Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 68 (2004) 1825–1831
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Effective metabolism and long intracellular half life of the anti-Hepatitis B agent adefovir in hepatic cells

Adrian S. Ray*, Jennifer E. Vela, Loren Olson, Arnold Fridland

Department of Drug Metabolism, Gilead Sciences, Inc., Foster City, Ca 94404, USA

Received 22 March 2004; accepted 8 July 2004

Abstract

Adefovir dipivoxil (ADV) is esterolytically cleaved to the 2′-deoxyadenosine monophosphate (dAMP) analog adefovir, subsequent phosphorylation leads to the formation of the anti-Hepatitis B virus (HBV) agent adefovir-DP. To better understand the mechanism of action of ADV, metabolism studies were done in Hep G2, Huh-7 and primary human hepatocytes. Separation of radiolabeled adefovir metabolites after incubation in Hep G2 cells suggested that adefovir in its mono- and di-phosphorylated forms are the only metabolites formed from adefovir. Incubation of 10 μM adefovir with hepatic cell lines and fresh monolayers of primary human hepatocytes from two donors and analysis of intracellular metabolites by liquid chromatography coupled to tandem mass sepctrometery resulted in adefovir-DP levels of approximately 10 pmol/million cells. Adefovir was more efficiently phosphorylated in primary hepatocytes than cell lines with adefovir-DP accounting for 44% versus 26% of total intracellular adefovir after 24 h. Egress studies showed adefovir-DP to have a half-life of 33 ± 3 h, 10 ± 1 h, 48 ± 3 h and 33 ± 2 h in Hep G2, Huh-7, and primary hepatocytes from two separate donors, respectively. The markedly shorter half-life in Huh-7 cells was inferred to be transport dependent based on its sensitivity to the transport inhibitor MK-571. Effective phosphorylation coupled with a long intracellular half-life and small competing dATP pool sizes in primary hepatocytes forms the cellular metabolic basis for the efficacy of adefovir dipivoxil in the treatment of chronic hepatitis B.

Keywords: HBV; HCV; Adefovir; Hepsera; Transport and nucleoside metabolism

1. Introduction

The World Health Organization estimates that approximately 400 million people world wide are chronically infected with HBV and at least 30% will develop cirrhosis and/or hepatocellular carcinoma. ADV (Hepsera®, Gilead Sciences, Inc.) is a 10 mg once daily oral treatment for HBV infection [1]. It has been shown to be effective in both HBV e antigen-positive and -negative patients [2,3] and is efficacious in patients with lamivudine resistant virus [4]. A novel resistance mutation to adefovir has recently been identified with an infrequent occurrence in the treated population [5]. These findings demonstrate that ADV is both an efficacious and durable therapy for chronic hepatitis B.

ADV is an oral prodrug of adefovir, an acyclic 2′-deoxyadenosine monophosphate (dAMP) analog. The pivoxil moieties act to increase cellular permeability [6] and are rapidly cleaved, chemically and by esterase activity, to adefovir during absorption through the gut wall [7]. To exert an antiviral effect, adefovir has to enter cells and be phosphorylated to its active diphosphorylated form (adefovir-DP). Adefovir has been shown to be transported into cells by a saturable protein mediated process in Hela cells [8] and by fluid-mediated endocytosis in CCRF CEM T-lymphoblastoid cells [9]. After entry into the cell, adefovir is phosphorylated to its monophosphate form (adefovir-MP) by adenylate kinase 2 [10,11]. Many enzymes have been shown to be able to phosphorylate nucleotide analogs to their triphosphate forms [12]. However, the

Abbreviations: ADV, adefovir dipivoxil; adefovir, 9-(2-phosphonomethoxyethyl)adenine; dA, 2′-deoxyadenosine; HBV, Hepatitis B virus; MK-571, 3-[[3-[2-(7-chloroquinolin-2-yl)vinyl]phenyl]-(2-dimethyl-carbamoyl-ethyl-sulfanyl)methylsulfanyl] propionic acid; CIP, calf intestinal phosphatase, the suffixes -MP, -DP or -TP are added to reflect the nucleoside or nucleotides mono-, di- or triphosphorylated forms, respectively.

* Corresponding author. Tel.: +650 522 5536; fax: +650 522 5266.
E-mail address: aray@gilead.com (A.S. Ray).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.07.010

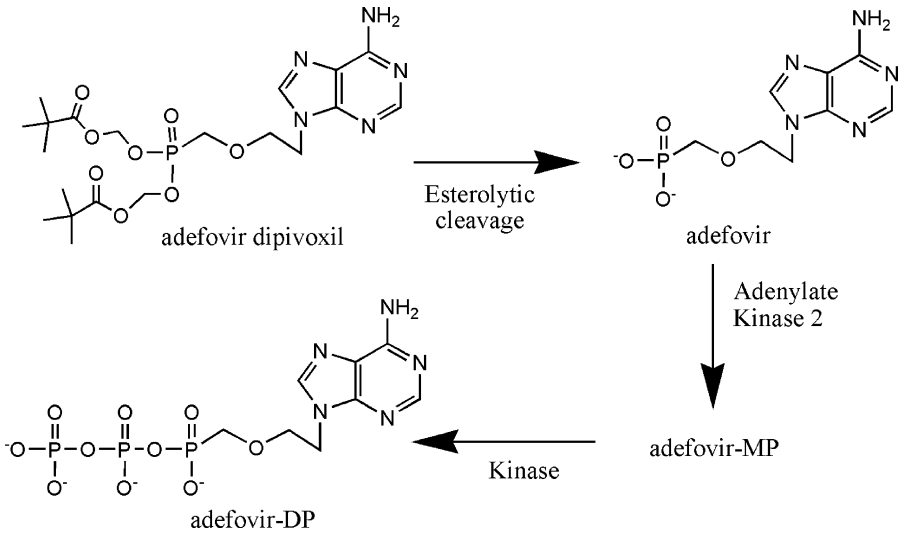

Fig. 1. Putative pathway for the activation of adefovir dipivoxil to the dATP analog adefovir-DP.

enzyme responsible for most efficiently catalyzing the phosphorylation of adefovir-MP is not known. A direct mechanism for the conversion of adefovir to adefovir-DP through the enzymatic addition of pyrophosphate has also been described [13]. The putative activation pathway for ADV is presented in Fig. 1. Upon phosphorylation, adefovir-DP competes with dATP for incorporation by the HBV reverse transcriptase. The lack of a 3′-hydroxyl group causes chain-termination when adefovir is incorporated into viral transcripts.

To better understand the pharmacological basis for the inhibition of HBV, cellular metabolism studies were done with adefovir in primary human hepatocytes and cell lines commonly used to study anti-HBV agents. Utilizing ion-pairing liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) it was found that adefovir is efficiently phosphorylated in hepatic cells. A long intracellular half-life serves to maintain levels of adefovir-DP sufficient to effectively compete with dATP and inhibit HBV replication. Taken together these results provide a metabolic profile consistent with the known efficacy of once daily administration of ADV in chronic hepatitis B treatment.

2. Materials and methods

2.1. Chemicals

Cell culture supplies were purchased from Irvine Scientific. [Adenine-2,8-^{3}H]-adefovir was obtained from Moravek Biochemicals, Inc. Adefovir was used fresh and double purified by the vendor to limit the appearance of radiolabeled adenosine metabolites (see results). Adefovir and adefovir-DP were provided by Gilead Sciences, Inc. Stable isotope labeled $^{13}C^{15}N$ dATP was purchased from Spectra Stable Isotopes. Adefovir-MP was synthesized by modification of an existing method for converting acyclic phosphonate nucleotide analogs into their diphosphorylated forms [14]. Briefly, adefovir was first treated with carbonyl diimidazole in dimethylformamide, followed by methanol at room temperature. A mixture of tetrabutylammonium phosphate and tributylamine was added to complete the formation of adefovir-MP. The resulting nucleotide was then purified using a linear gradient of triethyl ammonium bicarbonate and strong anion exchange chromatography on a mono Q HR 5/5 column purchased from Amersham Biosciences. Purity of nucleotides were verified by reverse phase ion-pairing HPLC (described below). The transport inhibitor 3-[[3-[2-(7-chloroquinolin to 2 to yl)vinyl]phenyl] and (2-dimethyl-carbamoyl-ethylsulfanyl)methylsulfanyl] propionic acid (MK-571) was obtained from Biomol Research Laboratories, Inc. All other chemicals used were the highest grade available from Sigma–Aldrich.

3. Cell culture

Hep G2 (American Tissue Type Culture number HB-8065) and Huh-7 cells (provided by Dr. Joseph Torresi, Royal Melbourne Hospital and The University of Melbourne) were maintained in Earle's modified essential medium supplemented with 10% heat inactivated fetal bovine serum, L-glutamine, nonessential amino acids and penicillin-streptomycin. Fresh monolayers of primary hepatocytes were obtained from Gentest pre-plated in 12 well tissue culture plates and experiments were done immediately using Gentest's recommended media (Hepatostim media). Cell lines were transferred to twelve well tissue culture plates by trypsonization and grown to confluency (0.88×10^{6} cells/well). For uptake experi-

ments, cells were continuously treated with 10 μM adefovir (1000 dpm/pmol ^{3}H adefovir for experiments using radioactive detection). At each time point the reaction was stopped by removing the drug containing media and washing the well two times with 5 mL ice cold phosphate buffered saline. Cells were then scraped into 1 mL 70% methanol and frozen overnight to facilitate the extraction of nucleotide metabolites. Cell lyses was inferred to be complete by the measurement of intracellular ribonucleotide concentrations similar to those found in the literature (data not shown). Samples to be analyzed by LC/MS/MS had 2 pmol tenofovir diphosphate (tenofovir-DP)/1 × 10^5 cells added to the 70% methanol to serve as an internal standard. Egress studies were done similarly but after 24 h incubation with media containing adefovir, the adefovir was removed from the well by washing two times with 5 mL warm media and the well replenished with media lacking adefovir. Time points were then taken as described for uptake. All samples were then dried under vacuum and resuspended in dH_2O (for radioactivity analysis) or 80 mM tetrabutylammonium hydroxide (TBAH) and 4 mM monobasic ammonium phosphate (for LC/MS/MS analysis). Recovery during sample preparation of all measured analytes from lysed cell matrix in 70% methanol was observed to be greater than 95% in control experiment.

3.1. Calf intestinal phosphatase (CIP) treatment of radioactively labeled intracellular metabolites

In order to distinguish whether phosphorylated radiolabeled metabolites formed in Hep G2 cells arose from adefovir or adenosine, dephosphorylation reactions were done. To intracellular metabolites extracted from 1 × 10^5 Hep G2 cells 10 units of CIP (Sigma–Aldrich) was added with provided CIP buffer. After 2 h at 37 °C reactions were analyzed by ion-pairing reverse phase HPLC (described below). While phosphorylated metabolites are not easily separated, the difference in retention between adefovir and adenosine allows for unambiguous identification of which gave rise to phosphorylated metabolite peaks observed in the absence of CIP treatment.

3.2. Effect of MK-571 on adefovir egress from Huh-7 cells

To assess the role of cellular transporters in the rapid egress of adefovir and its phosphorylated metabolites from Huh-7 cells, experiments were done in the presence of MK-571. MK-571's inhibition of the multi drug resistance proteins-4 and -5 has been characterized in the literature [15] but it is likely that its inhibition of transport is not limited to these two transporters. Side by side egress experiments were done in the presence or absence of 200 μM MK-571 essentially as described above. To study the effects of MK-571 wells were washed twice with 5 mL warm media containing MK-571 and then media was replenished containing MK-571 but lacking adefovir. Time points were taken as described above and metabolites analyzed by LC/MS/MS (described below).

4. Analysis

Studies using radiolabeled adefovir for detection were analyzed using reverse phase ion-pairing chromatography and a Phenomenex Prodigy 5u ODS C18 column (Phenomenex). The gradient was run with 2 min isocratic buffer A (5% acetonitrile, 5 mM tetrabutylammonium bromide (TBAB), 25 mM potassium phosphate (pH 6.0)) followed by a linear gradient to 50% buffer B (60% acetonitrile, 5 mM TBAB, 25 mM potassium phosphate (pH 6.0)) over 25 min. The column was then washed with 100% buffer B and re-equilibrated before injection of the next sample. Fractions were collected every minute and subject to scintillation counting.

Transient ion-pairing high performance liquid chromatography coupled to positive ion electrospray tandem mass spectrometery (LC/MS/MS) was used to analyze adefovir and its phosphorylated forms for uptake and egress studies. Methods will be described in detail in a separate manuscript. Briefly, earlier methods for nucleoside triphosphate analysis using TBAH as an ion pair [16] were modified to include an acetonitrile step gradient in order to attain sufficient retention of adefovir and adefovir-MP on an Xterra MS, C18, 3.5 μM, 1.0 × 100 mm reverse phase column (Waters Corporation). Mobile phases included 0.25 mM TBAH, 4 mM ammonium phosphate, pH 6.0 and concentrations of acetonitrile ranging from 6 to 20% using a flow rate of 50 μL/min. Parent/daughter mass transitions of 274.2/162.4, 354.0/162.4, 434.0/162.4 and 448.3/176.4 (all in atomic mass units) for adefovir, adefovir-MP, adefovir-DP and tenofovir-DP (internal standard) were monitored on an API-4000 triple quadrupole mass spectrometer purchased from Applied Biosystems/MDS Sciex. Concentrations were determined based on calibration curves covering three orders of magnitude with linearity in excess of r^2 equal to 0.99. Typical lower limits of quantitation of 20–60 fmol on column (0.2–0.6 pmol/million cells in a 10 μL injection of lysate from 1 × 10^5 cell) were achieved. Stable isotope methods for the measurement of dATP will be described in detail along with the adefovir detection methods elsewhere (manuscript in preparation). Briefly, standard curves in cellular matrix were done utilizing stable isotopically labeled dATP (mass transition of 506.7/145.6 amu) while concurrently the amount of dATP (mass transition of 492.1/136.2 amu) in the cellular sample was monitored. LC/MS/MS methods described above were used for these analyses. Data from uptake and egress experiments were fitted using nonlinear regression and the program Kaleidagraph (Synergy Software).

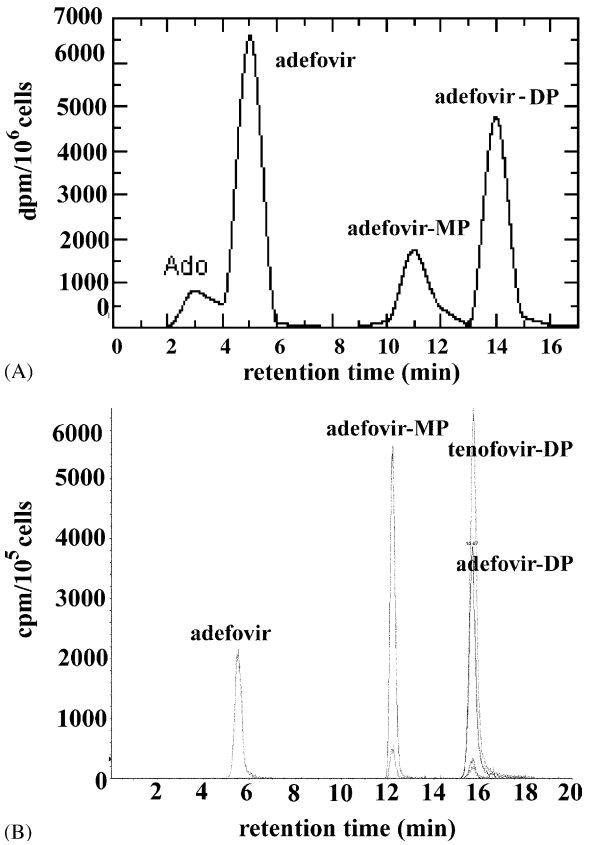

Fig. 2. Analysis of metabolites from cellular incubations with adefovir. (A) Metabolism of 10 μM adefovir after a 24 h incubation in Hep G2 cells showing 1.4, 14, 4.1 and 8.8 pmol/million cells of adenosine, adefovir, adefovir-MP, and adefovir-DP, respectively. (B) Separation of 2 pmol adefovir, adefovir-MP, adefovir-DP and tenofovir-DP (used as internal standard) by ion-pairing LC/MS/MS methods. Sample was generated by adding 200 nM of each analyte to Hep G2 lysate at a concentration of 1×10^5 cells/10 μl TBAH injection buffer and injecting 10 μl.

5. Results

5.1. Metabolism of [^{3}H] adefovir in Hep G2 cells

Metabolites formed from incubation of Hep G2 cells with 10 μM [^{3}H] adefovir (1000 dpm/pmol) for 24 h (metabolic pathway for ADV activation to adefovir shown in Fig. 1) were assessed by HPLC and radioactivity detection. Adenosine, adefovir, adefovir-MP and adefovir-DP were found to be the main radiolabeled peaks formed (Fig. 2A). Dephosphorylation with CIP can be used to clearly identify the phosphorylated peaks as arising from adenosine or adefovir because of their distinct analytical retention times. Dephosphorylation of radiolabeled nucleotides showed that after 24 h a majority of the radioactively labeled metabolites were due to adefovir. However, at early time points most radiolabeled peaks arose from adenosine in its mono-, di- and triphosphorylated forms (94% at 2 h, data not shown). Careful HPLC analysis of the radiolabeled adefovir showed a minor contaminant (<1% of total radiation) that coeluted with an adenine standard. These results illustrated the necessity of developing a more accurate method of quantitation using LC/MS/MS (Fig. 2B, manuscript detailing analytical method being prepared separately).

5.2. Analysis of adefovir uptake in cell lines and primary hepatocytes

To study the kinetics of uptake and phosphorylation of adefovir in different cell types, incubations were done over 24 h with cells continuously exposed to 10 μM adefovir in the media. Incubations in hepatic cells lines (Hep G2 and Huh-7) led to similar levels of adefovir and its phosphorylated metabolites in each cell type. After 24 h of incubation 26% of the total adefovir taken up into the two cell types was converted to adefovir-DP (Table 1). The uptake in Hep G2 cells showed a slow accumulation of adefovir only approaching equilibrium after 24 h and linear increases in adefovir-MP and -DP (Fig. 3A). Similar to cell lines, analysis of uptake and metabolism in primary hepatocytes from two donors showed a slow accumulation of adefovir in cells and linear increases in adefovir-MP and -DP. adefovir-DP was formed more efficiently in primary hepatocytes relative to either of the cell lines, comprising 44% of the total intracellular adefovir after 24 h in each of the two separate donors (Table 2).

5.3. Half-life of adefovir and its phosphorylated metabolites

After exposure of cells for 24 h to 10 μM adefovir, the rates of decay of intracellular adefovir, adefovir-MP and

Table 1
Uptake of 10 μM adefovir in hepatic cell lines

Hep G2 (pmol/million)				Huh-7(pmol/million)			
Time (h)	Adefovir	Adefovir-MP	Adefovir-DP	Time (h)	Adefovir	Adefovir-MP	Adefovir-DP
2	4.54 ± 0.65[a]	BLOQ[b]	0.34 ± 0.02	2	4.36 ± 0.28	BLOQ	0.23 ± 0.06
4	5.94 ± 0.21	0.62 ± 0.27	0.83 ± 0.08	4	5.43 ± 0.52	BLOQ	0.80 ± 0.05
8	9.61 ± 1.55	1.53 ± 0.12	2.17 ± 0.14	8	7.94 ± 0.20	0.61 ± 0.15	2.00 ± 0.15
24	21.8 ± 0.9	3.63 ± 0.75	8.95 ± 0.80	24	19.1 ± 0.7	1.74 ± 0.59	7.20 ± 0.39

[a] Values are the mean ± S.D. of three experiments.
[b] BLOQ = below limit of quantitation. Defined as signal less than five-fold greater than background. For these analytes this was defined as 0.2–0.6 pmol/million (20–60 fmol on column).

Table 2
Uptake of 10 μM adefovir in primary hepatocytes

Donor 1 (pmol/million)				Donor 2 (pmol/million)			
Time (h)	Adefovir	Adefovir-MP	Adefovir-DP	Time (h)	Adefovir	Adefovir-MP	Adefovir-DP
2	2.91 ± 0.41[a]	0.84 ± 0.27	1.11 ± 0.03	2	2.20 ± 0.34	BLOQ[b]	0.39 ± 0.02
4	4.74 ± 0.33	1.42 ± 0.15	2.22 ± 0.13	4	2.46 ± 0.24	0.37 ± 0.11	0.84 ± 0.05
8	6.45 ± 1.56	2.82 ± 0.28	5.36 ± 0.34	8	3.94 ± 0.15	0.99 ± 0.12	2.03 ± 0.02
24	12.4 ± 1.2	6.32 ± 1.13	14.8 ± 1.8	24	6.47 ± 0.33	2.69 ± 0.31	7.21 ± 0.27

[a] Values are the mean ± S.D. of three experiments.
[b] BLOQ = below limit of quantitation. Defined as signal less than five-fold greater than background. For these analytes this was defined as 0.2–0.6 pmol/million (20–60 fmol on column).

adefovir-DP were studied following the removal of drug from the media. Plotting the intracellular concentrations of adefovir, adefovir-MP and adefovir-DP in Hep G2 cells over 72 h showed a slow egress from cells consistent with a single exponential decay curve (Fig. 3B). Similar data were obtained from egress experiments done in Hep G2 cells using radiolabeled adefovir for detection of metabolites (data not shown). Single exponential decay curves fitted to the data from Hep G2, Huh-7 and the 2 primary hepatocyte donors are summarized in Table 3. In general, Hep G2 and primary hepatocytes showed similar apparent half-lives for each of the phosphorylated metabolites ranging from 19 ± 6 h (adefovir-MP in Hep G2 cells) to 48 ± 3 h (adefovir-DP in primary hepatocytes from donor 1). However, Huh-7 cells exhibited a more rapid egress of adefovir and its phosphorylated metabolites. The most marked change was on adefovir which had a half-life approximately 10-fold less than that found for the other cells.

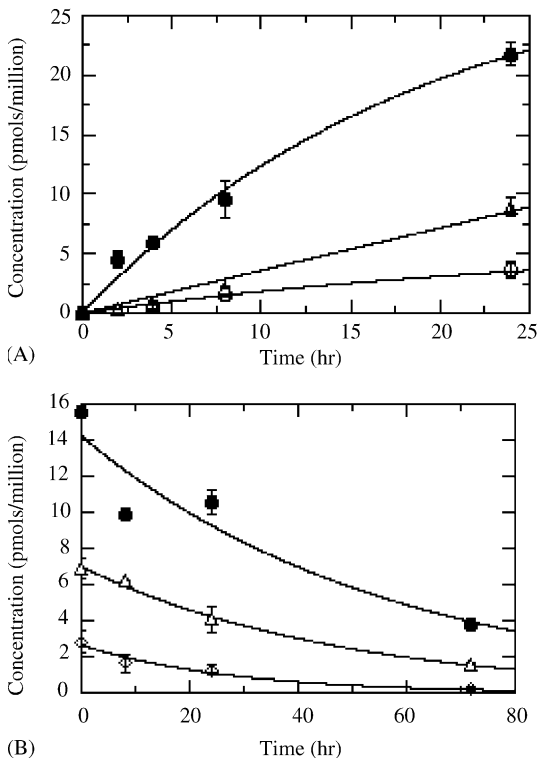

Fig. 3. Uptake (A) and egress (B) of metabolites formed from10 μM adefovir incubations in Hep G2 cells. Fitting data for adefovir (●), adefovir-MP (○) and adefovir-DP (△, values represent mean ± S.D. of three experiments) to a single exponential decay curve resulted in calculated half lives of 38 ± 15 h, 19 ± 6 h and 33 ± 3 h for adefovir, adefovir-MP and adefovir-DP, respectively.

5.4. Effect of a transport inhibitor on the rapid efflux observed in Huh-7 cells

To better understand the efflux of adefovir and its phosphorylated metabolites from Huh-7 cells, studies were done in the presence or absence of the transport inhibitor MK-571. Plotting the concentration over time showed that the efflux of adefovir, adefovir-MP and adefovir-DP were all markedly reduced by the presence of MK-571 (Fig. 4). Rate determination showed that adefovir, adefovir-MP and adefovir-DP left the cells approximately 3-fold more slowly in the presence of MK-571 (Table 4).

5.5. Assessment of competing dATP pool size in different cell types

Adefovir-DP competes with dATP for incorporation by the HBV reverse transcriptase. The level of dATP was measured in each of the cell types studied utilizing a stable isotope method and LC/MS/MS detection (manuscript in preparation). Consistent with published reports [17], it was found that primary hepatocytes from the two donors had 5.4- to 32-fold less dATP than either of the cell lines (Table 5).

Table 3
Half-lives for adefovir and its phosphorylated metabolites in different cell types

Cell type	Apparent $t_{1/2}$ (h)		
	Adefovir	Adefovir-MP	Adefovir-DP
Hep G2	38 ± 15[a]	19 ± 6	33 ± 3
Huh 7	3.7 ± 0.4	13 ± 4	10 ± 1
Primary hepatocytes donor 1	33 ± 2	38 ± 3	48 ± 3
Primary hepatocytes donor 2	34 ± 1	22 ± 1	33 ± 2

[a] Errors represent the mean ± S.D. of three experiments fit to a single exponential decay curve by non-linear curve fitting.

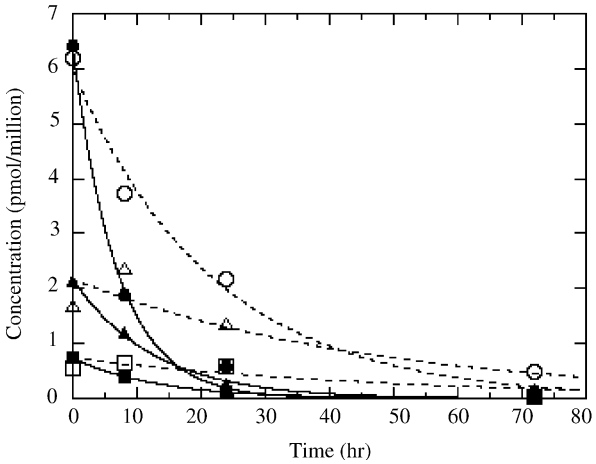

Fig. 4. Efflux in the absence or presence (closed or open symbols, respectively) of the transport inhibitor MK-571 of adefovir (● or ○), adefovir-MP (■ or □) and adefovir-DP (▲ or △) from Huh-7 cells. Fitting the data to single exponential decay curves showed that MK-571 caused an approximate three-fold increase in the half-lives for adefovir and its phosphorylated metabolites.

Table 4
Effect of 200 μM MK-571, an inhibitor of MRP 4 and 5, on adefovir and its phosphorylated metabolites efflux from Huh-7 cells

Conditions	Apparent $t_{1/2}$ (h)		
	Adefovir	Adefovir-MP	Adefovir-DP
Huh-7 – MK-571	4.8 ± 0.6[a]	9.2 ± 0.5	8.7 ± 0.1
Huh-7 + MK-571	15 ± 1	35 ± 15	32 ± 14

[a] Errors represent the mean ± S.D. of three experiments fit to a single exponential decay curve by non-linear curve fitting.

Table 5
Cellular concentration of dATP as determined by stable isotope LC/MS/MS

Cell type	dATP (pmol/million)
Hep G2	12.1 ± 0.3[a]
Huh 7	19.4 ± 0.4
Hepatocyte donor 1	0.605 ± 0.026
Hepatocyte donor 2	2.23 ± 0.03

[a] Values represent the mean ± S.D. of six injects of extracted intracellular material.

6. Discussion

In order to determine the number of metabolites generated intracellularly from adefovir, radiolabeled adefovir was incubated with Hep G2 cells. Dephosphorylation showed that adenosine metabolites (AMP, ADP and ATP) predominated at early time points (first 10 h) while adefovir phosphorylated metabolites accounted for a majority of the radiation thereafter. This radiation profile is consistent with a contaminant in the radiolabeled adefovir pulse labeling the adenosine nucleoside and nucleotide pools. Consistent with this hypothesis, analysis of the radiolabeled adefovir showed a minor contaminant with a retention time consistent with adenine (data not shown). Based on these findings adefovir-MP and adefovir-DP appear to be the only true metabolites formed from adefovir in Hep G2 cells. Similar conclusions were reached based on previous studies of adefovir metabolism in lymphocytes [18]. These data illustrate the metabolic stability of adefovir and suggest that it should have a simple activity profile based on only itself and its phosphorylated anabolites.

Adefovir was efficiently phosphorylated in each of the hepatic cell lines studied. In primary hepatocytes the antiviral active anabolite, adefovir-DP, was the predominant metabolite after 24 h. Efficient phosphorylation was accompanied by long intracellular half-lives for adefovir and its metabolites in Hep G2 cells and primary hepatocytes. The observation of long intracellular persistence of adefovir-DP in hepatic cells is consistent with previous studies in T-cells [19]. These in vitro observations suggest that pharmacologically effective levels of adefovir and its metabolites should persist in hepatic tissue and are consistent with the observed efficacy of once daily ADV therapy.

A number of efflux pumps have been implicated in the transport of nucleotide analogs including the multi-drug resistance proteins (MRPs) -2 [20], -4 and -5 [21,22] and breast cancer resistance protein (BCRP) [23] in cell culture. However, the physiological importance of these transporters during anti-viral therapy is still unclear [24]. The sensitivity of Huh-7 cell efflux of adefovir and its phosphorylated metabolites to MK-571 suggests that a transporter maybe involved. MK-571 has been shown to be an inhibitor of MRP-4 and -5 in transfected HEK293 cells with 50% inhibition constants of approximately 10 and 40 μM, respectively [24]. However, MK-571 inhibition of other transporters has not been well characterized and it is difficult to ascertain the identity of the specific transporter(s) responsible from these studies. In light of the importance of Huh-7 cells containing hepatitis C replicons in the screening of agents for anti-Hepatitis C virus activity [25], the presence of high levels of a cellular transporter could be an important factor to take into consideration. Clearly, further studies into the identity of the transporter responsible for adefovir efflux from Huh-7 cells, the potential effects of this transport mechanism on other compounds, and the establishment of its presence in different stocks and replicon transfected lines of Huh-7 cells are warranted.

Adefovir-DP competes with dATP for incorporation by HBV reverse transcriptase in order to exert its antiviral effect. The level of dATP in a given cell type is therefore important in defining the ability of adefovir to inhibit viral replication. Our measurements showed that in cellular samples the amount of dATP ranged from 0.605 to 19.4 pmol/million cells, with a rank order of Huh-7 > Hep G2 ≫ hepatocyte from donor 1 > hepatocyte from donor 2. Interestingly, in primary hepatocytes where the metabolism of adefovir was most efficient the levels of competing dATP were also the lowest. This suggests that because of a higher ratio of adefovir-DP to dATP, that

adefovir should have the greatest competitive advantage in the physiologically relevant cell type and that anti-HBV activity determined in cell lines may underestimate the ability of ADV to inhibit HBV in vivo.

In conclusion, this report demonstrates that adefovir is effectively phosphorylated in Hep G2, Huh-7 and primary hepatocytes accumulating high levels of the dATP analog adefovir-DP. Furthermore, in Hep G2 and primary hepatocytes, adefovir-DP has a long intracellular half-life. In Huh-7 cells we've identified evidence for a transport mechanism which may have implication for hepatitis C drug discovery efforts. Most importantly, these data illustrate, in the appropriate cellular system for HBV infection, that therapy with ADV should generate levels of adefovir-DP in the liver sufficient to compete with dATP for incorporation by HBV reverse transcriptase and that the amount of active metabolite should not vary greatly between doses. Overall these data provide a metabolic basis for ADV's efficacy in the clinical treatment of chronic hepatitis B.

Acknowledgement

We would like to thank Carol L. Brosgart for supporting the research and carefully reviewing the manuscript, Robert L. St. Claire III for advice on the LC/MS/MS methods used, Alan Huang for the synthesis of adefovir-MP, and William Delaney and Joseph Torresi for providing Huh-7 cells.

References

[1] Gilead Sciences, Inc., Hepsera (adefovir dipivoxil) full prescribing information, September 2002.
[2] Marcellin P, Chang TT, Lim SG, Tong MJ, Sievert W, Shiffman ML, et al. Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. N Engl J Med 2003;348:808–16.
[3] Hadziyannis SJ, Tassopoulos NC, Heathcote EJ, Chang TT, Kitis G, Rizzetto M, et al. Adefovir dipivoxil for the treatment of hepatitis B e antigen-negative chronic hepatitis B. N Engl J Med 2003;348:800–7.
[4] Dando T, Plosker G. Adefovir dipivoxil: a review of its use in chronic hepatitis B. Drugs 2003;63:2215–34.
[5] Angus P, Vaughan R, Xiong S, Yang H, Delaney W, Gibbs C, et al. Resistance to adefovir dipivoxil therapy associated with the selection of a novel mutation in the HBV polymerase. Gastroenterology 2003;125:292–7.
[6] Srinivas RV, Robbins BL, Connelly MC, Gong YF, Bischofberger N, Fridland A. Metabolism and in vitro antiretroviral activities of bis(-pivaloyloxymethyl) prodrugs of acyclic nucleoside phosphonates. Antimicrob Agents Chemother 1993;37:2247–50.
[7] Naesens L, Balzarini J, Bischofberger N, De Clercq E. Antiretroviral activity and pharmacokinetics in mice of oral bis(pivaloyloxymethyl)-9-(2-phosphonylmethoxyethyl)adenine, the bis(pivaloyloxymethyl) ester prodrug of 9-(2-phosphonylmethoxyethyl)adenine. Antimicrob Agents Chemother 1996;40:22–8.
[8] Cihlar T, Rosenberg I, Votruba I, Holy A. Transport of 9-(2-phosphonomethoxyethyl)adenine across plasma membrane of HeLa S3 cells is protein mediated. Antimicrob Agents Chemother 1995;39:117–24.
[9] Olsanska L, Cihlar T, Votruba I, Holy A. Transport of adefovir (PMEA) in human T-lymphoblastoid cells. Collect Czech Chem Commun 1997;62:821–8.
[10] Robbins BL, Greenhaw J, Connelly MC, Fridland A. Metabolic pathways for activation of the antiviral agent 9-(2-phosphonylmethoxyethyl)adenine in human lymphoid cells. Antimicrob Agents Chemother 1995;39:2304–8.
[11] Krejcova R, Horska K, Votruba I, Holy A. Phosphorylation of purine (phosphonomethoxy)alkyl derivatives by mitochondrial AMP kinase (AK2 type) from L1210 cells. Collect Czech Chem Commun 2000;65:1653–68.
[12] Krishnan P, Fu Q, Lam W, Liou JY, Dutschman G, Cheng YC. Phosphorylation of pyrimidine deoxynucleoside analog diphosphates: selective phosphorylation of L-nucleoside analog diphosphates by 3-phosphoglycerate kinase. J Biol Chem 2002;277:5453–9.
[13] Balzarini J, De Clercq E. 5-Phosphoribosyl 1-pyrophosphate synthetase converts the acyclic nucleoside phosphonates 9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine and 9-(2-phosphonylmethoxyethyl)adenine directly to their antivirally active diphosphate derivatives. J Biol Chem 1991;266:8686–9.
[14] Kappler F, Hai TT, Cotter RJ, Hyver KJ, Hampton A. Isozyme-specific enzyme inhibitors. 11. L-Homocysteine-ATP S-C5′ covalent adducts as inhibitors of rat methionine adenosyltransferases. J Med Chem 1986;29:1030–8.
[15] Reid G, Wielinga P, Zelcer N, De Haas M, Van Deemter L, Wijnholds J, et al. Characterization of the transport of nucleoside analog drugs by the human multidrug resistance proteins MRP4 and MRP5. Mol Pharmacol 2003;63:1093–4.
[16] St. Claire III RL. Positive ion electrospray ionization tandem mass spectrometry coupled to ion-pairing high-performance liquid chromatography with a phosphate buffer for the quantitative analysis of intracellular nucleotides. Rapid Commun Mass Spectrom 2000;14:1625–34.
[17] Traut TW. Physiological concentrations of purines and pyrimidines. Mol Cell Biochem 1994;140:1–22.
[18] Bronson JJ, Ho HT, De Boeck H, Woods K, Ghazzouli I, Martin JC, et al. Biochemical pharmacology of acyclic nucleotide analogues. Ann N Y Acad Sci 1990;616:398–407.
[19] Balzarini J, Hao Z, Herdewijn P, Johns DG, De Clercq E. Intracellular metabolism and mechanism of anti-retrovirus action of 9-(2-phosphonylmethoxyethyl)adenine, a potent anti-human immunodeficiency virus compound. Proc Natl Acad Sci USA 1991;88:1499–503.
[20] Miller DS. Nucleoside phosphonate interactions with multiple organic anion transporters in renal proximal tubule. J Pharmacol Exp Ther 2001;299:567–74.
[21] Schuetz JD, Connelly MC, Sun D, Paibir SG, Flynn PM, Srinivas RV, et al. MRP4 A previously unidentified factor in resistance to nucleoside-based antiviral drugs. Nat Med 1999;5:1048–51.
[22] Wijnholds J, Mol CA, van Deemter L, de Haas M, Scheffer GL, Baas F, et al. Multidrug-resistance protein 5 is a multispecific organic anion transporter able to transport nucleotide analogs. Proc Natl Acad Sci USA 2000;97:7476–81.
[23] Wang X, Furukawa T, Nitanda T, Okamoto M, Sugimoto Y, Akiyama S, et al. Breast cancer resistance protein (BCRP/ABCG2) induces cellular resistance to HIV-1 nucleoside reverse transcriptase inhibitors. Mol Pharmacol 2003;63:65–72.
[24] Wielinga PR, van der Heijden I, Reid G, Beijnen JH, Wijnholds J, Borst P. Characterization of the MRP4- and MRP5-mediated transport of cyclic nucleotides from intact cells. J Biol Chem 2003;278:17664–71.
[25] Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999;285:110–3.