Efavirenz in combination with n-acetyl cysteine shows improved outcome in treatment of hepatitis B.

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Abstract

Hepatitis B virus (HBV) infection is one of the leading causes of liver disorders such as liver cirrhosis and hepatocellular carcinoma. The prognosis of hepatitis B in patients with HIV co-infection is poor and chances of spontaneous clearance of HBV infection is less likely. To investigate if n-acetyl cysteine (NAC) would protect liver from toxic effects of efavirenz (EFVZ) as a possible combination therapy for hepatitis B. In vitro studies were performed using the HepG2 2.2.15 cell lines and duck model of hepatitis B (Guangxi Small Partridge; n=48), animals were randomly divided into eight groups (n=6 in each group). The drug groups were treated with three dosages of efavirenz (25, 50, 100 mg/kg/day) and three dosages of three dosages of efavirenz (25, 50, 100 mg/kg/day) plus N-acetyl cysteine (NAC) (20 mM). Both efavirenz and NAC inhibited the growth of HepG2.2.15 cells at high concentrations in a dose-dependent manner. The treatment of HepG2.2.15 cells transfected with HBV, with efavirenz for 24 h also exhibited a dose dependent inhibitory effect on the secretion of HBsAg and HBeAg antigens. In duck HBV infection model, efavirenz plus NAC not only significantly reduced serum HBV DNA levels but also improved ALT and AST profile (p=0.03). Our study provides useful evidence that co-administration of NAC with efavirenz protects the toxic effect of efavirenz, in patients with hepatitis B infection.

Keywords: HIV, Hepatitis B, Antioxidant.
Materials and Methods

Chemicals
Efavirenz (EFVZ) (99.0% purity) was purchased from Xing Cheng Chemphar (China). Milli-Q water from a Millipore Water Purification System was used where distilled water was required. Efavirenz was dissolved in DMSO (≤ 1%) and then diluted with culture medium for in vitro experiments. For animal experiments efavirenz was dissolved in distilled water and later diluted with normal saline for the animal tests. Lamivudine (LMVD) was obtained from GlaxoSmithKline (Fujian, P.R. China) and served as the positive control.

Cell culture
HepG2.2.15 (clonal cells derived from human hepatoma cell line G2) cells were obtained from the Chinese Academy of Medical Sciences (China) and maintained in modified eagle medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 380 μg/ml of G418, 50 U/ml of kanamycin, and 0.03% L-glutamine (Mercator, China) at 37°C in a 5% CO₂ atmosphere with 100% humidity.

Cell toxicity studies
The cytotoxic effect of Efavirenz towards HepG2.2.15 was evaluated using the MTT assay (10). Logarithmically growing cells were seeded in 96-well culture plates at a density of 1 × 10⁵ cells/ml (200 μl/well). They were cultured for 24 h and then treated with various concentrations of efavirenz (20, 42.1, 63.5, 128.2, 182.4 μM). OD values were read at 450 nm after 24 h and the percent of cell death was calculated.

Treatment of HepG2.2.15 cells with efavirenz
HepG2.2.15 cells were seeded at a density of 1 × 10⁵ cells/ml (200 μl/well) in 96-well plates and maintained at 37°C for 24 h, followed by treatment with various concentrations (20, 42.1, 63.5, 128.2, 182.4 μM) of efavirenz or 40 μM lamivudine (3TC). After 24 h of treatment, the supernatants from each group were collected independently to determine the secretion of albumin and the levels of HBV surface antigen (HBsAg), HBV e antigen (HBeAg) and HBV DNA.

Detection of HBsAg and HBeAg
HBsAg and HBeAg were simultaneously detected using ELISA kits (Rongsheng Biotechnology Co. Ltd., Shanghai, China) according to the manufacturer's instructions. The plates were read directly with a microplate reader at a wavelength of 450 nm. The inhibition ratio (%) was calculated as follows: (OD control-OD sample)/OD control *100%.

Detection of HBV DNA by fluorescence quantitative-PCR assay
To further evaluate the inhibitory effects of efavirenz on HBV replication, the extracellular and intracellular HBV DNA levels were determined by fluorescence quantitative PCR (FQ-PCR).

The culture supernatants were used to measure extracellular HBV DNA and the cells were harvested for the analysis of intracellular HBV DNA. The viral DNA was extracted using Viral DNA Extraction Kit (TaKaRa, Dalian, China). Briefly, HBV DNA was extracted and amplified with a Step One Plus Real-Time PCR system using FastStart Universal SYBR Green Master (ROX). The forward primer was 5'-AAC CAT TGA AGC AAT CAC TAG AC-3', and the reverse primer was 5'-ATC TAT GGT GGC TGC TCG AAC TA-3' [10,11]. The PCR was carried out in a 25 μl reaction volume containing 12.5 μl 2 SYBR Green Master (ROX), 1.0 μl 10 μM forward primer, 1.0 μl 10 μM reverse primer 8.5 μl ddH₂O and 2.0 μl HBV DNA. The thermal program comprised of an initial denaturation at 95°C for 10 min followed by 40 amplification cycles with each of the two following steps: 95°C for 15 s and 60°C for 1 min. DHBV DNA was quantified using a standard curve. In this assay, the linear range was 1 × 10⁵-1 × 10¹¹ copies/ml. The inhibition ratio was calculated through the following formula: (negative control-treated test sample)/negative control × 100%.
**Animal studies**

All animal procedures were performed as per the guidelines of the Care and Use of Laboratory Animals and were approved by the Animal ethical Care and Use Committee of the Qilu Hospital of Shandong University, China. All experimental procedures were conducted in conformity with institutional guidelines and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals. Both male and female ducks (Guangxi Small Partridge) were used (n=48). Each one day old duck was injected into its tibial vein with 0.2 ml of serum from ducks with positive DHBV DNA serology [1]. The drug treatment experiment was carried out 7 days after ducks were infected with DHBV, and then the positive ducks were randomly divided into eight groups (n=6 in each group). The drug groups were treated with three dosages of efavirenz (25, 50, 100 mg/kg/day) and three dosages of three dosages of efavirenz (25, 50, 100 mg/kg/day) plus N-acetyl cysteine (20 mM). The positive control group received 50 mg/kg/day LMVD and the control group received normal saline. Drug efavirenz and LMVD were administered orally once daily for 14 days continuously. Animals in the efavirenz+NAC treatment group received ad lib drinking water supplemented with 20 mM NAC and remained on supplemented water until sacrifice. Blood samples were taken at initiation of treatment (T0), the seventh day of treatment (T7), the fourteenth day of treatment (T14) and the third day (P3) of post-treatment follow-up. The serum was stored at 80°C until analysis. At the end of the experiment, the ducks were sacrificed, and the liver tissues were removed and fixed in a 10% formalin solution for histopathologic examination.

**Detection of serum Duck Hepatitis B Virus (DHBV) DNA**

DHBV DNA was detected at 0, 7, 14 days, and 3 days after cessation of treatment by FQ-PCR as described previously. The serum DNA was extracted using Viral DNA Extraction Kit (TaKaRa, Dalian, China). HBV DNA inhibition rate (%)=(copy number of the control-copy number of the study sample)/copy number of the control *100%.

**Determination of serum ALT and AST**

Serum samples were collected from each group at days 0, 7, 14 of the treatment and day 3 post-treatment. The serum levels of alanine transaminase (ALT) and aspartate aminotransferase (AST) were measured by AST and ALT detection kits (Shanghai Zhicheng Bioengineering Institute, Shanghai, P.R. China).

**Histopathological examination of duck liver**

The DHBV-positive ducks were treated with efavirenz and LMVD (i.g.) once daily for 14 days. On day 3 of post-treatment, the animals were euthanized and the liver tissues were removed. A portion of each harvested liver tissue was instantly fixed in 10% phosphate-buffered formalin. Then, this portion was processed by embedding in paraffin, sectioning into 5 μm pieces. The samples were stained with haematoxylin and eosin (H & E), and examined using light microscopy for histopathological examination.

**Statistical analysis**

Statistical analysis was performed using SPSS 16.0 software for windows. One-way analysis of variance (ANOVA) was used to compare the means among different groups, and the Tukey’s test was used to conduct multiple post-hoc comparisons. Results are expressed in means ± S.D. A P value <0.05 was considered statistically significant.

**Results**

**Cytotoxic effect of efavirenz on HepG2 2.2.15 cell line**

The cytotoxicity of efavirenz was determined using fresh culture medium containing serial 1:2 dilutions of efavirenz. The inhibition ratio of the HepG2.2.15 cells exposed to different concentrations of efavirenz is shown in Figure 1. There was an increase in the inhibition ratio percentage when NAC (20 mM) was added to the same concentration of EFVZ.
in HepG2.2.15 cells (P=0.061). The 50% cytotoxic concentration (TC_{50}) was 295.3 μM, and the maximum nontoxic concentration (TC_{0}) was 8.1 μM. These results were used to determine the dose range of efavirenz for the subsequent experiments. The maximum inhibition achieved by EFVZ was 58% and EFVZ plus NAC was 63.6% at dose of 300 mM of EFVZ (P=0.046).

Figure 3. Inhibitory effect of efavirenz on secretion of HBsAg (A) and HBeAg (B) in ducks plasma. Data were expressed as mean ± S.D. (n=6), P value <0.05, was considered statistically significant.

Figure 4. Inhibition of DHBV DNA in ducks plasma in the efavirenz and efavirenz+NAC treatment group. Data expressed as mean ± S.D.(n=6), P value <0.05 was considered statistically significant.

Antiviral effects of efavirenz in HepG2.2.15 cells

Treatment of HepG2.2.15 cells with efavirenz at various concentrations for 24 h resulted in significant reduction of HBsAg and HBeAg secretion in a dose-dependent manner, with IC_{50} values of 17.4 μM and 63.9 μM, respectively (Figure 2A). At the concentration of 42.1, 63.5, 128.2 and 182.4 μM, efavirenz showed significant inhibitory effects on HBsAg secretion, with the highest inhibition at 89.3%. Similarly to the HBeAg secretion, the secretion of HBeAg was significantly reduced with the highest inhibition at 79.5%. In the same experiment, lamivudine (40 μM) suppressed the secretion of HBsAg and HBeAg by 48.5% and 46.8%, respectively.

Figure 5. Effect of efavirenz on serum ALT. Three groups of hepatitis B infected ducks were administered 25, 50, 100 mg/kg/day EFVZ or EFVZ in combination with n-acetyl cysteine (NAC), one group was administered Lamivudine (LMVD) 50 mg/kg/day for 14 days and control group received no treatment. Serum ALT was measured on Day 7, Day 14 and post treatment day 3. A) ALT levels in ducks administered EFVZ alone B) ALT levels in ducks administered EFVZ in combination with NAC, *represents P<0.05.

In vivo anti-HBV activity of efavirenz in duck model

We fed the ducks with efavirenz at various concentrations once daily for 14 days, no toxicity was observed. To examine the in vivo anti-HBV activity of efavirenz, we first examined the HBsAg and HBeAg secretion in serum. To investigate the anti-HBV activity of efavirenz in ducks, the plasma DHBV DNA levels of the infected ducks with and without treatment were evaluated by FQ-PCR. During this experimental period, no significant side effects were observed in animals receiving antiviral therapy, while one third of ducks in the control group died. After treatment with efavirenz and LMVD for 7 and 14 days, the level of DHBV DNA of each group decreased significantly (P<0.05). As shown in Figure 4, the treatment
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with efavirenz at the dose of 25, 50 and 100 mg/kg/day for 14 days exhibited a time and dose dependent inhibitory effect on DHBV DNA level. The rebound of the DHBV DNA levels in efavirenz plus NAC treated ducks was to a less extent as compared with the LMVD treated group. No significant differences were observed in the DHBV DNA levels of any of the controls (Figure 3).

Figure 6. Effect of efavirenz on serum AST; Three groups of hepatitis B infected ducks were administered 25, 50, 100 mg/kg/day EFVZ or EFVZ in combination with n-acetyl cysteine (NAC), one group was administered Lamuvidine (LMVD) 50 mg/kg/day for 14 days and control group received no treatment. Serum AST was measured on Day 7, Day 14 and post treatment day 3. A) AST levels in ducks administered EFVZ alone B) ALT levels in ducks administered EFVZ in combination with NAC; *represents P<0.05.

Analysis of ALT, AST levels in serum

DHBV infection resulted in a significant hepatic damage. The ALT, AST levels of model control group increased evidently compared to normal group. Oral administration of efavirenz at three different doses for 7 and 14 days resulted in lower levels of ALT and AST in serum (Figures 5 and 6).

Histopathological examination of the duck livers

Typical photographs of liver sections by light microscopy show normal hepatocyte structure, and single layer of cells arranged around the central vein in a radical pattern. The following typical pathological characteristics were obvious in the model group: revealing massive ballooning degeneration, hepatocyte, loosening necrosis and inflammatory cell infiltration. Samples treated with efavirenz, on the other hand, exhibited dose dependent improvement of the hepatocellular architecture (Figure 7). It is worth noting that efavirenz at 50 and 100 mg/kg/day resulted in a more significant improvement than 3TC at 50 mg/kg/day. The results of the evaluation of all of the samples are summarized in Table 1.

Figure 7. Histopathological changes in duck liver (H&E staining at 40X) A) Duck liver in control group-no drug administered showing hydropic and fatty degeneration (B) Duck liver showing ballooning degeneration in hepatocytes in lamivudine administered group (50 mg/kg) (C) Efavirenz (100 mg/kg) administered duck liver showing megalocytosis and hyropic degeneration (D) Efavirenz (100 mg/kg) plus NAC administered duck liver showing improvement in hepatocellular hydropic degeneration.

Table 1. Summary of histopathological changes in duck livers.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals (n)</th>
<th>Pathological Scope</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No lesion</td>
<td>Light (&lt;1/3)</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Lamivudine group</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Efavirenz group</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Efavirenz and n-acetyl cysteine group</td>
<td>6</td>
<td>3</td>
</tr>
</tbody>
</table>

The liver sections were evaluated primarily on the basis of their hepatocytic degeneration, chronic inflammation and cell infiltration. Efavirenz treatment samples exhibited dose-dependent improvement of the hepatocellular architecture. Although treatment with 25 mg/kg/day efavirenz did not have a significant effect compared with 3TC treatment, efavirenz at.
50 and 100 mg/kg/day resulted in a more significant improvement than 3TC at 50 mg/kg/day.

Discussion

The anti-HIV drugs such as nevirapine and efavirenz are known to cause hepatocellular damage [12-14]. Therefore, in spite of showing efficacy in clearing hepatitis B virus (HBV) there drugs have limited usefulness in clinical setting in HIV patients with HBV co-infection. In addition, patients with HBV already have varied extend of liver damage and possess treat of relapse after cessation of therapy [15]. Compared with traditional nucleoside drugs, efavirenz is a non-nucleoside reverse transcriptase inhibitor (NNRTI) and inducer of the cytochrome P450 system therefore metabolised by liver. Efavirenz is used as part of highly active antiretroviral therapy (HAART) regimen, in combination with other retroviral drugs. In patients with deranged liver function this often causes serious adverse events [16].

In the present study, the hepatoprotective effect of N-acetyl cysteine (NAC) was utilised to evaluate the beneficial effect on lowering efavirenz induced hepatotoxicity, in vitro and in vivo for the first time. Using HepG2 2.2.15 cells, we showed that cytotoxicity was observed after the administration of low concentrations of efavirenz with TC0 of 9.7 μM. Both efavirenz and NAC inhibited the growth of HepG2.2.15 cells at high concentrations in a dose-dependent manner. The treatment of HepG2.2.15 cells transfected with HBV, with efavirenz for 24 h also exhibited a dose dependent inhibitory effect on the secretion of HBsAg and HBeAg antigens. Treatment with efavirenz, as well as efavirenz with NAC for 24 h, showed lower levels of extracellular and intracellular HBV DNA than negative control. Compared with LMVD (40 μM), efavirenz and concomitantly administration of NAC showed stronger inhibitory activity at the concentration of 128.2 or 182.4 μM.

To demonstrate the in vivo anti-HBV activity of efavirenz, and investigate if concomitant administration of NAC has hepatoprotective effect, we investigated the duck model of hepatitis B, a widely used system for the study of histopathological score. It is also important to note that efavirenz (50, 100 mg/kg/day) plus NAC group showed marked improvement in histopathological score.

Conclusion

Our study provides useful evidence that co-administration of NAC with efavirenz protects the liver from the toxic effect of efavirenz mono or combination therapy, in patients with hepatitis B infection. This can further be useful in treatment of patients with HIV and HBV co-infection. One limitation of this study is the absence of NAC alone group, which could have helped in identifying the contribution of NAC towards therapeutic efficacy. Further studies and validation in clinical settings are required to include NAC in the treatment protocol for HIV positive patients with hepatitis B infection.

Acknowledgement

This study was supported by the Foundation for Outstanding Young Scientist in Shandong Province (BS2011YY018)

References


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