Available online at www.sciencedirect.com
SCIENCE DIRECT
Antiviral Research 68 (2005) 147–153
Antiviral Research
www.elsevier.com/locate/antiviral

Antiviral therapeutic efficacy of foscarnet in hepatitis B virus infection

Yan-Xing Han [a,1], Rong Xue [b,1], Wei Zhao [b], Zhen-Xian Zhou [b], Jian-Nong Li [a], Hong-Shan Chen [a], Xiang-Hong Chen [a], Yan-Ling Wang [c], Yu-Huan Li [a], Yin-Wei Wu [b], Xue-Fu You [a], Li-Xun Zhao [a], Jian-Dong Jiang [a,*]

[a] *Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing 100050, China*
[b] *Division of Infectious Diseases, Nanjing Second Hospital, Nanjing 210003, China*
[c] *Department of Medicine, Mount Sinai School of Medicine, New York, NY 10029, USA*

Received 7 March 2005; accepted 5 September 2005

Abstract

Foscarnet (PFA), a viral DNA polymerase inhibitor, is a clinical agent for herpes viruses. The goal of the study was to evaluate the therapeutic efficacy of PFA in hepatitis B virus (HBV) infection. Intravenous infusion of PFA (1 g/day) for 4 weeks significantly reduced serum HBeAg ($p < 0.01$) and HBV DNA copies ($p < 0.05$) in 31 patients who were diagnosed with active chronic HBV infection (CHB) and had not received antiviral treatment previously. Alanine aminotrans-aminase (ALT), aspartate aminotransaminase (AST) and gamma glutamyl transpeptidase (γ-GT) of the patients declined ($p < 0.001$, 0.001 and 0.01, respectively). Kidney function (blood creatinine and urea nitrogen) remained unchanged. Another 21 lamivudine-resistant CHB patients with mutations at the tyrosine–methionine–aspartate–aspartate motif (YMDD) displayed a response to PFA similar to that mentioned above, with reductions in HBeAg ($p < 0.05$), HBV DNA ($p < 0.01$) and liver enzymes (ALT and AST, $p < 0.001$; γ-GT, $p < 0.05$). Moreover, PFA reduced serum HBeAg ($p < 0.01$), HBV DNA ($P < 0.05$), AST ($p < 0.05$) and ALT ($p < 0.02$) in a cohort of 13 severe CHB patients with advanced liver damage. PFA was also evaluated in vitro and in vivo. PFA inhibited HBV DNA replication in HBV-transfected human HepG2 cells (2.2.15 cells) with reduced amount of HBV RC-DNA and DS-DNA. In the duck HBV-infected ducklings, PFA reduced viral DNA and duck HBsAg in the serum ($p < 0.01$ for both).

Keywords: HBV infection; Foscarnet; Antiviral therapy

1. Introduction

Hepatitis B virus (HBV) infection remains to be one of the major medical problems worldwide, especially in China. Patients with chronic replicative HBV infection are at high risk of developing cirrhosis and primary hepatocellular carcinoma (PHC). Alpha-interferon is only partially effective for clinical use and is limited by its side effects. Lamivudine suppresses HBV through inhibition of reverse transcriptase, but the treatment often fails due to the emergence of mutations within the catalytic site of HBV DNA polymerase (tyrosine–methionine–aspartate–aspartate, YMDD motif), which leads to drug-resistance (Buti et al., 2001; Lai et al., 2003) in patients. Adefovir has been only recently introduced in hospitals in mainland China. Very few agents have proven effective in managing HBV infections (Lai et al., 2003). It is highly desirable to discover safe and effective anti-HBV agents with new structures and distinct mechanisms of action.

Foscarnet (PFA), which is effective in inhibiting DNA polymerases of viruses, is currently applied in clinic mainly for the treatment of acyclovir-resistant herpes simplex virus (HSV) and ganciclovir-resistant cytomegalovirus (CMV) infections (De Clercq, 2004). This small molecule has a structure distinct from the known antiretroviral nucleoside analogs and a unique binding site on viral DNA polymerase (Hess et al., 1980). It has provoked our interest as to its therapeutic efficacy for HBV infection. Although treating chronic replicative HBV infection with PFA has been explored in two clinical studies in Europe (Bain et al., 1989; Schvarcz et al., 1994), the conclusion is far from satisfactory, because (1) only few patients were included in the studies (three and eight patients, respectively); (2) PFA was given only for a short term (1 and 2 weeks, respectively); and (3) no reference groups were used as controls. It remains to

* Corresponding author. Tel.: +86 10 63165290; fax: +86 10 63017302.
E-mail address: jiandong.jiang@mssm.edu (J.-D. Jiang).
[1] These authors made equal contribution to this work.

0166-3542/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2005.09.002

be questioned as by which extent PFA inhibits HBV replication, whether PFA effective in lamivudine-resistant HBV infection and whether or not PFA can be used in HBV(+) patients with severe liver injury.

The aim of the present study was to investigate PFA as a single agent in the treatment of HBV infection. PFA was evaluated in patients with active chronic hepatitis B (CHB), including those who were not treated with any antiviral agent, or those who were treated with lamivudine and carried HBV YMDD mutants with resistance phenotype, or those who had suffered from severe CHB with advanced liver damage. The changes of HBV antigens and antibodies, viral DNA copies as well as liver enzymes in the plasma were determined before and after PFA treatment. PFA was also tested both in vitro and in vivo in order to promote our understanding of its therapeutic effect in HBV patients. We consider these results informative for the clinical application of PFA in antiviral therapy.

2. Materials and methods

2.1. Subjects and clinical study

Three cohorts of patients were enrolled at the Nanjing Second Hospital, Nanjing, China, between 2002 and 2004. All of these patients with positive readings of HBsAg, HBeAg and HBV DNA in the serum were diagnosed as having active CHB according to the diagnostic criteria for hepatitis in China (Wen, 2001). Detailed clinical information of the patients is shown in Table 1. The first cohort (CHB, $n = 42$) was enrolled from an outpatient clinic under the principle that none of these patients had been previously treated with anti-HBV chemotherapy. Out of the 42 patients, 31 (median age of 35 with a range between 23 and 72; 26 males and 5 females) were randomly assigned to receive PFA therapy (1 g/day, i.v. infusion, b.i.d., for 28 days) and 11 patients (median age of 31 with a range between 25 and 52; 7 males and 4 females) were assigned to take Gan-Le-Xin (4 g/day, oral, t.i.d., for 28 days), an anti-hepatitis Chinese herb formula from Suzhou Pharmaceutical Co. Ltd. (Datong, China). This group served as a reference in this study to compare PFA with the herb regimen commonly used in Chinese hospitals.

The second cohort consisted of those previously treated with lamivudine (lamivudine-resistant CHB, $n = 21$). These 21 patients (median age of 38 with a range between 17 and 78; 15 males and 6 females) had been on lamivudine therapy for 1–3 years, and the treatment was terminated due to failure in response to lamivudine. Sequences of the catalytic domain of HBV DNA polymerase were determined by sequencing analysis of the DNA samples isolated from patient serum. Briefly, a DNA sample was isolated from patient serum with QIA-amp DNA blood mini kit (Foster City, CA, USA). The target region of HBV DNA polymerase was amplified in a PCR reaction. The following set of primers was employed to amplify the YMDD-containing sequence: forward primer, 5′-ACTTGTATTCCCAT CCCATCA-3′; backward primer, 5′-GGTTCAAATGTATTCCCAAAGAC-3′. The amplified sequences were then examined with a conventional sequencing analysis using a commercial kit (USB sequence kit, Cleveland, OH, USA). Out of the 21 patients, 14 had the YIDD and 7 had the YVDD mutant. These patients were assigned to receive PFA therapy about 4 weeks after the lamivudine was terminated. The regimen was identical to that mentioned above (1 g/day, i.v. infusion, b.i.d., for 28 days).

The patients in the third cohort ($n = 13$, median age of 28 with a range between 17 and 54, 11 males and 2 females) were diagnosed with severe CHB with elevated serum bilirubin (>170 mmol/ml), prolonged prothrombin time (<40%) and reduced serum albumin (<30 g/L). These 13 patients were on therapy with herb formula, and not treated with lamivudine before they entered into the study.

Blood samples taken before and after therapy were examined to determine the blood levels of HBV as well as liver and kidney functions with conventional methods used in Chinese hospitals. HBV infection markers (HBsAg, anti-HBsAg, HBeAg, anti-HBeAg and anti-HBcAg) were qualitatively measured with the commercial test kits from Abbott (Chicago, USA), and the numerical reading values of the tests were used for statistical analysis in this study. HBV DNA in the blood was quantified using a commercial kit from Shenzhan PiJi bioengineer Co. Ltd. (Shenzhen, China). Briefly, 100 μl of serum sample was treated with DNA purification buffer followed by centrifugation. The DNA sample was then mixed with the amplification buffer containing HBV PCR reaction solution and Taq enzyme. The reaction was performed under the conditions recommended by the vendor. The final reaction product was measured in a fluores-

Table 1
Study cohorts

Patient cohorts and characteristics	HBV/wt ($n = 42$)		HBV/YMDD (+)	Severe CHB[a]
	PFA	Gan-Le-Xin		
N	31	11	21	13
Age (year)[b]	35 (23–72)	31 (25–52)	38 (17–78)	28 (17–54)
Male/female	26/5	7/4	15/6	11/2
Clinical diagnosis	CHB[a]	CHB	Lamivudine-resistant CHB	Severe CHB
Serum HBV DNA (copies/ml, ×10E6)[b]	4 (0.02–640)	6.6 (0.5–500)	6.9 (0.15–560)	11 (0.03–970)
ALT (IU/L)[b]	251 (22–1560)	380 (99–527)	289 (61–839)	286 (20–2098)
Previous anti-HBV treatment	None	None	Lamivudine	Chinese herbs

[a] Chronic Hepatitis B infection (active).
[b] Median (range).

cent PCR qualitative reader and the result was expressed as HBV DNA copies per ml serum. The liver enzymes (alanine aminotransferase, ALT; aspartate aminotransferase, AST; and gamma glutamyl transpeptidase, γ-GT) of the patients were determined with the kits from Randox (England). Blood creatinine (Cr) and urea nitrogen (BUN) were examined using conventional methods in the hospitals. All participants gave informed consent.

2.2. Anti-HBV activity of PFA in cell culture

2.2.15 cells, a HBV-transfected human HepG2 cell line (Sells et al., 1987), were kindly provided by Dr. Y.C. Cheng (Yale Medical School, New Haven, CT, USA). Cell viability was assessed by the MTT assay [3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide; Rubinstein et al., 1990]. TC50 of PFA, defined as the concentration that inhibited 50% cellular growth in comparison to untreated controls, was over 2400 μg/ml in 2.2.15 cells. Cells at 2×10^4 cells per well (96-well microplates, Falcon, Oxnard, CA, USA) were treated with PFA at 37 °C for 9 days in a concentration range from 0.1 to 1000 μg/ml, nontoxic to 2.2.15 cells. HBV-relaxed circular DNA (RC-DNA) and double-strand DNA (DS-DNA) were quantified by Southern blot described below.

Lamivudine (kindly given by Dr. B Öberg, Medivir, Sweden) was used as a positive control in the experiments, and ribavirin (Hubei Pharmaceutical Institute, Wuhan, China) as a negative reference.

2.3. Inhibition of duck HBV (DHBV) replication by PFA in vivo

Duck serum positive for duck HBsAg (DHBsAg) at 1:1000 of dilution was used for infection. Ducklings at 1 day of age (Beijing Nanyan Fowl Inc., Beijing) were i.v. infected with DHBV at a dose of 0.2 ml DHBV(+) serum per duckling (containing 5.7×10^6 cpm of DHBV DNA equivalents). Seven days later, PFA was administrated (62.5, 125, or 250 mg/kg, i.p., b.i.d.) for 14 days. DHBV DNA and DHBsAg were measured once a week by Southern blot and ELISA, respectively (see below).

The animal and human studies were approved by the Research Committees of the Institute of Medicinal Biotechnology and Nanjing Second Hospital.

2.4. Southern blot analysis for HBV DNA

2.2.15 cells were collected for DNA extraction with a method described before (Jiang et al., 1998). DNA (10 μg/sample) was loaded onto a 1% agarose gel for electrophoresis, which was followed by transfer to a nitrocellulose filter. The filters were prehybridized in a prehybridization buffer (5 × SSC, 0.1% SDS, 5 × Denhardt's solution, 100 μg/ml denatured salmon sperm DNA, and 50% formamide) at 42 °C for 4 h. Hybridization was done in the prehybridization buffer plus 5×10^7 cpm of 5′-[alpha-^{32}P] deoxycytidine-labeled full length HBV genomic DNA (from psp65-HBV5.1 plasmid kindly provided by Dr. J.T. Guo, Peking Union Medical College, Beijing) for 24 h at 42 °C with agitation. The nitrocellulose filter was rinsed three times with 0.1 × SSC (containing 0.1% SDS) at 22 °C, and twice at 65 °C. The filter was then exposed to X-ray film (Kodak, Rochester, NY, USA). Relative band intensities on the membrane were quantified by absolute integrated optical density (IOD) scanning using a Gel-Analyzer Version 3.0 software (Media Cybernetics, Silver Spring, MD, USA).

2.5. Southern blot analysis for DHBV DNA

For the measurement of DHBV DNA in the serum, a previously reported method was used with modifications (Chen et al., 1983; Yao et al., 2001). Briefly, 50 μl of duckling serum was directly spotted on the nitrocellulose membrane, and DHBV DNA was detected with 5′-[alpha-^{32}P] deoxycytidine labeled full-length DHBV genomic DNA (from plasmid DHBV-PUC18, kindly provided by Dr. W.G. Yang, Peking Union Medical College, Beijing). Quantitative measurement was done by examination of IOD unit.

2.6. ELISA detection of DHBsAg

Ninety-six-well plates were coated with 100 μl of purified anti-DHBsAg capture antibody (polyclonal IgG from rabbit, 1:100, from Dr. J.T. Guo, Peking Union Medical College, Beijing), and incubated at 4 °C for 16 h. After removing the coating solution, the plates were blocked with 200 μl of blocking buffer (1% BSA in PBS) at room temperature (RT) for 60 min. After washing in PBS, 100 μl of 1:50 diluted serum samples were added to the wells, followed by 1-h incubation at RT. The plates were then washed in PBS (five times) and reacted with 100 μl per well of 1:100 diluted anti-DHBsAg polyclonal IgG at RT for 1 h. After wash, the plates were treated with 100 μl of 1:1000 diluted goat anti-rabbit IgG labeled with horseradish peroxidase (Sigma, St. Louis, MO, USA) for 1 h at 37 °C. Color was developed after 20 min of exposure to 100 μl of ABTS substrate solution (Sigma) at RT. The reaction was stopped by adding 50 μl of H_2SO_4 (2N) per well. The optical density (OD) was obtained at 405 nm. The mean OD value in naive duckling control serum ($n = 18$) was 0.042 ± 0.011.

2.7. Statistics

To compare the values before and after treatment, a paired student *t*-test was employed. Differences in the mean of duck serum DHBV markers among study groups were tested by unpaired student *t*-test for equal or unequal variances depending on a preliminary *F*-test for the homogeneity of variance.

3. Results

3.1. Anti-HBV effects of PFA in patients with CHB

The results are shown in Table 2. In the 31 CHB patients who were not treated with lamivudine before, PFA significantly lowered the serum levels of HBeAg ($p < 0.01$) and HBV DNA ($p < 0.05$), both of which are important clinical markers for active HBV replication. The levels of HBsAg and serum

Table 2
Therapeutic effect of PFA in patients with chronic active hepatitis B virus infection

Measurement (unit, normal range)[a]	PFA treatment ($n = 31$)		Gan-Le-Xin treatment ($n = 11$)	
	Before	After	Before	After
Anti-HBV effect				
HBsAg (S/N, <2)	159 (51.3–2141.5)	132 (0.6–321)	260 (93–356)	216 (98–368)
Anti-HBsAg (mIU/ml, 0–10)	3.1 (0–12.5)	2.5 (0–13.5)	0.8 (0–10.1)	0.6 (0–10.5)
HBeAg (PEIU/ml, <0.28)	125 (0.32–5064)	10 (0.025–2984)**	203 (1.2–6700)	192 (1.3–6897)
Anti-HBeAg (S/CO, >1)	1.04 (0–27)	0.8 (0–28)	1.44 (0.24–9.35)	1.54 (0.12–3.2)
Anti-HBcAg (S/CO, >1)	0.067 (0.015–0.72)	0.075 (0.03–0.9)	0.096 (0.077–0.122)	0.1 (0.078–0.091)
HBV DNA (copies/ml, <5 × 10E2)	4 (0.02–640) × 10E6	0.012 (0–140) × 10E6*	6.6 (0.5–500) × 10E6	4.3 (0.24–500) × 10E6
Liver function				
ALT (IU/L, 0–40)	251 (22–1560)	43 (17–395)***	380 (99–527)	333 (28–882)
AST (IU/L, 0–40)	161 (25–817)	38 (18–139)***	289 (26–986)	266 (29–880)*
γ-GT (IU/L, <50)	108 (16–360)	63 (3–288)**	ND	ND
Kidney function				
Cr (μmol/L, 53–97)	74 (65–110)	83 (67–105)	71 (56–95)	79 (60–101)
BUN (mmol/L, 2.86–8.2)	4.49 (2.05–7.06)	5.26 (3.25–6.7)	3.92 (2.7–6.9)	4.15 (3.0–7.3)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GT, gamma glutamyl transpeptidase; Cr, creatinine; BUN, blood urea nitrogen. *** $p < 0.0001$; ** $p < 0.01$; * $p < 0.05$; as compared to that before treatment. ND, not done.
[a] Median (range).

antibodies to HBsAg, HBeAg as well as HBcAg remained statistically unchanged. In concordance with the anti-HBV effect, liver function was largely improved by significant reductions of ALT ($p < 0.001$), AST ($p < 0.001$) and γ-GT ($p < 0.01$). PFA was well-tolerated by all subjects. Kidney function was examined by measuring Cr and BUN before and 4 weeks after PFA treatment. As shown in Table 2, Cr and BUN were both within the normal range and without significant change after PFA therapy ($p = 0.1$ and 0.23, respectively). In the Gan-Le-Xin group ($n = 11$), the reductions of the HBV markers were not significant; liver transaminases declined after treatment, with a significant reduction in AST ($p < 0.05$), but not ALT ($p = 0.12$).

3.2. PFA treatment of lamivudine-resistant CHB

To evaluate the anti-HBV efficacy of PFA in patients with resistance to lamivudine, 21 patients with mutations in the HBV YMDD motif were enrolled in the study after the lamivudine treatment was terminated and were treated with PFA. The therapeutic regimen was identical to that mentioned above, and a significant reduction of HBV replication was observed in these patients 4 weeks after PFA treatment (Table 3). The serum levels of HBeAg as well as HBV DNA copies in these patients were significantly decreased ($p < 0.01$ for both) after 4 weeks on PFA therapy; serum HBsAg was without change. Liver function was also monitored, and ALT, AST as well as γ-GT declined significantly with p values less than 0.001, 0.001 and 0.05, respectively. These results were consistent with that of the patients treated upfront with PFA (Table 2).

3.3. Antiviral effect of PFA in patients with severe CHB

To learn whether, or not, PFA could be used in HBV(+) patients with advanced liver damage, 13 patients diagnosed with severe CHB were enrolled. Subjects in this cohort were treated with PFA at 1 g per day (i.v. infusion). As shown in Table 3, PFA significantly reduced the serum levels of HBeAg ($p < 0.01$) and HBV DNA copies ($P < 0.05$). Accordingly, liver function of the patients was significantly improved, as indicated by the reduction of AST ($p < 0.05$) and ALT ($p < 0.02$). All of the patients had their jaundice reduced. Their serum bilirubin declined from above 170 mmol/ml to the range between 60 and 95 mmol/ml after 4 weeks on PFA therapy.

3.4. Inhibition of HBV replication in 2.2.15 cells and DHBV-infected ducklings

The anti-HBV activity of PFA was then tested in vitro and in an animal model in an attempt to correlate its primary action on DNA polymerase with the clinical outcome. It has been demonstrated that the HBV-transfected HepG2 cell line (2.2.15 cells) can support the assembly and secretion not only of several replicative intermediates of HBV DNA, but also of Dane-like particles (Sells et al., 1987). This cell line is recommended as an in vitro model to study the life cycle of HBV (Price et al., 1989). As shown in Fig. 1, treating 2.2.15 cells with PFA inhibited the levels of intracellular HBV RC-DNA and DS-DNA, and the inhibition was dose-dependent. Lamivudine, as a positive control in the experiment, showed a significant inhibition on HBV DNA replication at a concentration as low as 0.23 μg/ml, whereas ribavirin, as a negative reference, showed no inhibition on HBV even when the dose was upto 290 μg/ml.

The effect of PFA on HBV replication was further evaluated in DHBV-infected ducklings. These ducklings were infected with duck HBsAg-positive serum via the intravenous route. The courses of the major virology indicators in the serum were consistent with those reported previously (Delmas et al., 2002). Serum samples were taken at 7-day intervals, and the peak levels of DHBV DNA and DHBsAg were observed between day 7 and 14 post-infection. PFA was given i.p. on day 7. As

Table 3
Antiviral effect of PFA in lamivudine-resistant CHB and severe CHB

Measurement (unit, normal range)[a]	PFA in Lamivudine-resistant CHB ($n = 21$)		PFA in Severe CHB ($n = 13$)	
	Before	After	Before	After
Anti-HBV effect				
HBsAg (S/N, <2)	205.31 (48.3-341.4)	209.3 (46.3–304.1)	192 (63–411)	181 (41–357)
HBeAg (PEIU/ml, <0.28)	134 (6–7283)	24 (0–560)**	3140 (42–7600)	35 (0–3186)**
HBV DNA (copies/ml, 5 × 10E2)	6.9 (0.15–560) × 10E6	0.021 (0.0005–17) × 10E6**	11 (0.03–970) × 10E6	0.0017 (0.0005–2.3) × 10E6*
Liver function				
ALT (IU/L, 0–40)	289 (61–839)	33.4 (12–644)***	286 (20–2098)	39 (25–200)*
AST (IU/L, 0–40)	172.4 (42–819)	38 (19–109)***	428 (25–2441)	48 (18–250)*
γ-GT (IU/L, <50)	94 (20–195)	52.6 (12.6–197)*	ND	ND

ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GT, gamma glutamyl transpeptidase. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$; as compared to that before treatment. ND, not done.
[a] Median (range).

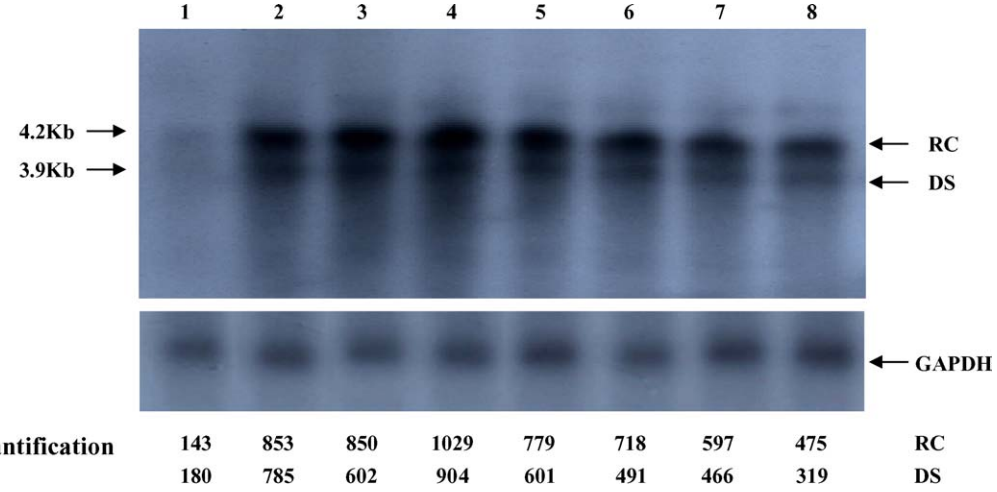

Fig. 1. Inhibitory effects of PFA on HBV replication in 2.2.15 cells. 2.2.15 cells were treated or untreated with reference agents or PFA for 9 days. Levels of HBV RC-DNA (RC) and DS-DNA (DS) were determined by Southern blot. GAPDH served as an internal control. Lane 1: lamivudine (0.23 μg/ml) as a positive reference; lane 2: untreated 2.2.15 cells; lane 3: ribavirin (290 μg/ml) as a negative reference; and lanes 4–8: PFA at a concentration of 0.86, 2.59, 7.78, 23.3, or 70 μg/ml, respectively. Relative band intensities for RC- and DS-DNA were quantified by density scanning, and are demonstrated in IOD units. The experiment was repeated three times.

shown in Fig. 2a, DHBV DNA levels in the PFA-treated groups were significantly lower than those of untreated controls on day 21 post-infection ($p < 0.05$ for 125 mg/kg group; $p < 0.01$ for 250 mg/kg group); the inhibition declined to a non-significant level when the dose of PFA was reduced to 62.5 mg/kg (data not shown). Accordingly, DHBsAg (Fig. 2b) went down to a similar extent as DHBV DNA. The body weights of the ducklings treated with PFA were without significant difference to that of the untreated ducklings ($p = 0.32$).

4. Discussion

PFA was selected for this study because of the following reasons. First, PFA is a small molecular weight inhibitor of viral DNA polymerase with a structure different from the conventional antiretroviral nucleoside analogs. Second, unlike lamivudine that interacts at a dNTP-binding pocket in the C subdomain of DNA polymerase (Lai et al., 2003), PFA binds to the enzyme at the pyrophosphate-binding site (Hess et al., 1980), suggesting its potential for the treatment of lamivudine-resistant HBV infection caused by YMDD alterations. Third, also different from lamivudine that requires a process of intracellular phosphorylation to convert the compound to the 5′-triphosphate form by host kinases for being effective in inhibiting DNA polymerase (Gray et al., 1995; Zemlicka, 2000), PFA directly inhibits DNA polymerase; therefore, the anti-HBV activity of PFA is theoretically independent of cellular functions as well as activities of host cell enzymes, suggesting that PFA might be a potential agent for treating CHB patients with severe liver injury.

After a 4-week treatment with PFA, a reduction of HBeAg and HBV DNA copies was achieved in CHB patients who had not been treated with lamivudine before. The major liver enzymes (ALT, AST and γ-GT) accordingly declined at a significant level, indicating a clinical improvement of liver function. Compared to the lamivudine therapies that have shown to lower HBV DNA copies to 99.9% with highly sensitive assays (Honkoop et al., 1998), the effect of PFA is also promising. Furthermore, the duration of PFA therapy in this study was con-

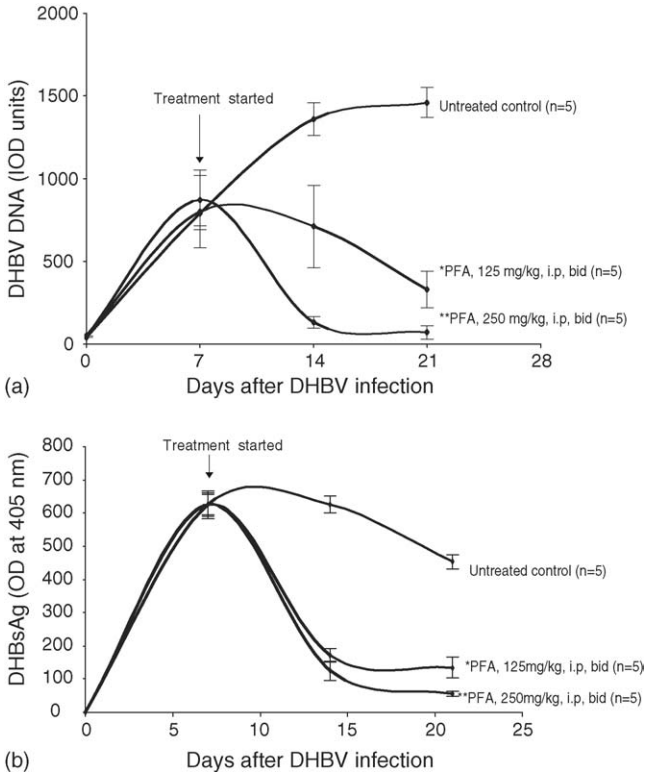

Fig. 2. Inhibition of DHBV proliferation in ducklings by PFA. DHBV-infected ducklings were treated with PFA for 14 days (see Section 2), and the levels of DHBV DNA (a) as well as DHBsAg (b) were determined. Values presented in the figure are means ± S.E. derived from five ducklings. $^{*}p < 0.05$, $^{**}p < 0.01$, as compared to the untreated control group.

siderably short as compared to that of lamivudine (Honkoop et al., 1998), and the regimen used in this study is a pilot trial for CHB; larger anti-HBV effects of PFA may be achieved by improvement of the treatment protocol. This notion is partially supported by our studies conducted in 2.2.15 cells and the duckling model. The dose-dependent activity of PFA on HBV proliferation was observed in the HBV-transfected cells and DHBV-infected ducklings. More importantly, for the CHB patients with genotypic evidence of lamivudine-resistance at the YMDD motif, PFA also demonstrated activity in decreasing serum HBeAg and HBV DNA copies, with a statistical significance similar to that with no lamivudine resistance. Whether HBV mutations can engender PFA resistance is under investigation in our laboratories.

In addition, PFA was also evaluated for its potential in severe CHB with advanced damage in liver function. After PFA infusion, a significant reduction of serum HBeAg and HBV DNA was achieved in severe CHB, paralleled with a decrease of liver enzymes and clinical improvement. As lamivudine might accelerate damage in hepatocytes, caution is recommended in many hospitals in China when lamivudine is applied in severe CBH with advanced liver damage (Chinese Clinical Group for HBV Infection, 2004; Kim et al., 2001; Luo et al., 2001); the observed therapeutic efficacy of PFA in severe CHB is therefore of particular significance. PFA was well-tolerated in all of the CHB patients; no adverse effects were associated with its intravenous infusion at the dose used (1 g/day, which is far below that used for HSV and CMV infections). Since nephrotoxicity is the potential side effect of PFA in a long-term therapy with high doses (Aschan et al., 1992; Honkoop et al., 1998; MacGregor et al., 1991), kidney function was monitored in the present study. Our data showed that PFA did not cause change in kidney function after 4-weeks treatment.

The inhibitory activity of PFA on HBV viral DNA polymerase is likely to account for its anti-HBV effect in CHB patients. Using HBV-transfected human hepatoma cell line 2.2.15 and a DHBV-infected duckling model, we demonstrated that PFA significantly inhibited HBV DNA replication, which is consistent with previous reports (Freiman et al., 1990; McMillan et al., 1995). The activity of PFA was dose-dependent both in cell culture and animal experiments. Since PFA binds to the viral DNA polymerase at the site different from that for lamivudine, the combination of PFA with lamivudine exhibited an additive effect against HBV in vitro (the results of combination therapy will be published separately).

Hepatitis B viral infection is a major medical problem in China, but the clinical choices of chemotherapy are few, leaving large number of HBV(+) patients going for alternative treatment that have not shown convincing therapeutic efficacy against HBV in clinic. The present study shows that PFA could be a new therapeutic choice for CHB patients, at various disease stages, whether or not they are resistant to lamivudine. The disadvantage of PFA is the route of administration (i.v. infusion). New techniques or strategies are needed to address this concern. Since the structure and action mechanism of PFA is different from that of lamivudine, PFA might be used to treat CHB patients in combination with lamivudine or other antiviral agents.

Acknowledgments

The project was supported by the Ministry of Sciences and Technology, P.R. China, and the National Foundation for Cancer Research (Bethesda, MD, USA).

References

Aschan, J., Ringden, O., Ljungman, P., Lonnqvist, B., Ohlman, S., 1992. Foscarnet for treatment of cytomegalovirus infections in bone marrow transplant recipients. Scand. J. Infect. Dis. 24, 143–150.

Bain, V.G., Daniels, H.M., Chanas, A., Alexander, G.J., Williams, R., 1989. Foscarnet therapy in chronic hepatitis B virus E antigen carriers. J. Med. Virol. 29, 152–155.

Buti, M., Sanchez, F., Cotrina, M., Jardi, R., Rodriguez, F., Esteban, R., Guardia, J., 2001. Quantitative hepatitis B virus DNA testing for the early prediction of the maintenance of response during lamivudine therapy in patients with chronic hepatitis B. J. Infect. Dis. 183, 1277–1280.

Chen, Y.J., Gu, J.R., Jiang, H.C., 1983. Direct detection of serum HBV DNA by spot hybridization. Chin. J. Infect. Dis. 1, 63–67.

Chinese Clinical Group for HBV infection, 2004. Clinical treatment of HBV with lamivudine. Chin. J. Hepatol. 12, 4–25.

De Clercq, E., 2004. Antiviral drugs in current clinical use. J. Clin. Virol. 30, 115–133.

Delmas, J., Schorr, O., Jamard, C., Gibbs, C., Trepo, C., Hantz, O., Zoulim, F., 2002. Inhibitory effect of adefovir on viral DNA synthesis and covalently closed circular DNA formation in duck hepatitis B virus-infected

hepatocytes in vivo and in vitro. Antimicrob. Agents Chemother. 46, 425–433.

Freiman, J.S., Murray, S.M., Vickery, K., Lim, D., Cossart, Y.E., 1990. Post-texposure treatment of experimental DHBV infection: a new therapeutic strategy. J. Med. Virol. 30, 272–276.

Gray, N.M., Marr, C.L., Penn, C.R., Cameron, J.M., Bethell, R.C., 1995. The intracellular phosphorylation of (−)-2′-deoxy-3′-thiacytidine (3TC) and incorporation of 3TC 5′-monophosphate into DNA by HIV-1 reverse transcriptase and human DNA polymerase gamma. Biochem. Pharmacol. 50, 1043–1051.

Hess, G., Arnold, W., Meyer zum Buschenfelde, K.H., 1980. Inhibition of hepatitis B virus polymerase by phosphonoformate: studies on its mode of action. J. Med. Virol. 5, 309–316.

Honkoop, P., de Man, R.A., Niesters, H.G., Main, J., Nevens, F., Thomas, H.C., Fevery, J., Tyrrell, D.L., Schalm, S.W., 1998. Quantitative hepatitis B virus DNA assessment by the limiting-dilution polymerase chain reaction in chronic hepatitis B patients: evidence of continuing viral suppression with longer duration and higher dose of lamivudine therapy. J. Viral Hepat. 5, 307–312.

Jiang, J.D., Davis, A.S., Middleton, K., Ling, Y.H., Perez-Soler, R., Holland, J.F., Bekesi, J.G., 1998. 3-(Iodoacetamido)-benzoylurea: a novel cancericidal tubulin ligand that inhibits microtubule polymerization, phosphorylates bcl-2 and induces apoptosis in tumor cells. Cancer Res. 58, 5389–5395.

Kim, J.W., Lee, H.S., Woo, G.H., Yoon, J.H., Jang, J.J., Chi, J.G., Kim, C.Y., 2001. Fatal submassive hepatic necrosis associated with tyrosine–methionine–aspartate–aspartate motif mutation of hepatitis B virus after long-term lamivudine therapy. Clin. Infect. Dis. 33, 403–405.

Lai, C.L., Ratziu, V., Yuen, M.F., Poynard, T., 2003. Viral hepatitis B. Lancet 362, 2089–2094.

Luo, k.Q., Jing, X.Y., Hua, J.S., 2001. Hepatic necrosis in HBV infection during lamivudine treatment. Chin. J. Lab. Clin. Virol. 4, 234–237.

MacGregor, R.R., Graziani, A.L., Weiss, R., Grunwald, J.E., Gambertoglio, J.G., 1991. Successful foscarnet therapy for cytomegalovirus retinitis in an AIDS patient undergoing hemodialysis: rationale for empiric dosing and plasma level monitoring. J. Infect. Dis. 164, 785–787.

McMillan, J.S., Shaw, T., Angus, P.W., Locarnini, S.A., 1995. Effect of immunosuppressive and antiviral agents on hepatitis B virus replication in vitro. Hepatology 22, 36–43.

Price, P.M., Banerjee, R., Acs, G., 1989. Inhibition of the replication of hepatitis B virus by the carbocyclic analogue of 2′-deoxyguanosine. Proc. Natl. Acad. Sci. U.S.A. 86, 8541–8544.

Rubinstein, L.V., Shoemaker, R.H., Paull, K.D., Simon, R.M., Tonisi, S., Skehan, P., Scudiero, D.A., Monks, A., Boyd, M.R., 1990. Comparison of in vitro anticancer-drug-screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. J. Natl. Cancer Inst. 82, 1113–1118.

Schvarcz, R., Hansson, B.G., Lernestedt, J.O., Weiland, O., 1994. Treatment of chronic replicative hepatitis B virus infection with short-term continuous infusion of foscarnet. Infection 22, 330–332.

Sells, M.A., Chen, M.L., Acs, G., 1987. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. Proc. Natl. Acad. Sci. U.S.A. 84, 1005–1009.

Wen, Y.M., 2001. Laboratory diagnosis of viral hepatitis in China: the present and the future. Clin. Chem. Lab. Med. 39, 1183–1189.

Yao, Y., Tang, N., Huang, A., 2001. Study on the replication of hepatitis B virus compared with that of duck hepatits B virus in primary duck hepatocytes. Zhonghua Yi Xue Za Zhi 81, 1157–1161.

Zemlicka, J., 2000. Enantioselectivity of the antiviral effects of nucleoside analogues. Pharmacol. Ther. 85, 251–266.