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The Potential Anti-HBV Effect of Amantadine in Combination with Ursodeoxycholic Acid and Biphenyl Dimethyl Dicarboxylate in HepG2 2.2.15 Cells

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Experimental studies have demonstrated that the triple combination of amantadine (A)/ ursodeoxycholic acid (UDCA, U)/ biphenyl dimethyl dicarboxylate (DDB, D) might have a preferential antiviral effect compared with that observed in interferon-induced antiviral signal pathways, such as those of STAT1α and the 6-16 genes. To confirm the results, this study examined whether the signal transduction for the antiviral activity in HepG2 2.2.15 was induced dependently or independently of interferon. To accomplish this, the correlation between the STAT1α and 6-16 genes, and nitric oxide, for the mediation of the antiviral activity was assessed. The increase in nitric oxide in the UDCA groups suggests that the inhibition of viral gene replication was enhanced by the amantadine combinations (AU and AUD), and might be more effective if incubated for longer periods. It was found that STAT1α was activated by the amantadine combination, although to a lesser extent than that of interferon-α, and the primary endpoints examined for the inhibition of gene expression (HBsAg and HBcAg) were remarkably well regulated. This suggests that the amantadine triple, or at least the double, combination had better clinical benefits than those of IFN-α and the nucleoside analogue single treatment. This demonstrates that the amantadine combination might be a substitute for the existing HBV therapy if the results of *in vivo* and *in vitro* studies concur.

Key words: Hepatitis B virus, HepG2 2.2.15, Interferon, Amantadine, UDCA, DDB

INTRODUCTION

The hepatitis B virus (HBV) is a causative agent of acute and chronic liver disease, and a major health problem around the world, particularly in Asia and Western Pacific regions (Davey, 1996). It can also lead to liver cirrhosis and has a strong association with hepatocellular carcinomas (Beasley, 1988). Regardless of the importance of a HBV infection to human health, no HBV specific drugs are available because the virus uses the body as a host for proliferation, even though several antiviral agents exist. Genealogically, the compounds applicable for HBV treatment are acyclovir for the acyclic guanosine analogues, azidothymidine (AZT) for the pyrimidine dideoxynucleoside analogues, 1-(2'-dedoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodocytosine (FIAC) for the 2'-fluoro-substituted arabinosyl-pyrimidines, dideoxyadenosine (ddA) for the purine dideoxynucleoside analogues, and (*S*)-9-(3-hydroxy-2-phosphonyl methoxypropyl) adenine (HPMPA) for the acyclic nucleoside phosphonates. To date, the drugs formally approved for treating chronic hepatitis B are interferon (IFN)-α, lamivudine (3TC) and adefovir dipivoxil. However, IFN and lamivudine have side effects and high resistance rate, up to 66% after four years of therapy, although a recent anti-HBV agent, adefovir, has much lower resistance (Clercq, 1999, 2004). Therefore, there is a need to develop antiviral agent(s) to replace, or at least to augment, existing drugs to lower the side effects and resistance rates to acceptable minimums whilst keeping or enhancing their antiviral efficacy.

Many studies have focused on the development of new or combination therapies that have an enhanced efficacy with minimal side effects and resistance (Asmuth *et al.*, 2004). Non-toxic natural substances, such as ursodeoxycholic acid (UDCA) and biphenyl dimethyl dicarboxylate (DDB), have recently been examined to determine if they have pharmacological effects on viral hepatitis (Fabris *et al.*, 1999).

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In a previous study, DDB, which is an active dibenzocyclooctadiene compound from the fruit of *Schizandra chinensis*, was found to play a potential role in lowering the gamma-glutamyl transferase ALT level, which is known to be an index of hepatitis, down to normal base line levels within 2-12 weeks (Huber *et al.*, 2004). *Schizandra chinensis* is a traditional Chinese herb that has been used as an astringent and for the treatment of viral and chemical hepatitis (Hancke *et al.*, 1999; Stuyver *et al.*, 2002). Moreover, DDB has been shown to have protective effect against CCl_4-induced hepatotoxicity (Chiu *et al.*, 2003). UDCA is a nontoxic, hydrophilic bile acid that has been shown to have protective effects on cell membranes *in vitro,* so might also have immunomodulatory effects. Although UDCA alone, or in combination with interferon, has failed to improve either the virological response rate or the histological features in HCV patients (Bonkovsky *et al.*, 1997), biochemical improvements have been reported in several trials of UDCA, with IFN-α, for the treatment of chronic hepatitis (Abdelmalek *et al.*, 1998). Moreover, as UDCA has great pharmacological potential in cytoprotection, membrane stabilization, antioxidation, immunomodulation and anti-apoptosis (Trauner *et al.*, 1999), it appears to have many synergistic outcomes when combined with potential antiviral agents. A water soluble antiviral agent, amantadine, has been known to be effective against influenza A and is used widely in the treatment of Parkinson's disease. In addition, it blocks the viral membrane matrix protein, M2, which is essential for the internalization of the virus by endocytosis (Wharton *et al.*, 1994).

In a previous study, amantadine-IFN combination therapy was compared with amantadine alone in IFN non-responders. The study showed both higher biochemical and virological responses rates with the combination therapy. However, further investigation will be needed for confirmation (Findor *et al.*, 1997).

This study examined the potential role of the amantadine/UDCA/DDB triple combination for its antiviral activity compared with a single dose of nitric oxide (NO), as NO plays a potential role in mediating the antiviral activity (Lowenstein *et al.*, 1994; Reiss and Komatsu, 1998). The inducible isoforms are present in macrophages and hepatocytes, and synthesize NO upon activation by cytokines, such as IFN-α/γ, TNF-α and IL-1α (Guidotti *et al.*, 2000). In addition, in order to confirm the antiviral activity in relation to the level of signal transduction, this study investigated the 6-16 gene and STAT1α, which are related to the induction of IFN-α-induced antiviral efficacy (Zhu *et al.*, 2003). The final assessment of the efficacy of the tested samples was confirmed by the HBV antigen, including the viral core (HBcAg) and viral surface antigens (HBsAg).

MATERIALS AND METHODS

Stock preparations

Stock ursodeoxycholic acid (UDCA), interferon-α (IFN-α), DDB and amantadine were prepared by dissolving in micro-filtered (0.2 μm) culture media, at 0.5, 25 and 50 μg/mL, 50, 100 and 200 μg/mL, 5, 10 and 20 μg/mL, and 100, 500 and 1000 IU/mL for UDCA, DDB, amantadine and IFN-α, respectively. The solutions were either kept at -20°C, for future use, or diluted directly in a 1-5% FBS RPMI1640 culture medium, for cell culture experiments.

Cell culture

The HepG2 2.2.15 human hepatoblastoma cell line (Sells *et al.*, 1987), into which a head-to tail dimmer of HBV DNA (strain ayw) had been stably transfected, was grown in RPMI 1640 (Invitrogen, USA), supplemented with 10% fetal bovine serum (FBS) (HyClone, USA), at 37°C under 5% CO_2. The cultures were refreshed with 2% FBS medium in the main studies.

Nitric oxide assay and reversed transcription PCR (RT-PCR)

The amount of NO produced by the HepG2 2.2.15 cell line under different sample conditions was determined by examining culture supernatants for the stable end product, nitrite, using an automated procedure based on the Griess reaction. Briefly, an aliquot (100 μL) of the culture medium was incubated with 50 μL of 0.1% sulfanilamide in 5% phosphoric acid and 50 μL of 0.1% *N*-1-naphthyl-ethylenediamine dihydrocholate (Sigma, USA). After a 10 min incubation period at room temperature the absorbance was measured at 540 nm. For RT-PCR, the cells, at a density of 1.5×10^5/mL, were plated in 6-well plates and treated with each sample for the designated culture times (one to five days). All sample concentrations were varied, as described above, and the experiments performed under various conditions, in either single or combination treatments, from 24 to 120 h (5 day). After the designated culture time, the total RNA was obtained from 1×10^7/mL cells using Trizol reagent (Life Technologies, USA). Briefly, the cells were lysed using 1 mL Trizol reagent, 200 μL chloroform added after 1 min incubation at room temperature, and the mixture centrifuged at 13500 rpm for 15 min. The aqueous layer (about 450 μL) was transferred to another tube, and the RNA precipitated by centrifugation, with 450 μL isopropanol, at 13500 rpm for 10 min. The RNA pellets were washed in 700 μL cold Et-OH (70% in DEPC water) and dried in air. The total RNA (0.5 μg) from each group was examined by RT-PCR (MJ Research, USA) using the primers specific for HBsAg, HBcAg, the 6-16 gene and GAPDH (Table I) in a total reaction volume of 20 μL. The amplified cDNA was separated on a 1.2%

Table I. Oligonucleotide primer sequences used for the PCR

Target	Primer	Amino acid sequence
HBsAg (380bp)	5' Primer	5' - gaa gca ccc aag tgt cct gg -3'
	3' Primer	5'- aaa cgg act gag gcc cac tc -3'
HBcAg (423bp)	5' Primer	5'- tcc gtg atc tgc tcg aca cc -3'
	3' Primer	5'- acc ttc gtc tgc gag gcg ag -3'
6-16 (553bp)	5' Primer	5'- caa gct taa ccg ttt act cgc tgc tgt -3'
	3' Primer	5'- tgc ggc cgc tgc tgg cta ctc ctc acc t -3'
GAPDH (576bp)	5' Primer	5'-cca tca cca tct tcc agg ag-3'
	3' Primer	5'-cct gct tca cca cct tct tg-3'

agarose gel and stained with ethidium bromide. The PCR band intensities are expressed as OD values using the UVIDocMw program.

Western blot analysis

The cells were scraped and centrifuged for 1 minute at 1250 rpm. The cell pellets were washed with PBS, collected and lysed with RIPA buffer. The protein samples (30 μg) extracted from the cytosol were heated in SDS loading buffer at 90°C for 5 minutes, prior to separation on the 12-15% SDS-polyacrylamide mini-gels, and transferred to a PVDF membrane (Appligene, USA). The membranes were blocked using 5% skimmed milk in TBS-T buffer solution and probed with the primary antibodies directed against each protein; STAT1α (mouse anti-STAT1α; Zymed Lab, USA) and Actin (goat polyclonal, sc-1615; Santa Cruz, USA). The bound antibodies were detected using species-specific secondary antibodies conjugated to HRP.

Statistical analysis

The data are reported as the mean ± S.D. Statistical significance was determined using the Student's t-test. Any significant differences were identified using the SPSS software (ver. 10).

RESULTS

Amantadine combination elevates the production of nitric oxide

At the various incubation times (1~5 day), marked elevations in the amount of NO produced in the amantadine combinations were found, particularly in those with UDCA (Fig. 2). Although no distinctive comparison between the groups was available on day 1, the NO level was elevated considerably after day 3, which continued, time dependently, up to day 5 in the AUD, AU and UDCA single groups, whereas that in the other groups was not affected. These three groups had significantly elevated levels of

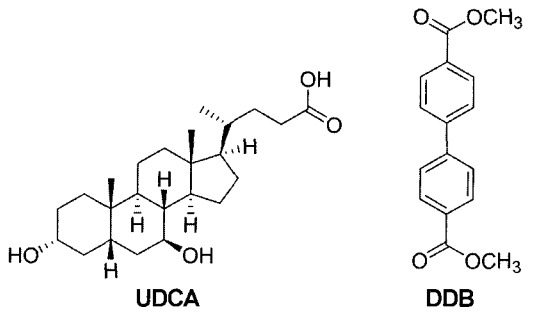

Fig. 1. Chemical structures of Ursodeoxycholic acid (UDCA) and Biphenyl dimethyl dicarboxylate (dimethyl biphenyl-4,4'-dicarboxylate; [1,1'-Biphenyl]-4,4'-dicarboxylic acid dimethyl ester)

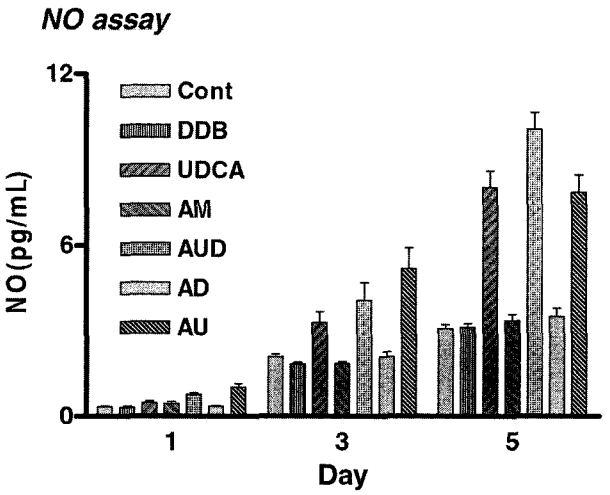

Fig. 2. Comparison of the NO production in response to each treatment group. DDB and amantadine, and their combinations, did not affect the NO production, whereas UDCA and its combination (AUD and AU) increased the NO production. NO was analyzed using the Greiss reagent system, employing a spectrophotometer at 540 nm. When the cells reached approximately 80% confluence, each sample group was treated, and analysis carried out on day 5 in a 96 well plate, as described in materials and methods. The data represent the mean±SD of three separate experiments.

NO production, up to 2 to 2.5 fold, on days 3 and 5 compared with the control group. This pattern continued with longer incubation times (7 day) (data not shown).

DDB enhances the antiviral effect of amantadine

In order to determine if the amantadine combination had antiviral potential when applied to a human hepatocyte cell line (HepG2 2.2.15), the 6-16 gene expression level and STAT1α activation was compared (Figs. 3~4). The 6-16 gene was markedly expressed in the presence of IFN-α at all doses (100 to 1000 IU/mL) ($p>0.05$), whereas the gene was not expressed in the AUD group, with the exception of the high dose group (AUD3). At the most applicable combination dose (20/50/100 μg/mL, AUD), which was selected from a preliminary study, the 6-16 gene was

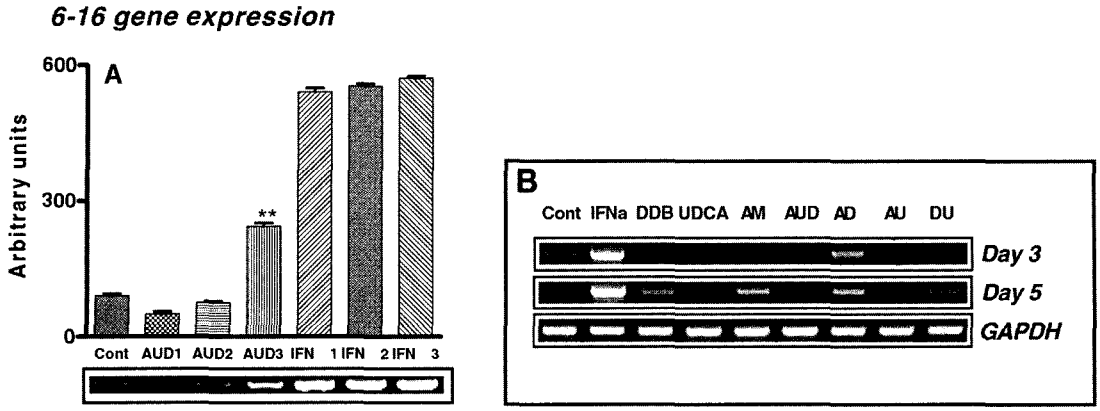

Fig. 3. The 6-16 gene induced by IFN-α treatment in the HepG2 2.2.15 cell line. (A) The HepG2 2.2.15 cells were treated with 100 (IFN1), 500 (IFN2) and 1000 (IFN3) IU/mL of IFN-α and 0.5/5/50 (AUD1), 10/25/50 (AUD2) and 20/50/200 (AUD3) μg/mL of the combination for 3 day as the preliminary verification of gene expression. (B) The cells were then cultured for 3 and 5 day, under the same conditions, with the most appropriate dose of the combination (20/50/100, AUD). The GAPDH gene was used as the control.

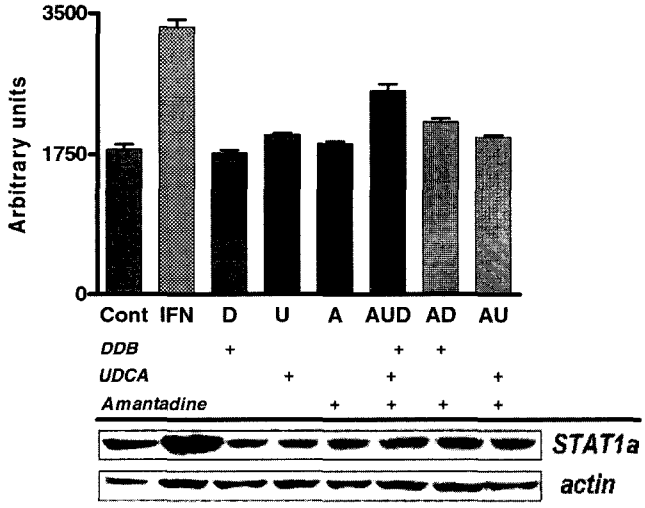

Fig. 4. The IFN-α and amantadine-DDB combination induced STAT1α in HepG2 2.2.15 cells. The cells were incubated for 3 day after adding each sample to the media, and after completing the co-incubation, the cell lysates were analyzed by western blot using a STAT1α mouse monoclonal antibody. β-actin was used as the internal standard. The STAT1α bands were quantified by densitometry and normalized, with the results from repeated experiments shown in the above panel.

found to be expressed when treated with amantadine and DDB, and this expression was maintained when the two agents were combined (Fig. 3B). The changes in the expression levels were not significant between day 3 and 5, but the 6-16 gene was expressed in the amantadine single and DDB-UDCA groups when incubated for a longer time (over 5 day). This suggests that in this system, the expression of the 6-16 gene can be induced by amantadine and DDB, as well as by IFN-α. In conjunction with this, the STAT1α gene was observed after 3 day incubation. As shown in Fig. 4, the STAT1α gene was activated in the presence of IFN-α, but less so in the AUD and AD groups, which were linked to the results of 6-16 gene expression.

AUD combination inhibits HBsAg and HBcAg expression

The inhibition of HBsAg and HBcAg expressions by the amantadine combinations was examined. The total RNAs were isolated after days 3 and 5, and compared with the band density calculated *via* densitometry (Fig. 5A, 5B). HBsAg was clearly observed in the AUD group, despite the extended incubation time, but no steady state inhibition of the two genes was found in the other groups, even though the AU group inhibited the expression of HBsAg on day 5 (Fig. 5A). In contrast to the surface gene, the core gene was distinctively comparable between the tested groups (Fig. 5B). A statistically significant inhibition of the gene was observed in the AUD ($p<0.05$), AD ($p<0.01$) and DU groups ($p<0.05$), but not in the AU group, which showed a similar pattern to the UDCA and amantadine single groups.

DISCUSSION

This study established that two clinically important natural products, UDCA and DDB, have adjuvant potential in anti-HBV therapy when combined with the antiviral agent, amantadine. This study examined whether these two agents have anti-HBV potential resulting from either the direct or indirect activity, when combined with amantadine, on the HBV *in vitro* model as they have been used as an adjuvant therapy in HBV infections to normalize the pathological indices, such as SGOP and SGPT, and for the possibly of their antiviral effect (Neuman *et al.*, 2001). IFN is the product secreted by virus-infected cells, which is capable of preventing further infection of the exposed

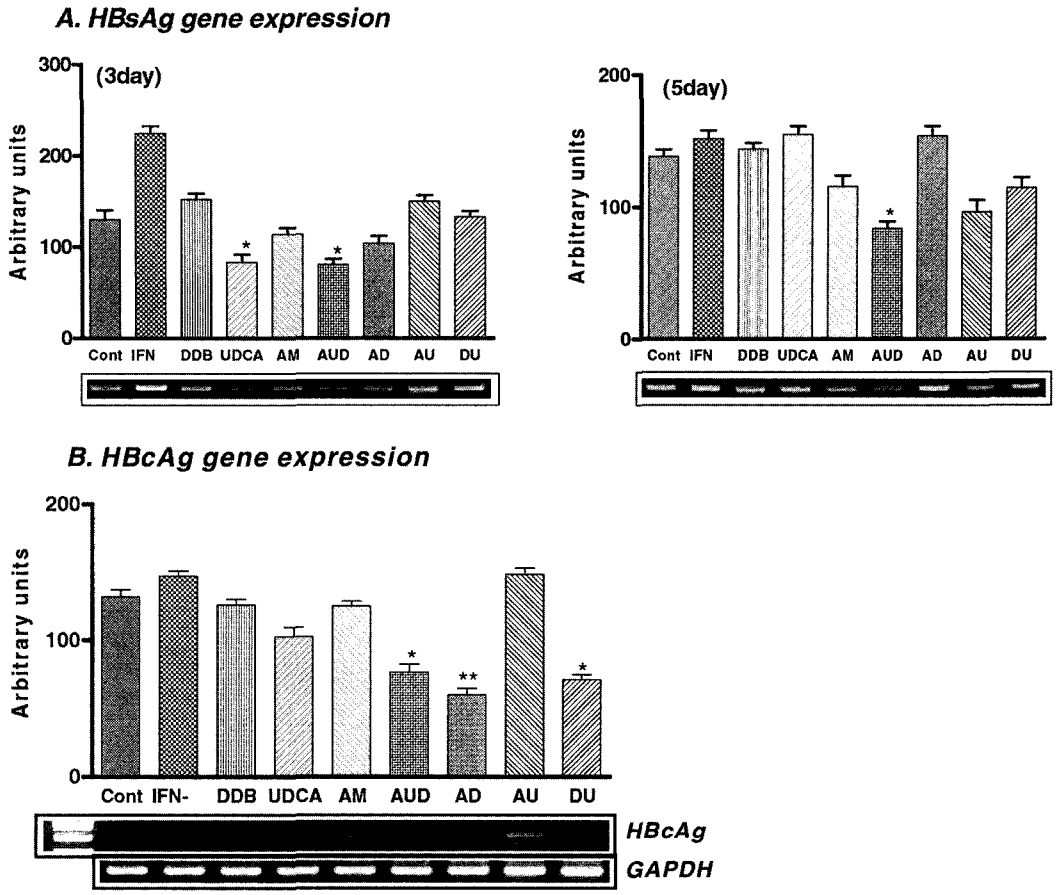

Fig. 5. RT-PCR for the human viral core (HBcAg) and viral surface antigens (HBsAg) in HepG2 2.2.15 cells. The cells were cultured for 3 day, as described in materials and methods. After treatment, the total RNA was obtained, and then analyzed by RT-PCR analysis using the HBsAg and HBcAg specific and GAPDH primers. Cont; control (culture media), IFN-α (interferon alpha, 1000 IU/mL), DDB (biphenyl dimethyl dicarboxylate, 100 μg/mL), UDCA (ursodeoxycholic acid, 50 μg/mL), AM (amantadine, 20 μg/mL), AUD (amantadine-UDCA-DDB, 20/50/100 μg/mL). The data represent the means±SD. *, $p<0.05$; **, $p<0.01$ (vs. control).

cells (Lindenmann, 1982). In recent years, the demand for new antiviral strategies has increased. However, the severe side effects and viral mutation following several years of medication have attracted clinical concern (Clercq, 2004). No completely safe and effective antiviral agents for HBV are available, despite the many trials of combination antiviral therapy, to silence the physical symptoms of the side effects and avoid drug-resistant HBV mutants (Locarnini *et al.*, 1999; Cotonat *et al.*, 2000).

This study investigated the potential role of amantadine in combination with UDCA and DDB for its antiviral effect by comparison with their single doses and positive controls. Moreover, an attempt was also made to determine if the combination was correlated with the STAT1α signal pathway and 6-16 gene expression, which is induced by IFN (STAT1α-mediated IFN-inducible gene) (Zhu *et al.*, 2003). For the other possible antiviral effects, this study examined NO, which mediates the antiviral activity of IFN-γ *via* the inhibition of viral gene replication (Guidotti *et al.*, 2000), the activity of which is not induced during natural infection (Bose *et al.*, 2003). As shown in Fig. 2, UDCA, in combination with amantadine, enhanced the NO production in the HepG2 2.2.15 in a time dependent manner. However, the amantadine single and AD combination was unaffected. Therefore, the STAT1α and 6-16 genes were examined to determine if those antiviral indicators were associated with NO production (Figs. 3, 4). No particular association was found between STAT1α/6-16 and NO production. However, the HBsAg gene expression was significantly inhibited by treatment with AUD and AU with longer incubation times (Fig. 5), suggesting that NO mediates the antiviral activity of the amantadine combination. In turn, the increase in the NO level, which can be both injurious and protective in inflammation, can have an antiviral effect and; thereby, inhibit viral replication, as observed with IFN-γ (NO dependent). In addition, the high-level of NO induction due to the overproduction of the proinflammatory cytokines, TNF-α, IL-1β and IL-6, through the activation of Kupffer cells in the early stage of acute hepatitis B, may damage infected hepatocytes, which is

another mechanism for stopping viral replication (Koulentaki *et al.*, 2004). More precise and in-depth experiments should be performed on the effect of UDCA in HBV infection, but UDCA in combination with amantadine may offer a good choice for long-term antiviral therapy in preventing viral replication. The function of the 6-16 gene is still unknown, but is regarded as either an IFN-α responsive or a primary response gene that enhances the IFN-α antiviral efficacy (Zhu *et al.*, 2003). Fig. 3 shows the 6-16 gene expression in the AUD and IFN-α groups by increasing the concentration of each single dose (Fig. 3A). This result was re-evaluated in fig. 3B by examining the expressions of 6-16 mRNA on day 3 and 5 to ensure the time dependency. Unlike the NO production in response to DDB treatment, DDB was effective at expressing the 6-16 gene up to day 5. Interestingly, the DDB and amantadine single groups expressed the 6-16 gene on day 5 (Fig. 3B). In the IFN-α induced antiviral activity, expression of the STAT1α gene is closely related because it is activated by the stimulation of the viral IFNs induced by viral infection (Darnell *et al.*, 1994). Activated STAT1α, after the activation of receptor-associated tyrosine kinase, translocates to the nucleus, activating the transcription of the target genes, including antiviral proteins, such as the PKR protein kinase, MxA and 2'-5' oligoadenylate synthetase (Platania and Fish, 1999). This study found that STAT1α was significantly elevated in the IFN-α, AUD and AD groups (Fig. 4), indicating that the AUD triple combination has potential antiviral activity benefit through the STAT-dependent pathway, even though the effect is not as great as that of IFN-α. Finally, it was found that expression of the HBcAg gene was inhibited under the AUD and AD treatments, suggesting that the amantadine triple and AD combination therapies can effectively regulate the replication of the viral gene. Overall, the antiviral activity of amantadine in the HepG2 2.2.15 cells may be affected by DDB and UDCA, which have both been used to treat hepatic inflammation. The well-proven safety of compounds is an important benefit in antiviral therapy. Moreover, the mediation of the antiviral activity of UDCA and DDB, through NO production and STAT1α-mediated antiviral activity, is synergistically enhanced in combination with amantadine.

In conclusion, the amantadine combination might have clinical benefits for use in anti-HBV therapy. The treatment with a single agent was not as responsive in elevating the levels of NO, the STAT1α and 6-16 gene expression levels, or in inhibiting the surface and core viral antigens, whereas the inhibition by the amantadine combination was marked. In particular, the 6-16 gene induced by STAT1 activation was elevated in the AUD and AD groups. This suggests the 6-16 gene was responsive to these groups, which is likely to have resulted in the IFN-induced antiviral activity. This potential anti-HBV activity was supported by the final endpoint of the HBcAg gene expression, which was well regulated in the AUD, AD and DU groups. Therefore, it is possible that the amantadine triple combination, or at least the double combination (i.e. AD & AU), may be clinically beneficial to type B hepatitis compared with the current anti-HBV agents that have problematic physical side effects and drug resistances. More precise and in-depth investigations are needed, but possible anti-HBV activity of the combination treatment may result from the UDCA and DDB's cell specific effect on NO production (inhibition of viral gene replication) and the STAT1α-mediated antiviral activity (elevation of antiviral components).

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