Determination of Pharmacokinetics and Pharmacodynamics of Lamivudine After Highdoses in Duck Hepatitis B Virus-Infected Pekin Ducks

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Abstract

Purpose: The Duck Hepatitis B Virus (DHBV)-infected Pekin duck model has been shown to be a reference model for the evaluation of anti-HBV treatments. The purpose of the current study was to characterize the pharmacokinetic and pharmacodynamic profiles of lamivudine, a potent nucleoside inhibitor of HBV replication, in DHBV-infected Pekin ducks.

Methods: Lamivudine serum concentrations were measured by LC-ESI-MS/MS following the administration of 80 mg/kg IV, 200 mg/kg IM and 480 mg/kg PO of lamivudine to DHBV-infected Pekin ducks. Whereas, DHBV viremia levels were measured by real-time-PCR before, during and after 6-week lamivudine treatment of 40 mg/kg IM daily, or 100 or 200 mg/kg PO daily.

Results: The average apparent total body clearance and volume of distribution of lamivudine were 0.29 L/hr/kg and 6.5 L/kg, respectively. The average area under the concentration-time curve was 318, 661 and 1344 µg/hr/mL for 80 mg/kg IV, 200 mg/kg IM and 480 mg/kg PO of lamivudine, respectively. 6-week lamivudine treatment of 100 mg/kg and 200 mg/kg PO were indifferently able to significantly lower DHBV titers compared with control and 40 mg/kg IM groups. However, the latent suppression of DHBV titers after the termination of lamivudine treatment was significantly more in 200 mg/kg PO compared with 100 mg/kg PO.

Conclusions: Our results suggest that the optimum dose of lamivudine against chronic HBV is higher than the current recommended dose in human.

Introduction

It is estimated that approximately two billion individuals have been infected with Hepatitis B Virus (HBV) at some point in their lives. Of these, over four hundred million develop chronic HBV infection, with persistent HBV DNA and HBV Surface Antigen (HBsAg) in their serum [1]. As a consequence of chronic HBV infection these patients are at high risk of developing cirrhosis, Hepatocellular Carcinoma (HCC), and liver failure resulting in over a million deaths each year [1,2]. The efficacy of current chronic HBV treatments, namely interferon-α and nucleoside analogues, is limited by the high barrier of drug-resistant mutants in addition to the persistence of intranuclear Covalently Closed, Circular Viral DNA (cccDNA), which is responsible for viral relapse as a consequence of treatment withdrawal [3-7].

There are six nucleoside analogues approved for the treatment of HBV, namely lamivudine, adefovir, entecavir, telbivudine, tenofovir and emtricitabine. Lamivudine [(−)-2′,3′-dideoxy-3′-thiacytidine] has been also approved for the treatment of human immunodeficiency virus (HIV) but at higher daily oral dose of 300 mg, compared with 100 mg for HBV. Lamivudine is phosphorylated inside the body to lamivudine-5′-triphosphate, which is a potent inhibitor of viral replication by acting as a chain terminator of viral DNA synthesis and also by competitively inhibiting viral reverse transcriptase [8-12]. In chronic HBV patients, there is a marked reduction in viremia during lamivudine treatment followed by a reversal of T cell anergy, resulting in enhancement of the HBV-specific cytotoxic T cell activity and CD8+ T-cell proliferation [13,14]. However, lamivudine has a low genetic barrier of resistance, placing lamivudine as an alternative line of chronic HBV treatment according to WHO guidelines for the prevention, care and treatment of persons with chronic hepatitis B infection [15]. Several reports have argued that lamivudine dose has not been properly adjusted and increasing the daily dose of lamivudine to 300 mg would impair the development of...
resistance and enhance the therapeutic outcome of lamivudine [16-18]. Though, lamivudine is still in the first-line of treatment in HBV/ 
HIV-coinfected adults, adolescents and children [15].

While lamivudine has been used in different animal models for 
HBV, the duck hepatitis B virus (DHBV)-infected Pekinduck.model has been shown to be a reference model for the evaluation of novel 
anti-HBV approaches and for testing their ability to clear viral cccDNA [19-22]. DHBV infection of newly hatched ducklings results in nearly 
100% chronic infection, similar to neonatal HBV infection of humans. 
Therefore, this approach was used in the present study to establish a 
flock of chronic DHBV carriers of the same age. The objectives of the 
current study were first to characterize the pharmacokinetic profile 
of lamivudine in DHBV-infected Pekinduck following Intravenous 
(IV), Intramuscular (IM) and Oral (PO) administration. The second 
objective was to quantify the effect of three lamivudine treatments of 
different pharmacokinetic/pharmacodynamic indices on DHBV titer 
in the serum of DHBV-infected Pekin duck.

Materials and Methods

Chemicals

Lamivudine was purchased from Royal Pharm (Hangzhou, 
China). The D4T (2′,3′-Didehydro-2′-deoxythymidine) compound 
that was used as an internal standard was purchased from Sigma- 
Aldrich (St. Louis, MO). Acetonitrile, methanol, formic acid, 
ammonium formate (High-Performance Liquid Chromatography 
[HPLC] grade) and other chemicals (analytical grade) were 
purchased from Fisher Scientific (Toronto, ON). Oasis HLB 1cc (30 
mg) solid phase extraction cartridges were purchased from Waters 
(Mississauga, ON).

Animal studies

All experimental procedures involving animals were approved by 
the University of Alberta Health Sciences Animal Policy and Welfare 
Committee and performed in compliance with relevant institutional 
policies, the Association for the Accreditation of Laboratory Animal 
Care guidelines, the National Institutes of Health regulations and 
local, provincial and federal laws.

Infection of Ducklings with Clal strain of DHBV

All animals were screened for the presence of DHBV infection by 
dot blotting prior to use in these studies. The mutant virus, DHBV- 
Clal, was made as previously described [23], Clal DHBV strain 
contains a point mutation at nucleotide 1858 which introduces a 
Clal restriction site without altering any amino acid sequence [23]. 
The adult Pekin ducks used in the current study were inoculated with 
the Clal DHBV strain within 48 h of hatching, and the 100 
µL of inoculum (1.10 x 109 genomic copies per mL) was injected 
intravenously in the medial metatarsal vein using insulin syringes. 
Animals were considered chronically infected if viremia was detected 
by PCR at 8 weeks after inoculation.

Experimental design

In the current study, two experiments were performed: 1) an 
experiment to determine the pharmacokinetic profile of lamivudine 
and 2) an experiment to investigate the effect of lamivudine 
administration on DHBV serum levels.

With respect to lamivudine pharmacokinetic profile, 10 adult 
DHBV-infected Pekin ducks (mean weight of 4.13 ± 0.35 kg) were used. 
Ducks were administered 80, 200 and 480 mg lamivudine per Kg 
of body weight by IV, IM and PO routes, respectively. An additional 
duck was administered saline served as a control. Blood samples were 
collected prior to drug administration and at 0, 0.25, 0.5, 0.75, 1, 1.5, 
2, 3, 4, 6, 8, 12, and 24 h after dosing through venipuncture of the 
opposite medial metatarsal vein. The blood samples were centrifuged 
immediately after clotting at room temperature, and serum was 
stored at -80° C until analysis.

In the second experiment, 16 adult DHBV-infected Pekin ducks 
(mean weight of 4.13 ± 0.35 kg) were used. Groups of 4 ducks were 
treated with saline, 40, 100 or 200 mg lamivudine per Kg of body 
weight by IM or PO routes of administration for 6 weeks. Blood 
samples were collected 3-week before, during and 6-week after 
lamivudine treatment.

Measuring lamivudine serum concentration by LC-ESI-MS/ 
MS assay

Serum samples obtained from ducks were first extracted by solid-
phase extraction technique. Briefly, 10 µl of the internal standard 
(0.1 mg/ml of D4T) was added to 100 µl serum sample and an equal 
volume of methanol was added. After vortexing and centrifugation 
at 10,000 x g for 6 min, the supernatant was brought up to 1 mL 
with water. Oasis HLB cartridges were preconditioned with 1 mL 
of methanol and water, in sequence. Following that, cartridges were 
loaded and washed with 1 mL of 5% v/v methanol/water. Finally, 
samples were eluted twice with 1 mL of ice cold methanol, which was 
dried under vacuum, and re-suspended in 30 µl of 5% v/v methanol/
water with 0.1% formic acid (pH~4.0). Chromatographic separation 
was achieved with a 150mm x 3.0mm Grace All tech Platinum 
column. The mobile phase consisted of 40mM NH4COOH in 1% 
acetonitrile pH 4.3 (buffer: A), and 100% acetonitrile (buffer: B). The 
flow rate of the mobile phase was 430 µl/min, and the gradient was 
25%/to 5% A in 10 min, 5% A to 100% B in 1 min, then 100% B for 
19 min. Mass spectrometry detection was carried out using Applied 
Bio systems mass spectrometer AB Sciex QTRAP 2000 coupled with 
The Agilent 1100 HPLC instrument. Multiple reaction monitoring 
under positive-ion mode was used, (230+112) for lamivudine and 
(225+127) for D4T. The standard curves were linear over the ranges 
of 2.9 to 857 mg/mL. Inter- and Intra-day accuracy (%error) and 
precision (coefficient of variation) were assessed at 2.9, 285.7, and 857 
mg/ml, and they were less than 3.1% at all concentrations.

Quantitation of DHBV in duck serum

Serum (100 µL) was digested with 0.8 mg/mL proteinase K in 
1% SDS and TSE Buffer, and incubated at 55°C for 2 h. DHBV DNA 
was then isolated using the Macherey-Nagel Nucleospin Gel & PCR 
Clean-up Kit. DHBV DNA titers were measured by qPCR using 
BioRad’s CFX36 RT system, C1000 Thermal Cycler, with samples 
run in triplicate. Each reaction consisted of 2 µl of DNA sample, 10 
µL of ‘TaqMan’ Universal Master Mix II (with UNG, from Applied 
 Biosystems), 6 µL of 1.5 µM probe, and 2 µL of 1.5 µM of each primer. 
Primers used for DHBV were forward Primer: 5’ - GGG AAA GGA 
GAG CC CTA CA - 3’, reverse Primer: 5’ - TCT ATG GTG TCT GCT 
GCA ACT - 3’, probe: 5’ - CCA ACG TGC GGG CTC CCC TC - 
3’ with a 5’ FAM and 3’ Iowa Black quencher. The protocol started.
at 45°C for 2 min (for UNG activity), 95°C for 10 min for hot-start activation of the DNA Polymerase, then 43 cycles of 95°C for 15 sec and 60°C for 30 sec for DNA denaturation and annealing/extension. To build the standard curve, pALT-DHBV16 monomer plasmid (generously provided by Dr. Qingxia Yao) was used as template. The plasmid was diluted to 109, 108, 107, 106, 105, 104, 103, 102, 101 copies per µL.

Data analysis

Lamivudine serum concentration-time data fitting was performed using Graph Pad Prism (version 5.01; GraphPad Software, La Jolla, CA). One- and two-compartment models were tried, and optimal pharmacokinetic model was determined by the Akaike information criterion as a measure of the goodness of fit. The area under the serum concentration-versus-time curve (AUC) was calculated by linear-trapezoidal method. Absolute bioavailability was calculated as AUC*DoseIV/AUCIV*Dose. The Cmax and Tmax were determined from the original data. Lamivudine half-life (t1/2) was calculated as 0.693 x apparent volume of distribution (Vdap)/apparent total body clearance (Clap). The simulation of pharmacokinetic data and the calculation of pharmacokinetic/pharmacodynamics indices were performed using Wolfram Mathematica 10.1 (Wolfram Research, Inc., Champaign, IL). Regarding statistical analysis, one-way analysis of variance, followed by a Tukey’s post hoc test, was used. A result was presented as mean±S.E and was considered statistically significant where P < 0.05.

Results and Discussion

Despite the common use of DHBV model to assess the efficiency of antivirals against human HBV, characterization of the pharmacokinetic profile of lamivudine in ducks has not been reported previously. Therefore, we determined, in DHBV-infected Pekin ducks, the values of pharmacokinetic parameters of lamivudine following IV, IM and PO administration of three escalating doses (80 to 480 mg/kg) to confirm the linearity of its pharmacokinetics. The serum concentration-time curves of the three routes are showed in figure 1. We found that lamivudine pharmacokinetics follows one-compartment kinetics, and pharmacokinetic parameters values and S.E. are shown in table 1. The elimination of lamivudine in ducks was remarkably higher than what was reported for human and woodchuck (t1/2 values were 1.5, 4.3 and 3.3 h, respectively) [24, 25]. However, our determined values for Clap and Vdap fitted perfectly in the reported allometric relationship with species body weight [25, 26]. The absorption of lamivudine after IM administration was significantly faster than after PO route (Tmax values were 0.32 and 0.58 h, respectively). Also, the extent of lamivudine absorption after IM administration was higher than after PO route (F values were 0.83 and 0.71 h, respectively). Our F values following PO administration are

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\begin{array}{|c|c|c|c|c|c|c|c|c|}
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\text{Route} & \text{Dose (mg/kg)} & \text{Cmax (µg/mL)} & \text{Tmax (hr)} & \text{Kp (min-1)} & \text{Clap (L/hr/Kg)} & \text{Vdap (L/Kg)} & \text{AUC (µg.hr/mL)} & \text{t1/2 (hr)} & \text{F} \\
\hline
\text{IV} & 80 & - & - & - & 0.25±0.03 & 0.56±0.05 & 318±36.9 & 1.54±0.12 & - \\
\hline
\text{IM} & 200 & 351.5±76.5 & 0.25±0.07 & 431±101 & 0.28±0.03 & 0.61±0.05 & 661±58.1 & 1.48±0.11 & 0.83 \\
\hline
\text{PO} & 480 & 435±39.2 & 0.58±0.08 & 0.04±0.01 & 0.35±0.05 & 0.79±0.09 & 1344±258 & 1.57±0.14 & 0.71 \\
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Figure 1: Concentrations of lamivudine in serum following single administration of 80mg/kg IV, 200 mg/kg IM and 480 mg/kg PO of lamivudine toDHBV-infected Pekin ducks. Results are presented as mean and SE (n=3).
on DHBV decay in vivo. The concentration-time curves of 40 mg/kg IM, and 100 and 200 mg/kg PO daily for 6 weeks were simulated, and are shown in figure 2A. In addition, the pharmacokinetic/pharmacodynamic indices of the three lamivudine treatments for the 6-week duration were calculated (Table 2). Our results showed that both 100 mg/kg and 200 mg/kg treatments were able to significantly suppress but not eliminate DHBV titers at all time points compared with either control or 40mg/kg groups (Figure 2B). A plateau of DHBV titers was reached by the fourth week of lamivudine treatment in case of 100 and 200 mg/kg doses, whereas, it was apparently reached by the second week for 40 mg/kg dose (Figure 2B). There was no significant difference in DHBV titers after 200 mg/kg compared with 100 mg/kg of lamivudine at any time point (Figure 2B). Interestingly, after 6 week of the termination of lamivudine treatment, 200 mg/kg dose led to a significantly lower serum DHBV titers compared with 100 mg/kg dose (Figure 3). With respect to the pharmacokinetic/pharmacodynamics indices, the ~2-fold increase in lamivudine dose from 40 to 100 to 200 mg/kg led to small increase in the total time the serum concentration of lamivudine was above the IC50 value of 0.1 µM (36) (t >IC50); during the 6-week duration of treatment t >IC50 was 706, 830, and 896 hr for 40, 100 and 200 mg/kg, respectively, which is correlated with the observed direct effect of lamivudine on DHBV titer (Table 2). In contrast, the AUC and Cmax showed ~2-fold increase by increasing lamivudine dose from 40 to 100 to 200 mg/kg, which is more correlated with the observed latent effect of lamivudine on DHBV titer (Table 2).

In summary, the current study is the first to report the pharmacokinetic and pharmacodynamic profiles of lamivudine in DHBV-infected Pekin ducks. Lamivudine still has a clear cost advantage over the other competitors. In the current situation of continuous emerging resistance against lamivudine as well as its competitors, the clinical dose of lamivudine has to be adjusted to get the maximum efficiency out of it. Our results came to support previous reports suggesting that the optimum dose of lamivudine against chronic HBV is higher than the current recommended dose (16-18). 600 mg PO daily of lamivudine was previously reported to be tolerable in human [24]. Therefore, giving 600 mg daily in

Figure 2: A: Simulation of concentration-time curves of single dose and 6-week treatment of lamivudine of 40mg/kg IM, or 100 mg/kg or 200 mg/kg PO daily toDHBV-infected Pekin ducks. B: Viremia levels in Pekin ducks before and following 40mg/kg IM, or 100 mg/kg or 200 mg/kg PO daily of 6-week lamivudine treatment to DHBV-infected Pekin ducks. Results are expressed as DHBV DNA copies in serum normalized to the baseline DHBV DNA copies before treatment, and are presented as mean and SE (n=4).

Figure 3: Viremia levels in Pekin ducks after 6 weeks of the termination of 100 mg/kg or 200 mg/kg PO daily of lamivudine treatment to DHBV-infected Pekin ducks. Results are expressed as DHBV DNA copies in serum normalized to the baseline DHBV DNA copies before treatment, and are presented as mean and SE (n=4).

intermittent fashion, switching between 300 mg and 600 mg daily, would further enhance the tolerability and make use of the latent effect of lamivudine high doses on viral serum titers.

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References


Table 2: The pharmacokinetic/pharmacodynamic indices of 40 mg/kg IM, and 100 and 200 mg/kg OP once daily for 6 weeks in Pekin ducks.

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