Background: Clevudine (CLV) is a nucleoside analogue of the unnatural L-configuration that has demonstrated potent activity against hepatitis B virus (HBV) in vitro and in Phase III clinical studies. Human hepatoma cell lines are the only liver-derived cells in which CLV metabolism has been investigated. Here, we examine CLV metabolism in primary human hepatocytes, which better represent CLV metabolism in the liver.

Methods: HPLC analysis of primary human hepatocyte extracts incubated with radiolabelled CLV was used to determine the time course of CLV phosphorylation. Effects of the exogenous cell concentration of CLV on phosphorylation were assessed and the half-life of the CLV phosphorylated forms was determined.

Results: The major CLV metabolite formed in human primary hepatocytes was 5′-monophosphate, whereas in the hepatoma cell lines the major metabolite was 5′-triphosphate. The level of CLV 5′-triphosphate was similar in both cell types. In primary hepatocytes the conversion of CLV 5′-monophosphate to the corresponding 5′-diphosphate was the rate-limiting step in CLV phosphorylation; the level of CLV phosphorylation was dependent upon exogenous drug concentration and exposure time. CLV 5′-triphosphate accumulated rapidly with peak levels observed after ~8 h. When cells were incubated with 1 μM CLV, the approximate maximal plasma concentration achieved in individuals receiving the 30 mg dose, the average intracellular concentration of CLV 5′-triphosphate was 41.3 ± 8.4 pmols/10^6 cells (~10 μM). The initial half-life of CLV triphosphate was ~11 h.

Conclusions: These results are consistent with once daily CLV dosing currently being used in Phase III clinical studies.

Original article
Clevudine is efficiently phosphorylated to the active triphosphate form in primary human hepatocytes

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Introduction

Hepatitis B virus (HBV) infection constitutes a major worldwide health threat. In addition to the morbidity associated with acute clinical infection, chronic liver disease, cirrhosis, and primary hepatocellular carcinoma (HCC) are recognized sequelae [1,2]. It is estimated that there are 350 million chronic carriers worldwide [2]. To date, interferon-α, lamivudine, adefovir, entecavir and, more recently, telbivudine have received approvals for the treatment of chronic HBV infection. However, the development of new antiviral agents for the treatment of chronic hepatitis B remains a major goal with the hope of obtaining a sustained virological response post-treatment.

Clevudine, also known as 1-(2′-deoxy-2′-fluoro-β-L-arabinofuranosyl)-5-methyluracil, 1-(2′-deoxy-2′-fluoro-β-L-arabinofuranosyl)thymine, L-FMAU and CLV, is a nucleoside analogue of the unnatural β-L configuration that has potent anti-HBV activity in vitro and in vivo with a favourable toxicity profile in all species tested [3–13]. In HBV-infected patients, CLV has demonstrated potent antiviral efficacy and significant biochemical improvement after only 24 weeks of therapy [8,12,13]. These effects have been sustained in a significant portion of patients when therapy was stopped with no viral rebound occurring in ~3% and ~16% of patients who were positive or negative for hepatitis B e antigen (HBeAg), respectively [8,12,13]. The mechanism of action of CLV involves the preferential inhibition of the DNA-dependent activity of the HBV DNA polymerase by CLV 5′-triphosphate [10,14,15]. Cytosolic thymidine kinase, deoxycytidine kinase and mitochondrial deoxyxypirimidine kinase are the major enzymes responsible for phosphorylating CLV to the 5′-monophosphate derivative. Subsequent phosphorylation to di- and triphosphate is catalyzed by thymidylate kinase and 3-phosphoglycerate kinase, respectively [14,16,17]. Previous metabolism studies performed in human hepatoma cells (HepG2 and
HepG2 2.2.15 cells) showed that CLV was readily phosphorylated by these cells to the 5'-triphosphate and the triphosphate was the major metabolite [10,14]. When CLV was removed following a 24 h exposure of human hepatoma cells to the drug, 90% of the triphosphate disappeared in the first 8 h; in the subsequent 16 h the rate of decay of the triphosphate was slower [10]. Here, we describe the metabolism of CLV in primary human hepatocytes and show that the metabolic profile of CLV is different from that seen in human hepatoma cells. The results of these studies provide further support for once daily dosing with CLV.

Methods

Compounds and cells

CLV was synthesized by Pharmasset, Inc. (Princeton, NJ, USA). [3H]-CLV (specific activity = 13.6 Ci/mmol) was synthesized by Moravek Biochemicals, Inc. (Brea, CA, USA). Primary human hepatocytes in six-well collagen-coated plates were purchased from CellzDirect (Pittsboro, NC, USA) or Cambrex (Walkersville, MD, USA). Cells were incubated 16 h prior to exposure to the drug. All incubations were carried out at 37˚C in a humidified 5% CO2 atmosphere.

Time course study

To determine the time course of phosphorylation of CLV, cells from three different donors were incubated with 10 μM [3H]-CLV (2,000 DPM/pmol) for up to 48 h. After 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 8, 12, 24 and 48 h of incubation with CLV cells were harvested by trypsinization. The cells were harvested, extracted with methanol and the extracts were analysed by HPLC as described below. Kinetic simulation of the rate of intracellular phosphorylation of CLV was performed using KinTekSim version 3.20 (KinTek Corporation, Austin, TX, USA).

Dose response study

To determine the effect of exogenous CLV concentration on CLV phosphorylation, cells from two different donors were incubated at 37˚C for 24 h with [3H]-CLV at concentrations ranging from 0.1 to 500 μM. At the end of the incubation the cells were harvested, extracted and analysed as described below.

Half-life determination

To determine the half-life of the phosphorylated forms of CLV, cells from three different donors were incubated with 10 μM [3H]-CLV (2,000 DPM/pmol) for 24 h. Following the incubation, the cells were washed three times with warm drug-free medium. Additional washes were performed throughout the course of the experiments to avoid reutilization of excreted CLV. Cells were harvested and extracted at 0, 1, 2, 4, 6, 8, 12, 24, 48 and 72 h following the removal of CLV. The half-life of CLV was calculated by fitting the data to a single exponential decay equation using GraFit program version 5 (Erithacus Software, Horley, Surrey, UK).

Sample preparation

At the selected times, extracellular medium was removed and the cell layer washed three times with cold phosphate-buffered saline (PBS). The cells were removed by trypsinization and brought up to 1 ml in cold PBS and counted. The cells were then pelleted by centrifugation at 1,200 RPM for 5 min and the pellet was resuspended in 1 ml of cold 60% methanol followed by an overnight incubation at -20˚C. The extracts were centrifuged at 14,000 RPM at 4˚C for 2 min. Supernatants were dried under a gentle flow of filtered nitrogen and stored at -20˚C until analysis. Residues were resuspended in 100 μl of water and 50 μl aliquots analyzed using HPLC.

Identification of intracellular metabolites

CLV and the phosphorylated derivatives of CLV were separated by ion-exchange HPLC using a Series 200 HPLC system (PerkinElmer, Wellesley, MA, USA) and a Whatman 10 μm SAX column (Whatman, Maidstone, UK) coupled to a radiometric detector (610TR Radiometric Flow Scintillation Analyzer, PerkinElmer, Wellesley, MA, USA). The mobile phase consisted of buffer A (0.02 M KH2PO4) and buffer B (1 M KH2PO4, pH 3.5). Elution was performed using a linear gradient of buffer B from 0 to 100% for 90 min. CLV and the respective phosphorylated forms of CLV were identified on the basis of the retention time of synthesized standards of the phosphorylated forms of CLV.

Results

Metabolic profile of CLV in primary human hepatocytes

HPLC analysis of extracts prepared from primary human hepatocytes, following 24 h incubation with 10 μM CLV, was performed as described above. All three phosphorylated derivatives, CLV mono-, di- and triphosphate (CLV-MP, CLV-DP and CLV-TP, respectively), were detected (Figure 1A) and the retention time for each radiolabelled phosphate species corresponded to the retention times of the unlabelled reference compounds. The predominant phosphorylated derivative detected in primary human hepatocytes was CLV-MP (Figure 1A). The mean intracellular concentration of CLV-MP in hepatocytes from 10 donors incubated with 10 μM CLV was 393 ±70 pmol/10⁶ cells (Table 1), whereas the levels of
CLV-DP and CLV-TP were lower with a mean intracellular concentration of 23.6 ± 7.2 and 85.8 ± 25.5 pmol/10⁶ cells, respectively (Table 1). This profile was different from that seen with the human hepatoma cell line Huh7. The predominant phosphorylated form of CLV in Huh7 cells incubated with 10 μM CLV was CLV-TP (Figure 1B). The level of CLV-DP and CLV-TP in Huh7 cells (20.28 ± 1.31 and 80.07 ± 6.46 pmol/10⁶ cells, respectively) was similar to that formed in primary human hepatocytes (Table 1). However, the level of CLV-MP in Huh7 cells (25.8 ± 4.7 pmol/10⁶ cells) was 15-fold lower than the level of CLV-MP (Table 1) formed in stationary primary human hepatocytes.

Effect of exogenous drug concentration on CLV metabolism
CLV nucleotide formation was dependent upon the concentration of the drug in the medium (Figure 2). Increasing the exogenous concentration of CLV resulted in an increase in the intracellular concentration of each of the three phosphate derivatives of CLV. CLV-TP levels reached equilibrium at an extracellular CLV concentration of ∼10 μM and the levels of CLV-MP and CLV-DP reached equilibrium at concentrations of ∼100 μM and ∼50 μM, respectively (Figure 2).

To determine the level of CLV-TP that might be achieved in individuals receiving the 30 mg dose of CLV, we measured the level of CLV-TP after exposing hepatocytes to 1 μM CLV, which is approximately equal to the mean maximal plasma concentration (Cₘₐₓ) of CLV attained in individuals receiving the 30 mg dose. Using HPLC analysis following a 24 h exposure of hepatocytes obtained from four donors to 1 μM CLV, we determined a mean concentration for CLV-TP of 41.3 ± 8.4 pmols/10⁶ cells.

Kinetics of CLV phosphorylation in primary human hepatocytes
In order to determine the steady-state level of CLV and its phosphorylated derivatives, primary human hepatocytes from three donors were incubated with 10 μM clevudine (CLV) for 24 h. The mean concentrations of detected clevudine (CLV) metabolites after exposure to 10 μM clevudine for 24 h were 393 ± 70, 23.6 ± 7.2 and 85.8 ± 25.5 pmol/10⁶ cells for CLV-MP, CLV-DP and CLV-TP, respectively. For comparison, the mean concentrations in Huh7 cells were 25.8 ± 4.7, 20.3 ± 1.3 and 80.1 ± 6.5 pmol/10⁶ cells for CLV-MP, CLV-DP and CLV-TP, respectively. CLV-MP, clevudine monophosphate; CLV-DP, clevudine diphosphate; CLV-TP, clevudine triphosphate.

Table 1. Clevudine mono-, di-, and triphosphate formation in primary hepatocytes from different donors and in Huh7 cells exposed to 10 μM clevudine for 24 h

<table>
<thead>
<tr>
<th>Donor</th>
<th>Age, years</th>
<th>Gender</th>
<th>Race</th>
<th>CLV-MP, pmol/10⁶ cells</th>
<th>CLV-DP, pmol/10⁶ cells</th>
<th>CLV-TP, pmol/10⁶ cells</th>
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<tbody>
<tr>
<td>A</td>
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<tr>
<td>B</td>
<td>13</td>
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<td>African–American</td>
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<td>12.1</td>
<td>83.3</td>
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<tr>
<td>C</td>
<td>43</td>
<td>Male</td>
<td>African–American</td>
<td>394</td>
<td>23.1</td>
<td>88.9</td>
</tr>
<tr>
<td>D</td>
<td>62</td>
<td>Female</td>
<td>Caucasian</td>
<td>336</td>
<td>18.8</td>
<td>60.8</td>
</tr>
<tr>
<td>E</td>
<td>68</td>
<td>Female</td>
<td>Caucasian</td>
<td>321</td>
<td>20.9</td>
<td>105.4</td>
</tr>
<tr>
<td>F</td>
<td>71</td>
<td>Female</td>
<td>Caucasian</td>
<td>392</td>
<td>20.2</td>
<td>68.1</td>
</tr>
<tr>
<td>G</td>
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<td>Male</td>
<td>Caucasian</td>
<td>445</td>
<td>36.5</td>
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</tr>
<tr>
<td>H</td>
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<td>African–American</td>
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<td>66.1</td>
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<tr>
<td>I</td>
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<tr>
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<td>Hispanic</td>
<td>506</td>
<td>32.9</td>
<td>147.3</td>
</tr>
</tbody>
</table>

In primary human hepatocytes the mean concentrations of detected clevudine (CLV) metabolites after exposure to 10μM clevudine for 24 h were 393 ± 70, 23.6 ± 7.2 and 85.8 ± 25.5 pmol/10⁶ cells for CLV-MP, CLV-DP and CLV-TP, respectively. For comparison, the mean concentrations in Huh7 cells were 25.8 ± 4.7, 20.3 ± 1.3 and 80.1 ± 6.5 pmol/10⁶ cells for CLV-MP, CLV-DP and CLV-TP, respectively. CLV-MP, clevudine monophosphate; CLV-DP, clevudine diphosphate; CLV-TP, clevudine triphosphate.
10 µM CLV. At different times, cells were extracted and the extracts were analysed by HPLC; the concentration of the phosphorylated forms of CLV was determined. As the intracellular concentration of ATP, the likely phosphate donor in the anabolic pathway of CLV, is in excess over CLV and its metabolites, all phosphorylation events can be considered pseudo-first-order. Kinetics of the phosphorylation events were simulated using KinTekSim by fitting the data of the levels of the metabolites from the three different donors’ hepatocytes (Figure 3A) to a pseudo-first-order rate equation. On the basis of the simulated rate constants, apparent equilibrium constants were calculated as shown in Figure 3B. The kinetic simulation indicates that the fastest step in the anabolic pathway is the phosphorylation of CLV to CLV-MP and that the phosphorylation of CLV-MP to CLV-DP is the rate-limiting step. The simulation also indicates that the phosphorylation of CLV-DP to CLV-TP, although considerably slower than the initial phosphorylation step, is faster than the phosphorylation of CLV-MP to CLV-DP.

Intracellular stability of the phosphorylated derivatives of CLV

To determine the half-life of each of the phosphorylated forms of CLV, primary human hepatocytes from three different donors were incubated for 24 h with 10 µM CLV, at which point the medium containing CLV was removed and replaced with drug-free medium. At different time points up to 72 h, extracts were prepared and the intracellular concentrations of CLV-MP, -DP, and -TP were quantified by HPLC analysis.

Of the three phosphorylated forms of CLV, CLV-MP showed the most rapid decrease in intracellular concentration. The decrease in CLV-MP followed single-phase exponential decay kinetics with a mean half-life of 0.9 ± 0.1 h (Figure 4). Similarly, the decrease of CLV-DP followed single-phase exponential decay kinetics giving a mean half-life of 5.3 ± 2.1 h. CLV-TP, by contrast, had a different decay profile. The level of CLV-TP continued to increase for up to 2 h following the removal of extracellular CLV. Thereafter, CLV-TP levels declined following single exponential decay kinetics with a mean half-life of 10.8 ± 2.1 h.

**Figure 2. Dose dependency of the formation of CLV-MP, CLV-DP and CLV-TP**

Primary human hepatocytes were incubated for 24 h with extracellular clevudine (CLV) concentrations of 0.1, 0.5, 1, 5, 10, 50, 100, 250 and 500 µM. CLV-MP, clevudine monophosphate; CLV-DP, clevudine diphosphate; CLV-TP, clevudine triphosphate.
Metabolism of CLV in primary hepatocytes obtained from different donors

Over the course of this study, hepatocytes from 10 different donors were used in our experiments. We therefore compared the level of CLV-MP, -DP and -TP produced by the hepatocytes of each donor to see if there was significant individual variation in the metabolism of the drug. For consistency we chose to use the results from the 24 h incubations with 10 μM CLV. As shown in Table 1, there appears to be little variation in the phosphorylation of CLV in hepatocytes from the different donors. The one exception, donor J, had a concentration of CLV-TP that was ∼70% higher than the mean intracellular concentration for all 10 donors’ hepatocytes.

Discussion

CLV is a nucleoside analogue of the unnatural t-configuration that has been shown to have potent anti-HBV activity in vitro, in vivo using animal models and in the clinic [3–13]. In cell culture CLV is readily phosphorylated to the corresponding 5′-triphosphate form of the compound [10,14]. The mechanism of action of CLV involves the inhibition of HBV DNA synthesis by CLV-TP [10,14,18]. In previous metabolism studies, human hepatoma cell lines were the only liver-derived cells in which the metabolism of CLV was investigated. In these cell lines CLV was readily converted to the corresponding 5′-monophosphate species and maximal metabolite formation was observed within 8 h; CLV-TP was the abundant species [10]. The metabolism of CLV in primary human hepatocytes is more likely to be representative of the metabolism of CLV in the liver than hepatoma cell lines. Thus, we evaluated the metabolism of CLV in primary hepatocytes with three aims in mind: to determine if the metabolic profile of CLV was different in primary human hepatocytes compared with hepatoma cell lines; to ascertain the half-life of CLV-TP in human hepatocytes; and to determine if there was a difference in the metabolism of CLV in primary hepatocytes from different donors.

When we compared the metabolism of CLV in primary human hepatocytes with the metabolism of the drug in the human hepatoma cell line Huh7, we found that the metabolic profile in primary hepatocytes differed from that seen in Hep7 cells. The metabolic profile of CLV in Huh7 cells was similar to that reported for HepG2 and HepG2 2.2.15 cells with CLV-TP being the predominant phosphorylated species [10,14]. However, in primary human hepatocytes the predominant metabolite was CLV-MP. The level of CLV-MP in hepatocytes was 15-fold greater than that formed in Huh7 cells treated with the same concentration of CLV (Table 1). However, the level of CLV-TP was similar in the two cell types.

Formation of the active triphosphate of CLV increased with time and reached steady-state by ∼8 h after exposure to the parent compound. The mean intracellular concentration of CLV-TP after exposure to 10 μM CLV for 24 h was 82.8 pmol/10⁶ cells. With the exception of one donor’s hepatocytes, the intracellular concentration of CLV-TP showed little variation. In this one exception, the level of CLV-TP was ∼70% higher than the mean. We also determined the intracellular concentration of CLV-TP in hepatocytes from four donors exposed to 1 μM CLV, which approximates to the Cmax in patients receiving the 30 mg dose of CLV. The average concentration of CLV-TP was 41.3 ±8.4 pmols/10⁶ cells. Based on a 3 μl cell volume for normal human parenchymal cells [19], this would give an intracellular concentration of ∼10 μM for CLV-TP. Therefore, the intracellular concentration of CLV-TP would be about 80-fold higher than the inhibition constant (Ki) of 0.12 μM reported by Liu et al. [14] for CLV-TP in an endogenous polymerase assay with the HBV viral particles.

The levels of CLV-MP, -DP and -TP in hepatoma cells ranked from the highest concentration to the
lowest was triphosphate > monophosphate > diphosphate, whereas in primary human hepatocytes the intracellular concentration was monophosphate > triphosphate > diphosphate. This suggests that in hepatocytes the conversion of CLV-MP to CLV-DP is the rate-limiting step in the anabolism of CLV. Results from fitting the data from time-course experiments using the hepatocytes of three different donors to a pseudo-first-order equation showed that the rate of phosphorylation of CLV to CLV-MP was the fastest step in the anabolism of CLV and the conversion of CLV-MP to CLV-DP was the slowest step in the pathway. These different results may be due to different phosphorylation patterns in primary hepatocytes versus hepatoma cell lines. CLV is known to be phosphorylated in vitro to CLV-MP by both thymidine kinase-1 and -2 and also 2′-deoxycytidine kinase [14]. It is possible that the expression levels of these kinases in particular deoxycytidine kinase (the enzyme that is most likely to be responsible for phosphorylating CLV [14]), may be different in these two cell types. Such mechanistic studies are currently ongoing.

In vivo efficacy studies performed in the woodchuck model showed marked, rapid inhibition of virus replication and no significant toxicity [11,20]. In the woodchuck there was a dose-dependent delay in viral recrudescence and a reduction or loss of covalently closed circular DNA [11,20]. In Phase III studies in both HBeAg-positive and HBeAg-negative patients, 30 mg CLV given once daily demonstrated potent antiviral efficacy and significant biochemical
improvement after only 24 weeks of therapy [8,12,13]. A sustained antiviral response (SVR) was seen in a significant number of patients with no viral rebound occurring in ~3% and ~16% in HBeAg-positive and HBeAg-negative patients, respectively, at 24 weeks after stopping therapy [8,12,13].

The mechanism by which CLV exerts this sustained antiviral response has been the subject of much speculation. One thought is that CLV-TP has an extraordinarily long half-life in the liver. Here we show that the half-life of CLV in primary human hepatocytes is ~11 h. This would mean that approximately seven half-lives or 3.2 days would be required for the intracellular concentration of CLV-TP (10 μM) to decline to a concentration that is less than the Ki for CLV-TP (0.12 μM) reported by Liu et al. [14] against the viral polymerase.

Although the half-life is sufficiently long to support once daily dosing it is, nevertheless, too short to account for the continued suppression of viral replication that is seen following the cessation of CLV treatment. Therefore, this slow or lack of recrudescence most likely involves some other mechanism such as a reduction in covalently closed circular DNA, which has been observed with CLV in the woodchuck model [11,20], or an as yet to be identified immunological effect on the viral polymerase.

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Disclosure statement

The authors are employees of Pharmasset, Inc.

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Clevudine phosphorylation in primary human hepatocytes