton Street, Liverpool, L69 3GE and* [2] *Glaxo-Wellcome Research and Development, Greenford, Middlesex, UB6 0HE, UK*

Aims Lamivudine (3TC, 2′-deoxy-3′-thiacytidine) requires intracellular metabolism to its active 5′-triphosphate, 3TC-5′-triphosphate (3TCTP), to inhibit the replication of hepatitis B virus (HBV). We have investigated the activation of 3TC, in the presence and absence of a range of compounds, in HepG2 cells. The intracellular levels of the endogenous competitor of 3TCTP, 2′-deoxycytidine-5′-triphosphate (dCTP), were also determined and 3TCTP/dCTP ratios calculated.

Methods The effects of a number of compounds on 3TC (^{3}H; 1 μM) phosphorylation were investigated by radiometric h.p.l.c. dCTP levels were determined using a template primer extension assay. 3TCTP/dCTP ratios were calculated from these results.

Results The phosphorylation of 3TC was significantly increased in the presence of either hydroxyurea (HU), methotrexate (MTX), or fludarabine (FLU). For example, at 100 μM HU, control 3TCTP levels were increased to 361% of control, whereas at 100 μM FLU, control 3TCTP levels were increased to 155%. dCTP pools were significantly reduced in the presence of HU and FLU, at 100 μM concentrations only. However, for all the above three compounds investigated, the ratio of 3TCTP/dCTP was favourably enhanced (e.g. at 1 μM MTX, 255% of control). Neither ganciclovir (GCV), lobucavir (LCV), penciclovir (PCV), adefovir dipivoxil (ADV), nor foscarnet (FOS) had any significant effects on 3TC phosphorylation or dCTP pools.

Conclusions These results suggest that the activity of 3TC may be potentiated when combined with one of the modulators studied. The lack of an interaction between 3TC and the other anti-HBV agents is reassuring. These *in vitro* studies can be used as an initial screen to examine potential interactions at the phosphorylation level.

Keywords: 3TC, dCTP, drug interactions, HepG2 cells, phosphorylation

Introduction

Chronic hepatitis B virus (HBV) infection is a major cause of life-threatening liver disease, including cirrhosis and hepatocellular carcinoma [1, 2]. HBV infection is a very serious health problem, with over 300 million individuals (5% of the world's population) infected.

HBV is a DNA virus, but it replicates using an RNA intermediate. This requires the activity of a reverse transcriptase function that is present in the HBV DNA polymerase. The multistep mechanism of HBV replication begins with reverse transcription of pregenomic HBV RNA, mediated by the reverse transcriptase activity of HBV DNA polymerase. Next, the HBV DNA polymerase exerts its DNA-dependent DNA polymerase activity to synthesize the usually incomplete positive-sense DNA strand from the negative-sense strand template [3, 4].

The development of drugs for the treatment of HBV infection can be split into two main areas. Firstly, the use of interferon α, a naturally occurring compound that can exert an antiviral effect or act as an immunomodulator [5] and secondly, the use of nucleoside analogues that function

Correspondence: Dr Stephen Kewn, Department of Pharmacology & Therapeutics, New Medical Building, University of Liverpool, Ashton Street, Liverpool, L69 3GE, UK. Tel.: (0151) 794–5565; Fax: (0151) 794 5540.
E-mail: S.Kewn@liverpool.ac.uk.

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as inhibitors of viral replication [6–8]. Interferon α is currently the only approved therapy for chronic HBV. However, the combination of a poor response rate and emerging side-effects are often associated with its use [9, 10].

Lamivudine (2′-deoxy-3′-thiacytidine; 3TC) and penciclovir (PCV) are among the most promising nucleoside analogues for the treatment of HBV, and are currently undergoing clinical trials. The majority of the nucleoside analogues being considered as anti-HBV therapies were originally developed to treat other viral infections. For example, 3TC, the levorotatory isomer of a cytidine analogue, is used in the treatment of human immunodeficiency virus (HIV). In contrast, PCV, and other guanosine nucleosides such as ganciclovir (GCV) and lobucavir (LCV), are used for the treatment of herpes virus infections [11, 12]. All of the nucleoside analogues inhibit HBV replication via a common mechanism. They are phosphorylated by intracellular enzymes to their active 5′-triphosphates (TPs) which then inhibit the HBV DNA polymerase through competition with their respective endogenous 2′-deoxynucleoside-5′-triphosphates (dNTPs), and by acting as chain terminators [13–15]. However, the rapid rebound of virus and the emergence of drug resistant HBV mutants observed with 3TC therapy for example [16, 17], suggests that the use of such compounds as monotherapies will be limited. Therefore, it seems likely that the future of HBV therapy will involve the use of combination regimens, in a similar fashion to the current treatment of HIV infection.

The possibility of the use of combination therapy for the treatment of HBV infection may give rise to a number of problems. These include cross-resistance, overlapping toxicity profiles, and the potential for drug–drug interactions. The reliance on the formation of the active TPs stresses the importance of assessing drug interactions between different combinations of nucleoside analogues at the phosphorylation level.

The present study describes the determination of 3TC phosphates and 2′-deoxycytidine-5′-triphosphate (dCTP) pools in an hepatic cell line (HepG2). The ability of other anti-HBV agents (GCV, LCV, PCV, adefovir dipivoxil (ADV), foscarnet (FOS)) to alter these levels was investigated, and also a number of compounds that may be able to act as modulators (e.g. hydroxyurea (HU)). As inhibition of HBV replication by 3TC is partly dependent on competition between 3TCTP and dCTP, alteration to these levels and the subsequent 3TCTP/dCTP ratio in the presence of such compounds may result in a change in efficacy of 3TC. HepG2 cells are an immortal cell line derived from human hepatocytes and have previously been shown to be an excellent *in vitro* model for drug metabolism studies. HepG2 cells can also be transfected with HBV (2.2.15 cells), in order to assess the antiviral potency of these compounds [8, 15, 18–21].

Methods

Chemicals

HepG2 cells were obtained from Porton Down, Salisbury, U.K. Foetal calf serum and trypsin were purchased from Gibco Life Technologies Ltd, Paisley, Scotland, U.K. Penicillin/streptomycin and L-glutamine were acquired from Northumbria Biologicals, U.K.[2′,8′-^{3}H] 2′-deoxyadenosine-5′-triphosphate (dATP) (specific activity, 15–18 Ci mmol^{-1}) was purchased from Moravek Biochemicals Inc., Brea, CA, U.S.A. Sequenase 2.0 enzyme was obtained from Amersham Life Science, Inc., Ohio, U.S.A. The synthetic oligonucleotide primer 5′-TTGTTGTTGTTGTTGGGCGGTGGAGGCGGC-CGCCTCCACCGCC-3′ was acquired from MWG-Biotech GmbH, Germany, or Pharmacia, U.K. [^{3}H]-3TC, 3TC and 3TCTP were kindly donated by Glaxo-Wellcome, U.K. GCV was donated by Syntex Pharmaceuticals Ltd, Maidenhead, U.K. LCV was a gift from Bristol-Myers Squibb, Wallingford, CT, U.S.A. PCV was kindly donated by Smith Kline Beecham, U.K. ADV was a gift from Gilead Sciences, Foster City, CA, U.S.A. DE81 filter papers (25 mm DEAE paper) were purchased from Whatman, U.K. Liquid scintillation fluids (Flo-scint IV and Aqualuma Plus) were obtained from Packard, CT, U.S.A. All other drugs and chemicals were purchased from Sigma Chemical Company Ltd, U.K.

HepG2 cell maintenance

HepG2 cells were routinely maintained in 75 cm^2 flasks ($2–8 \times 10^6$ cells/flask), containing Basal growth media (Basal media supplemented with foetal calf serum (10%), L-glutamine (2 mM) and penicillin (5000 units ml^{-1})/streptomycin (5000 μg ml^{-1})) at 37°C in a humidified, 5% CO_2 gassed incubator. HepG2 cells are an attached cell line requiring trypsinization in order to remove cells from the flask surface. Firstly, the existing media was poured off and discarded, and the resulting monolayer of cells was washed with Versene (PBS plus 0.25% EDTA; 3 ml). Trypsin (0.5% solution; 3 ml) was then added, and discarded after 1min. The cells were suspended in fresh Basal growth media and maintained as required, then allowing 3 h for reattachment to occur. The HepG2 cells doubled approximately every 48 h.

HepG2 cell incubations with[^{3}H]-3TC

HepG2 cells ($6–8 \times 10^6$ cells/flask) were incubated with [^{3}H]-3TC (0.65μCi; 1 μM) in a final volume of 6 ml Basal

growth media. Cells were incubated at 37°C for 24 h in a humidified, 5% CO_2 gassed incubator. The effects of HU, methotrexate (MTX), fludarabine (FLU), GCV, LCV, PCV, ADV, and FOS on 3TC phosphorylation were investigated over therapeutically relevant concentrations. That is, they encompassed or were similar to the plasma concentrations of these compounds observed *in vivo* [22–28]. For example, HU was assessed by the addition of 60 μl of 1 mM and 10 mM solutions to each flask to give final concentrations of 10 and 100 μM, respectively. FLU and FOS were also studied at 10 and 100 μM. MTX was investigated at 0.1 and 1 μM. Finally, GCV, LCV, PCV, and ADV were assessed at 1 and 10 μM concentrations. Experiments were performed in duplicate on 4 separate occasions.

Cell collection, extraction and h.p.l.c. analysis

Following incubations, cells were trypsinized as described earlier and suspensions were transferred to 20 ml sterilin tubes, centrifuged (2772 *g*, 4min, 4°C) and the supernatant fraction discarded. The resulting cell pellets were washed with 5 ml PBS, re-centrifuged (2772 *g*, 4min, 4°C) and the supernatant fraction removed. Methanol (60%, 1 ml) was added to the cells, which were then vortexed and extracted overnight at 4°C. After extraction, samples were centrifuged and the methanol extracts of each set of flasks pooled. A 50 μl aliquot of the extract was counted in scintillant (4 ml) in a liquid scintillation counter to determine the total intracellular radioactivity. The 60% methanol was then evaporated under a steam of nitrogen. Samples were stored at −20°C until h.p.l.c. analysis.

Cell extracts were reconstituted in 50 μl of ddH_2O and 3TC and its phosphate metabolites were identified by radiometric h.p.l.c. using a protocol previously described [29, 30].

HepG2 cell incubations and dCTP determination

Incubations were performed using identical conditions as described previously, except 3TC was nonradiolabelled. All of the incubations were performed in duplicate on four separate occasions.

Following incubations, HepG2 cells were trypsinized as described earlier and aliquots from the resulting cell suspensions ($1–1.5 \times 10^6$ cells) were transfered to 1.5 ml microcentrifuge tubes. The tubes were extracted using perchloric acid and the acid extracts neutralized by the addition of freshly prepared 0.5N trioctylamine (27%) in 1′,1′,2′-trichlorotrifluoroethane (73%) (100 μl). Extracts were stored at −20°C until determination of dCTP pools by a similar procedure to that described previously [31]. Reaction mixtures (100 μl) contained 0.2μCi [^{3}H]-dATP, 0.25 μM synthetic oligonucleotide primer, 10 mM $MgCl_2$, 5 mM dithiothreitol, 50 mM Tris-HCl (pH 7.5) and 1 unit Sequenase 2.0 enzyme.

The reactions were performed at 37°C for 30 min (the time period to allow optimal incorporation of dNTPs). Duplicate aliquots from each reaction mixture were spotted onto Whatman DE81 paper circles (25 mm DEAE paper), which binds oligonucleotides. After drying, the paper circles were washed three times for 10 min each with 5% Na_2HPO_4 (1×8 ml, 2×4 ml) to remove unincorporated nucleotides, rinsed once with ddH_2O (8 ml) and once with 95% ethanol (3 ml). The paper circles were dried and then counted in Aqualuma Plus scintillant (4 ml).

Cell viability

Cell viability was assessed by the tetrazolium MTT (3,(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; MTT) assay. The MTT assay is based on the reduction of the soluble pale yellow tetrazolium salt to an insoluble blue formazan product by the mitochondria of cells [32, 33]. HepG2 cells were incubated with either HU, MTX, FLU, GCV, LCV, PCV, ADV, or FOS at the concentrations previously investigated in 96 well microtitre plates (1×10^5 HepG2 cells/well; 100 μl volume) at 37°C in a humidified, 5% CO_2 gassed incubator for 24 h. MTT (5 mg ml^{-1}; 25 μl) was added 2 h prior to terminating the incubation by the addition of 20% SDS (w/v) in 50% dimethylformamide (v/v). The plates were then incubated for a further 20 h (37°C; 5% CO_2), to lyse the cells and dissolve the formazan crystals within them, and then read at 570 nm.

Data manipulation and statistical methods

Cell counting and cell viability were also assessed at the conclusion of the incubations by the method of trypan blue exclusion, to aliquots of cells taken from each plate. The 3TC phosphorylation data and dCTP data were standardized to pmol per million viable cells. 3TCTP/dCTP were calculated using these data. Cell viability data as determined by the MTT assay were expressed as percentage change in formazan production. The data were analysed by analysis of variance (ANOVA) followed by a modified *t*-test (Bonferroni).

Results

3TC phosphorylation results

A typical h.p.l.c. profile for the detection of 3TC and phosphorylated anabolites, in HepG2 cells incubated for 24 h, is shown in Figure 1. 3TC-5′-diphosphate (3TCDP) was the predominant metabolite, accounting for

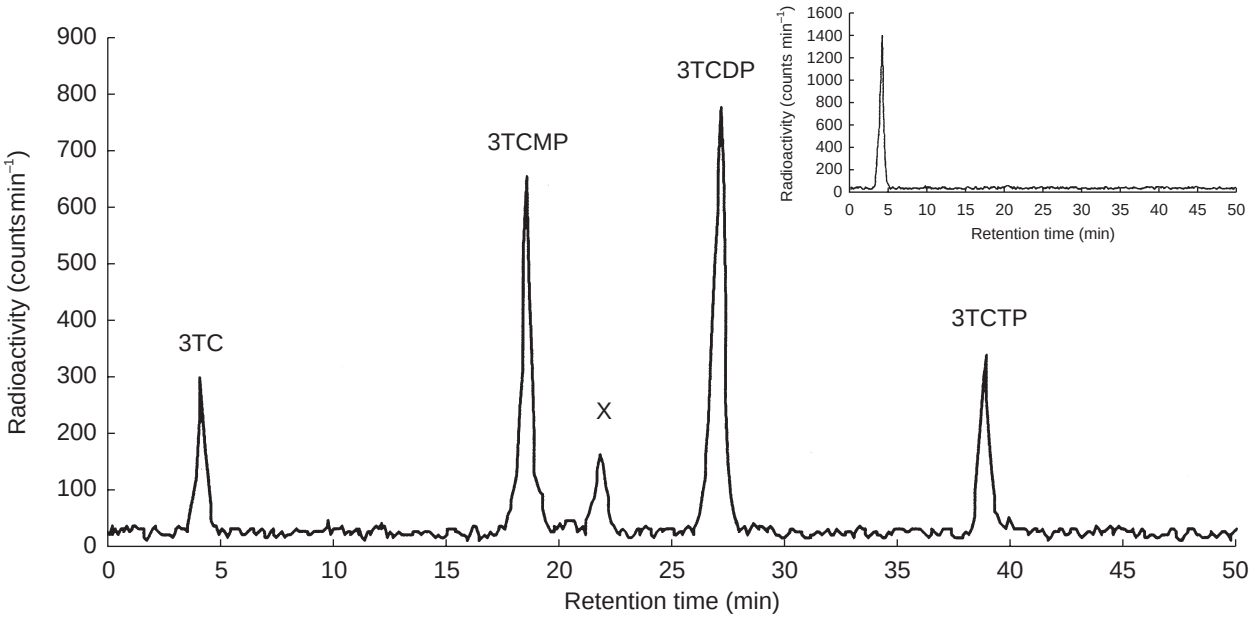

Figure 1 Typical radiochromatogram showing the intracellular phosphorylation of [^{3}H]-3TC (0.65μCi; 1 μM) in HepG2 cells incubated for 24 h. Inset: radiochromatogram shows HepG2 sample after treatment with acid phosphatase (0.5mg ml^{-1}, 37°C, 2 h). Metabolite X is postulated to be 3TCDP choline.

about 45% of the 3TC metabolites detected. 3TC-5′-monophosphate (3TCMP) and 3TCTP accounted for approximately 32% and 15% of the metabolites, respectively. The remaining 8% of the 3TC metabolites detected corresponded to a peak between 3TCMP and 3TCDP, which was labelled X. Complete hydrolysis of all the metabolites was achieved by the addition of acid phosphatase (0.5 mg ml^{-1}) to the extracts (Figure 1 inset). Assuming that the volume of a eukaryotic cell such as HepG2 is 1 pl [34], the intracellular concentration of 3TCTP from control cells ranged from 0.12 to 2.83 μM.

The effects of HU, MTX, and FLU on 3TC phosphorylation are shown in Table 1. There was a significant increase in 3TC phosphates by HU at a concentration of 100 μM (e.g. 361% of control 3TCTP production, $P<0.01$). MTX significantly enhanced the phosphorylation of 3TC at concentrations of 0.1 μM (e.g. 196% of control total phosphates; $P<0.05$), and 1 μM (e.g. 267% of control 3TCTP production; $P<0.01$). 3TC phosphorylation was also significantly increased in the presence of FLU at a concentration of 100 μM (155% of control 3TCTP production; $P<0.05$, and 193% of control total phosphates; $P<0.001$).

Neither GCV, LCV, PCV, ADV, or FOS significantly altered the phosphorylation of 3TC at any of the concentration studied (Tables 2 and 3).

dCTP and cell viability results

The effects of all the compounds previously investigated

Table 1 The effect of HU, MTX and FLU on 3TC phosphate levels and dCTP pools, in HepG2 cells incubated for 24 h.

Drug	*Total 3TC phosphates (pmol/10^6 cells)*	*Amount of 3TCTP (pmol/10^6 cells)*	*Amount of dCTP (pmol/10^6 cells)*	*3TCTP/dCTP ratio*
HU Control	5.02±0.47	0.79±0.53	4.48±1.61	0.176
HU 10 μM	8.51±6.14 (165)	1.08±0.56 (194)	4.04±1.30 (92)	0.267
HU 100 μM	12.22±5.50 (239)**	2.11±0.89 (361)**	3.37±1.36 (74)**	0.626
MTX Control	5.66±2.06	0.84±0.34	4.03±0.93	0.208
MTX 0.1 μM	10.56±3.37 (196)*	1.91±1.19 (219)*	5.27±2.23 (128)	0.362
MTX 1 μM	14.46±5.81 (258)**	2.45±1.45 (267)**	4.62±1.68 (113)	0.530
FLU Control	4.61±0.19	1.11±0.02	4.91±0.47	0.226
FLU 10 μM	4.98±0.84 (106)	1.10±0.29 (99)	4.95±0.45 (102)	0.222
FLU 100 μM	9.03±1.36 (193)***	1.72±0.46 (155)*	2.97±0.55 (61)***	0.579

3TC concentration was 1 μM. Data expressed as mean ± s.d. ($n=4$).% of control values in brackets. Data analysed by ANOVA and modified *t*-test (Bonferroni). *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Table 2 The effect of GCV, LCV and PCV on 3TC phosphate levels and dCTP pools, in HepG2 cells incubated for 24 h.

Drug	*Total 3TC phosphates (pmol/10^6 cells)*	*Amount of 3TCTP (pmol/10^6 cells)*	*Amount of dCTP (pmol/10^6 cells)*	*3TCTP/dCTP ratio*
GCV Control	5.98 ± 1.35	0.52 ± 0.45	5.01 ± 0.55	0.104
GCV 1 μM	5.95 ± 1.05 (102)	0.52 ± 0.49 (96)	5.04 ± 0.63 (101)	0.103
GCV 10 μM	5.42 ± 1.27 (91)	0.44 ± 0.39 (90)	5.15 ± 0.36 (103)	0.085
LCV Control	10.66 ± 1.54	2.31 ± 0.51	4.23 ± 0.26	0.546
LCV 1 μM	10.42 ± 1.66 (98)	2.50 ± 0.82 (107)	4.06 ± 0.95 (95)	0.616
LCV 10 μM	10.17 ± 2.02 (97)	2.30 ± 1.05 (99)	4.69 ± 0.21 (111)	0.490
PCV Control	9.68 ± 0.27	1.62 ± 0.33	4.68 ± 0.20	0.346
PCV 1 μM	10.04 ± 0.61 (104)	1.83 ± 0.37 (114)	4.64 ± 0.68 (99)	0.394
PCV 10 μM	9.68 ± 1.04 (100)	1.85 ± 0.43 (120)	5.01 ± 1.01 (107)	0.369

3TC concentration was 1 μM. Data expressed as mean ± s.d. ($n = 4$).% of control values in brackets. Data analysed by ANOVA and modified *t*-test (Bonferroni).

on 3TC phosphorylation, on dCTP levels, can also be found in Tables 1,2 and 3.

HU, at a concentration of 100 μM, significantly reduced the levels of dCTP (74% of control dCTP levels; $P < 0.01$; Table 1). The corresponding ratio of 3TCTP/dCTP was increased at this concentration (356% of control). HU 10 μM also caused an increase in the 3TCTP/dCTP ratio (152% of control). dCTP pools were not significantly affected by MTX, at any of the concentrations studied (Table 1). However, the 3TCTP/dCTP ratio was still increased at 0.1 and 1 μM MTX (e.g. at 1 μM, 255% of control). FLU, at a concentration of 100 μM, significantly reduced dCTP levels (61% of control dCTP levels; $P < 0.001$; Table 1). Consequently, the 3TCTP/dCTP ratio was increased at this concentration (256% of control).

The presence of GCV, LCV, PCV, ADV, or FOS had no effect on the levels of dCTP in HepG2 cells at any of the concentrations investigated (Tables 2 and 3). No alterations in the ratios of 3TCTP/dCTP were observed with these drugs.

Finally, the MTT assay was used to determine cell viability in the presence of all the compounds studied (data not shown). FLU significantly reduced formazan production at concentrations of 10 and 100 μM (e.g. at 10 μM, 94% of control formazan production; $P < 0.05$). Formazan production was also significantly decreased in the presence of MTX at concentrations of 0.1 and 1 μM (e.g. at 1 μM, 88% of control formazan production; $P < 0.001$). None of the remaining compounds investigated had any significant effects on formazan production at any of the concentrations investigated.

Discussion

The limitations of current therapies for the treatment of HBV infection suggest that the use of two or more drugs in combination will provide more benefits to patients in the future. Advantages of combination therapy include synergy, the targetting of different parts of the HBV lifecycle, and an action against mutants evolved or selected under monotherapy drug pressure. A number of *in vitro* studies have demonstrated additive to synergistic activities of combinations of FOS with purine nucleoside analogues (e.g. GCV) against both hepadnaviruses and herpes viruses [35–38]. In contrast, the combination of purine analogues with anti-HIV nucleosides (e.g. zidovudine (ZDV), didanosine (ddI)) has resulted in antagonism of activities against HIV infection [39]. However, little is known about

Table 3 The effect of ADV and FOS on 3TC phosphate levels and dCTP pools, in HepG2 cells incubated for 24 h.

Drug	*Total 3TC phosphates (pmol/10^6 cells)*	*Amount of 3TCTP (pmol/10^6 cells)*	*Amount of dCTP (pmol/10^6 cells)*	*3TCTP/dCTP ratio*
ADV Control	6.93 ± 0.84	0.75 ± 0.13	2.67 ± 0.57	0.281
ADV 1 μM	6.67 ± 0.60 (97)	0.83 ± 0.21 (111)	2.78 ± 0.79 (105)	0.299
ADV 10 μM	6.46 ± 0.81 (94)	0.69 ± 0.08 (94)	3.24 ± 1.12 (121)	0.213
FOS Control	6.29 ± 0.98	1.35 ± 0.33	4.77 ± 0.25	0.283
FOS 10 μM	6.04 ± 0.66 (99)	1.26 ± 0.53 (93)	4.70 ± 0.88 (99)	0.268
FOS 100 μM	5.74 ± 0.77 (94)	1.21 ± 0.43 (88)	4.57 ± 1.02 (96)	0.265

3TC concentration was 1 μM. Data expressed as mean ± s.d. ($n = 4$).% of control values in brackets. Data analysed by ANOVA and modified *t*-test (Bonferroni).

the effects of combinations of anti-HIV nucleosides (e.g. 3TC) with purine nucleosides against HBV infection.

The aims of these studies were to investigate the intracellular phosphorylation of 3TC in HepG2 cells, and to study the effects of various compounds on this activation. As the activity of 3TC against HBV (and HIV) is dependent on formation of the active TP, we were interested in investigating whether the presence of other compounds, which may be used in combination regimens with 3TC, could alter this activation. As the level of the competing dNTP is equally as important, the effects of these compounds on dCTP pools was also studied, so that 3TCTP/dCTP ratios could be calculated. Additionally, the effects of a number of modulators were also investigated for their ability to affect these levels in HepG2 cells. The use of such compounds may potentiate the activities of existing therapies.

The phosphorylation of 3TC in HepG2 cells is similar to that observed in previous studies with U937 cells and phytohaemagglutinin (PHA) stimulated peripheral blood mononuclear cells (PBMCs) [30]. The rate-limiting step for this activation again appears to be from 3TCDP to 3TCTP, as the levels of 3TCDP were the greatest. 3TC phosphorylation studies in 2.2.15 cells (HepG2 cells transfected with HBV) performed by Rahn *et al.* [21], showed that 3TCDP was also the largest anabolite formed. Although 3TCTP levels were higher than those observed in the work described here, the overall activation of 3TC was similar. However, an additional metabolite (X), postulated to be 3TCDP choline, was also observed, in a similar fashion to 3TC phosphorylation in U937 cells [30]. In comparison with previous findings in U937 cells or PHA-stimulated PBMCs, the levels of 3TC phosphates were lower in HepG2 cells. This may be due to lower amounts of intracellular enzymes compared with the other cell types investigated, or possibly may also involve the longer doubling time of HepG2 cells (observed once every 48 h).

HU (100 μM), significantly increased the phosphorylation of 3TC in HepG2 cells, with significant reductions in dCTP also noted at this concentration (Table 1). The resulting 3TCTP/dCTP ratio was increased dramatically. The mechanism behind this interaction involves the production of dCTP itself. dCTP is produced from two sources, the reduction of circulating cytidine-DP (CDP) to form dCDP, which is then phosphorylated to dCTP (the *de novo* pathway), and, the sequential phosphorylation of dC to dCTP (the dC salvage pathway). HU inhibits ribonucleotide reductase, the enzyme responsible for the reduction of CDP to dCDP, thereby halting the *de novo* pathway [40]. The corresponding fall in dCTP levels (as shown by the results) leads to activation of deoxycytidine kinase (dCK), resulting in an increased phosphorylation of 3TC, which utilizes the salvage pathway for activation. However, dCTP itself should also increase, but it is the balance of the two pathways that will ultimately determine the overall effect of HU on the levels of this metabolite.

MTX gave rise to significant increases in 3TC phosphorylation, whereas the size of the dCTP pool was unaffected at this same concentration (Table 1) MTX is thought to inhibit cell growth through an inactivation of dihydrofolate reductase [41]. A loss of cell viability was observed as determined by the MTT assay. This results in a disturbance of *de novo* thymidylate and/or purine biosynthesis [42]. Reductions in 2′-deoxythymidine-TP (dTTP) pools are thought to lead to a removal of feedback inhibition of dCMP deaminase activity, theoretically resulting in a reduction in dCTP levels. However, no change in dCTP was observed in the presence of this compound suggesting that dCK may have been activated to compensate for the loss of dCTP. An increase in 3TC phosphorylation supports this hypothesis. Consequently, the 3TCTP/dCTP ratio was still increased, suggesting that MTX may also have the ability to potentiate the activity of 3TC.

FLU significantly increased 3TC phosphorylation as well as significantly reducing dCTP levels, at a concentration of 100 μM (Table 1), resulting in an increased 3TCTP/dCTP ratio. Similarly, studies by Rahn *et al.* [21] demonstrated modest increases in 3TCDP and 3TCTP using FLU 5 μM, in 2.2.15 cells. The activity of FLU has been attributed to direct and indirect effects of the active moeity of FLU, fludarabine-TP (FaraATP), on dCK. FaraATP directly activates dCK, and also indirectly activates this enzyme through inhibition of ribonucleotide reductase (in a similar fashion to HU) [43]. The observed increase in 3TC phosphorylation is therefore expected, whereas the reduction in dCTP suggests that activation of dCK is unable to overcome the initial inhibition of ribonucleotide reductase.

The use of the above modulators again demonstrates their ability to increase the 3TCTP/dCTP ratio. This may result in a potentiation of the activity of 3TC, when used in combination with one of these compounds. Although further studies would need to be performed to assess the effectiveness of such combinations, the viability of this type of approach to therapy should not be ignored, especially as salvage therapy or pre-emptive therapy for groups at high risk of evolving a mutation.

The purine nucleosides GCV, LCV, and PCV, the nucleotide ADV, and FOS had no significant effects on both 3TC phosphorylation and dCTP pools (Tables 2 and 3). The lack of any interactions observed with these compounds suggests that the activity of 3TC may be unaltered when used in combination. However, further studies would need to be performed to assess whether 3TC has the ability to alter the activation of these compounds, and also to determine the effectiveness of combinations of

3TC with the purine nucleosides, and ADV and FOS, against HBV replication.

This work has shown that 3TC is phosphorylated in HepG2 cells, in a similar fashion to the other cell types previously investigated. The advantageous interaction between 3TC and various modulators in this cell line, and the lack of an interaction with other anti-HBV agents is reassuring. These studies may have implications for the design of combination regimens that may be used in the future treatment of HBV infection.

The next stage of the work is to determine 3TCTP and dCTP levels in liver biopsy samples from HBV-infected patients on therapy, and to determine whether the *in vitro* data are predictive of the situation *in vivo*.

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