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Interferon Signal Transduction of Biphenyl Dimethyl Dicarboxylate/Amantadine and Anti-HBV Activity in HepG2 2.2.15

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Biphenyl dimethyl dicarboxylate (DDB) is a hepatoprotectant, which is used as an adjuvant agent in a treatment for chronic hepatitis. Amantadine is an antiviral agent, which is utilized primarily in the treatment of influenza, but also, occasionally in the treatment of hepatitis C. In a previous study, we reported that DDB, coupled with amantadine, would exert an anti-HBV effect, *via* the induction of interferon-inducible gene expression in the HepG2 2.2.15 cell line. The primary objective of the present study was to determine whether or not DDB and/or amantadine exhibit anti-HBV properties, and what mechanisms of action might be involved in such properties. In our study, we were able to determine that DDB stimulates Jak/Stat signaling, and induces the expression of interferon alpha (IFN-α) stimulated genes, most notably 6-16 and ISG12. In addition, the antiviral effectors induced by IFN-α, PKR, OAS, and MxA, were regulated in the presence of DDB at its optimal concentration (250 μg/mL), to a degree commensurate with the degree of induction associated with the IFN-α treated group. Finally, we determined that the replication of pregenomic RNA and HBeAg was inhibited by DDB treatment, and this inhibition was maximized when coupled with the administration of amantadine (25 μg/mL). In conclusion, the results of this study demonstrated clearly that DDB, as well as the combination of DDB/amantadine, directly inhibited IFN-α signaling-mediated replication of HBV in infected hepatocytes, and thus may represent a novel treatment for chronic hepatitis B, which would be characterized principally by its improved safety over other treatment strategies.

Key words: DDB, Amantadine, Chronic hepatitis B, Interferon alpha, Jak/Stat, 6-16, ISG12

INTRODUCTION

The Hepatitis B virus (HBV) is a common cause of liver disease all over the world, and its epidemiology has been extensively addressed in Asian countries. Clinically, HBV represents a silent killer in patients with chronic HBV infection, resulting in the development of cirrhosis, liver failure or hepatocellular carcinoma (HCC) (Lee, 1997; Lok, 2002). Specifically, the incidence of hepatocellular carcinoma, which is profoundly associated with HBV, has increased in recent years, and this disease currently kills approximately 0.5 million people per year (Parkin *et al.*, 2001). In order to ameliorate the incidence of HBV infection, a vaccination strategy has been institutes, the results of which have been, in general, successful (Hershey *et al.*, 2005). However, as this disease is readily transmitted through the blood at any time, the complete elimination of the disease may prove impossible. Therefore, the development of an effective therapy for HBV is clearly warranted. Currently, two drug therapies are used for the treatment of chronic hepatitis B (CHB) involving either interferon alpha (IFN-α), or lamivudine. However, these therapies are somewhat clinically limited with regard to their use in cases of CHB, in that IFN-α is associated with undesirable flu-like side effects and lamivudine, a nucleoside analogue, has been associated with high rates of viral resistance, as well as the emergence of mutants (Liaw *et al.*, 2000; Perrillo *et al.*, 2000). Therefore, future therapies for CHB must necessarily involve a safe antiviral activity, with less side-effects and no drug-resistance. Conventional IFN-α has been discovered to possess a dual mode of immunomodulatory action, and is though to probably stimulate the immune system, as well as expressing an antiviral activity

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that suppresses viral replication (Guidotti *et al.*, 1994; Malik *et al.*, 2000). In response to viral infection, a number of signal transduction pathways are activated, and IFN-α is encoded as an early event. The IFN-α secreted from the infected host cells then engages in interactions with the neighboring cells *via* binding to cell surface IFN receptors, and induces the Janus kinase (Jak)/signal transducer, and activator of transcription (Stat) signaling (Samuel, 2001). These delayed events after the initiation of INF-α-IFN receptor interactions subsequently result in the induced production of double-stranded RNA-dependent protein kinase (PKR), 2′-5′-oligoadenylate synthase (OAS), and the MxA protein, all of which interfere with viral transcription and translation protocols (Jacobs and Langland, 1996; Fernandez *et al.*, 2003). Here, we report that the biphenyl dimethyl dicarboxylate (DDB) obtained from *Schizandra fructus* may induce a signal transduction similar to that associated with IFN, and its antiviral activity should be maximized *via* combination with amantadine (1-aminoadamantanamine), a water-soluble tricyclic amine, which has previously been used in clinical settings to prevent viral infections with influenza A in HepG2 2.2.15 cells. We reported previously that DDB may induce anti-HBV activity, and the efficacy of this effect can be optimized *via* combination with amantadine and ursodeoxycholic acid (Joo and Lee, 2005). Although mechanical evidence regarding the antiviral properties of DDB for antiviral activities is not yet available in any real abundance, studies conducted in this laboratory have suggested that the replication of viral DNA might be inhibited by DDB, and that this outcome may be induced *via* interferon-like signal pathways that effect the degradation of mRNA. Finally, our data suggested that DDB, when coupled with amantadine, may exert an anti-HBV effect predicated on the inhibition of viral DNA, with an optimal synergy inherent to the combination.

MATERIALS AND METHODS

Materials

The biphenyl dimethyl dicarboxylate was provided by Woori Pharmaceutical, Inc. (Seoul, Korea) and the amantadine was obtained from Sigma (MO, U.S.A.). The recombinant human Interferon alpha (rhIFN-α) was acquired from Biosource (CA, U.S.A.) and the Cy3 dye was provided by Amersham Biosciences (England, U.K.) and Sigma (MO, U.S.A.).

Cell culture

HepG2 2.2.15 cells, human hepatoblastomas stably transfected with genomic HBV DNA (Acs *et al.*, 1987), were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco Technologies, MD, U.S.A.) supplemented with 10% fetal bovine serum (FBS). The cells were seeded in either six-well plates or culture dishes, depending on the purpose of the specific study, at a density of 3×10^5 cells. The studied compounds were added to the medium approximately 3 days after cell inoculation. The cells were then allowed to grow for 3-9 days in the presence of drugs, with medium changes conducted every 3 days.

LDH assay

Cytotoxicity induced by DDB and amantadine was evaluated *via* measurements of lactate dehydrogenase (LDH) leakage into the culture medium. Subsequent to exposure to the drugs at various concentration dilutions, the culture medium was harvested and centrifuged for 10 minutes at 3000 rpm, in an attempt to generate cell-free supernatants. The LDH activity in the medium was assessed using a commercially available non-radioactive LDH assay kit manufactured by Promega (CytoTox 96®). LDH is a stable cytoplasmic enzyme which is present in all cells, and is rapidly released into cell culture supernatants upon the infliction of plasma membrane damage. In this study, LDH activity was evaluated *via* a coupled enzymatic reaction, in which tetrazolium salt was reduced to formazan with a microplate spectrophotometry system (Spectra Max 340, Molecular Devices, U.S.A.) operated at a wavelength of 490 nm. % Cytotoxicity was calculated using the formula: [(Experimental LDH release (OD_{490})/Maximum LDH release (OD_{490}) × 100].

RT-PCR

Total RNA from each of the samples was extracted *via* the Trizol method, and the cDNA was synthesized with a Reverse Transcription System (Promega, WI, U.S.A.) in accordance with the manufacturer's instructions. The reverse-transcribed cDNA was then amplified in a PCR Master Mix system (Promega, WI, U.S.A.) in order to maintain a constant PCR product. PCR reactions were conducted in DNA Engine Dyad® (MJ Research, U.S.A.). After 3 minutes of heating at 94°C, the reaction mixtures were cycled as described in Table I. The products were analyzed on ethidium bromide-stained 1.2% agarose gel, then quantified using a UVIdoc gel documentation system. The media DNA was precipitated with polyethylene glycol 8000 (Sigma, MO, U.S.A.) as described previously (Doong *et al.*, 1991).

Western blot

HepG2 2.2.15 cells were lysed in 1X RIPA buffer with protease inhibitors. After 15 minutes of centrifugation at 13500 rpm, equal volumes of lysates from an equivalent number of cells were fractionated *via* 10% SDS-PAGE. After being transferred to PVDF membranes, the blots were probed with 1:600 anti-Stat2 and -Jak1 (Santa Cruz Biotechnology, CA, U.S.A.) for 2 h at room temperature.

Table 1. Primer sequences for PCR

Target Genes	Primers	Nucleotide sequences	Ann. temp. [°C]	Cycles
6-16 (553bp)	5'Primer	CAA GCT TAA CCG TTT ACT CGC TGC TGT	56	30
	3'Primer	TGC GGC CGC TGC TGG CTA CTC CTC ACC T		
ISG12 (134bp)	5'Primer	AGT TGT GGC TGT GCC CAT GGT G	62	32
	3'Primer	CAA GGC TGC CCG AGG CAA CTC C		
PKR (575bp)	5'Primer	TGG CTG GTG ATC TTT CAG CAG G	56	32
	3'Primer	AGA GTT GCT TTG GGA CTC ACA C		
OAS (472bp)	5'Primer	TGA TGG GTC CAC CAT CCA GGT G	59	32
	3'Primer	CAG CAG GAT GTT CCT GAT GGT C		
MxA (581bp)	5'Primer	ACC AGC TGA GCC TGT CCG AAG C	59	32
	3'Primer	CCG GAC CAT ATC CGT CAC GGT G		
pgRNA (1.2kb)	5'Primer	GGC CCA CTC ACA GTT AAT GAG A	57	30
	3'Primer	ACA GGT GCA ATT TCC GTC CG		
HBeAg (253bp)	5'Primer	ACC TCA CCA TAC TGC ACT CAG G	59	30
	3'Primer	GGC TGG AGG AGT GCG AAT CCA C		
IFNAR (642bp)	5'Primer	GCG GCT CCC AGA TGA TGG TCG T	57	27
	3'Primer	TCC ATG ACG TAA GTA GTG CTG C		
GAPDH (576bp)	5'Primer	CCA TCA CCA TCT TCC AGG AG	58	27
	3'Primer	CCT GCT TCA CCA CCT TCT TG		

The blots were washed for 1 h in TBS-Tween solution, incubated for 2 hours with anti-mouse IgG-HRP (1:10000, Zymed, CA, U.S.A.) at room temperature, again washed in TBS-Tween, and developed using an enhanced chemiluminescence system.

RESULTS AND DISCUSSION

Cytotoxicity assessment of DDB and amantadine in HepG2 2.2.15

In order to evaluate the cytotoxicity produced by DDB and amantadine, necrosis, a form of cell death involving membrane permeabilization followed by cell destruction coupled with the extravasation of cellular components, was assessed on the basis of the levels of release of the intracellular enzyme lactate dehydrogenase (LDH) into the culture media. As is shown in Fig. 1, we determined that both of the drugs were cytotoxic at a dose of over 500 μg/mL for DDB and 100 μg/mL for amantadine. However, this associated cytotoxicity was reduced markedly after the administration of treatment with one-half of the maximal dose, and the next lower doses were found not to be as cytotoxic as was the control LDH level. In addition, this result provided us with an effective and non-cytotoxic dosage for each drug, namely, 250 μg/mL DDB and 25 μg/mL amantadine.

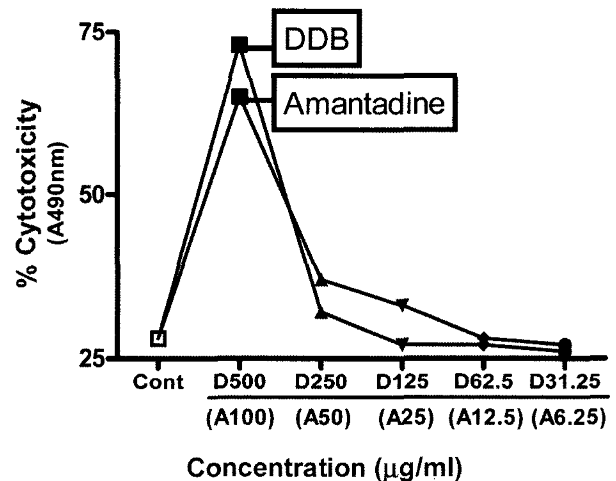


Fig. 1. Cytotoxicity of DDB and amantadine in HepG2 2.2.15. The cells were treated with DDB or amantadine in serial dilutions, and incubated for 24 h. After incubation, the supernatants were obtained and the levels of LDH leakage from cells were analyzed, as described in the Materials and Methods section. The data represent the mean values of three different experiments.

IFN-α receptor gene expression

As IFN-α receptor (IFNAR) in response to virus infection is crucial to delayed events within the interferon system following early events in viral invasion, we hypothesized that DDB and/or amantadine might engage in interactions with the IFN receptor, thus inducing downstream signaling pathways, including those involving Jak and Stat. Fig. 2 shows that both DDB and amantadine effected increases in the expression of IFNAR mRNA. Interestingly, we deter-

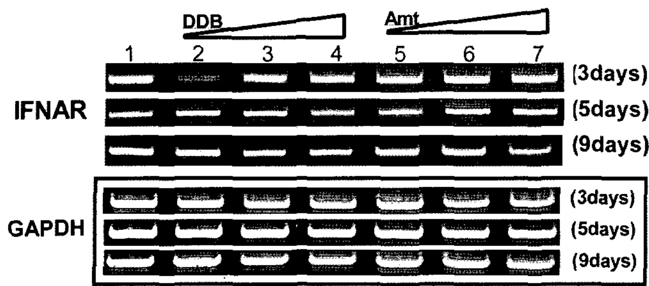


Fig. 2. RT-PCR analysis of IFN-α receptor gene expression. The cells were treated with serial concentrations of DDB and amantadine for 3, 5, and 9 days, after which the expression of IFN-α receptor mRNA at each of the time points was evaluated *via* the reverse transcription of total RNA to cDNA, then amplified *via* PCR. GAPDH was used as an internal control.

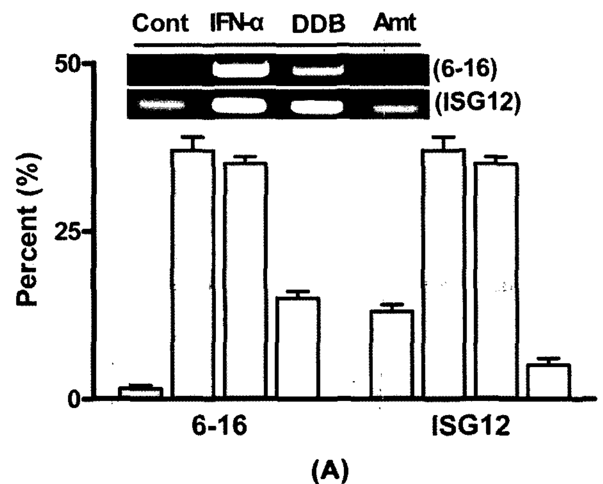
mined that IFNAR expression occurred in a reversed pattern between day 3 and day 9 when treated with DDB, and this effect was clearly dose-dependent. Conversely, amantadine effected an increase in IFNAR expression during the early phases of incubation at all concentrations, and this effect persisted over 9 days of incubation, except at higher concentrations. This suggests that both DDB and amantadine may rapidly induce the IFN-α signal pathway, although IFNAR expression returns to normal levels with elapsed time. Considering the strong association between IFNAR expression and successful IFN-α therapy, we hypothesized a synergistic antiviral effect when these drugs are administered in combination.

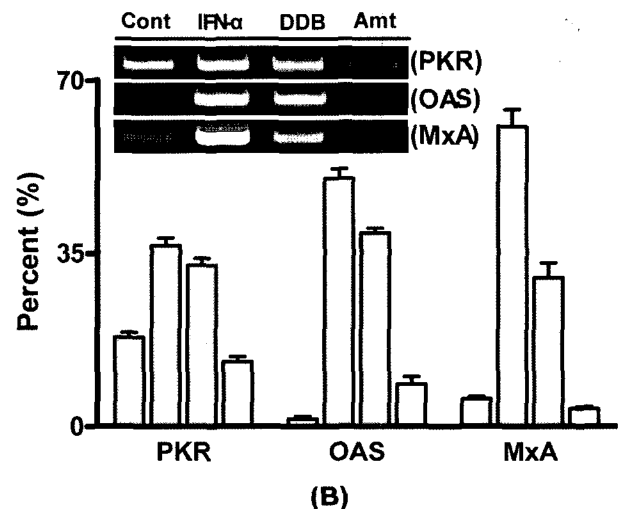
Gene expression over the interferon pathway

In order to confirm the theorized antiviral effects of DDB and amantadine, we conducted an investigation into the interferon-inducible responses which were discovered in a previous study (Joo and Lee, 2005). Primarily, we sought to determine whether the drugs stimulate 6-16 and ISG12 genes which were transcriptionally upregulated in a variety of cell types, including the HepG2 cell line, in response to IFN-α. We determined that the 6-16 and ISG12 genes (interferon stimulated gene) were both expressed intensely in the presence of either DDB or amantadine, whereas the control was expressed only weakly in ISG12 and was not detected in the 6-16 gene (Fig. 3A). These results are indicative of the notion that DDB and amantadine may stimulate the human 6-16 and ISG12 genes, and that the elevated expression of the 6-16 gene associated with DDB treatment might play an important antiviral role, just as IFN does. Moreover, upon comparison with the IFN-α-treated group, we uncovered compelling evidence to suggest that DDB and amantadine induced IFN-like responses.

Interferon-like antiviral responses

IFN-inducible gene expressions were examined in con-





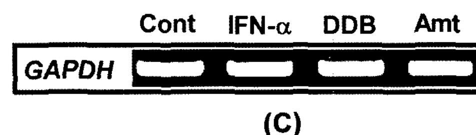


Fig. 3. IFN-α inducible genes and correlative antiviral effectors. Transcripts of the indicated human 6-16, ISG12, PKR, OAS and MxA genes were detected *via* RT-PCR. RNA was isolated 5 days after exposure to INF-α (1000 IU/mL), DDB (250 μg/mL), or amantadine (50 μg/mL). (A) The expression of 6-16 and ISG12 genes and (B) antiviral effectors (PKR, OAS and MxA) were compared in terms of percentage (%) reaching to the band density of a control. (C) PCR assays included, as an internal control, primers for GAPDH. Data are expressed as means ± SD.

junction with dsRNA-dependent enzymes, including protein kinase R (PKR), oligoadenylate synthase (OAS) and the MxA protein, all of which are known to be induced by IFN and to mediate antiviral activities. In order to confirm the IFN-like antiviral potencies of DDB and amantadine, we administered an INF-α treatment as a positive control,

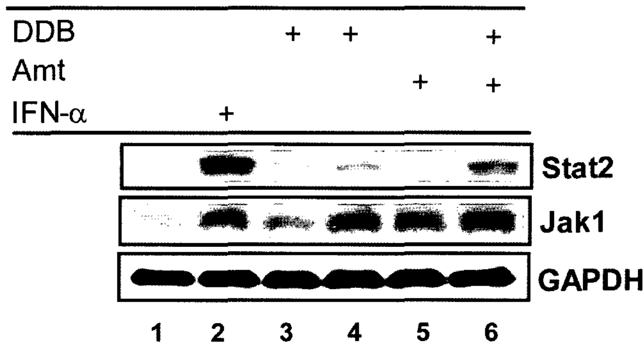


Fig. 4. Comparison of the efficacy of DDB and amantadine on Jak/Stat signaling. The cells were cultured for 5 days in the presence of either DDB (250 μg/mL), amantadine (50 μg/mL) or DDB/amantadine. IFN-α (1000IU) was added as a positive control, and compared with DDB and amantadine. The total cellular proteins (25 μg) were then extracted and subjected to Western blot analysis, as was described in the Materials and Methods section. Human polyclonal anti-GAPDH (1:10000) was used as an internal control.

and compared the efficacy of this treatment with the efficacy of the DDB and amantadine groups, with regard to levels of induced gene expression. Our RT-PCR analysis of IFN-inducible antiviral components to mRNA served to validate our previous report, namely that DDB coupled with amantadine generated an antiviral effect *via* IFN signal pathways (Joo and Lee, 2005). Fig. 3B shows that DDB and/or amantadine induced antiviral effects *via* the activation of at least three antiviral components, PKR, OAS, and MxA, as compared to what was observed with the IFN-treated group. These findings were further verified *via* an evaluation of the Jak/Stat signaling pathway via Western blot analysis. Fig. 4 demonstrates that DDB, rather than amantadine, regulates the IFN-induced Jak/Stat pathway to a degree commensurate with that of IFN-α, which stimulates Stat1 and Stat2, which become tyrosine-phosphorylated by Jak 1.

Inhibition of viral gene expression

In order to determine definitively whether DDB and/or amantadine effect a blockage of the posttranscriptional steps of HBV replicative intermediates, which are then prevented from facilitating the formation of viral proteins, we hypothesized that DDB and/or amantadine might inhibit the translation of HBV transcripts, such as pregenomic RNA (pgRNA), which is transcribed from covalently-closed-circular HBV DNA in the nuclei of hepatocytes (Nassal and Schaller, 1996), and thus may inhibit either the reverse transcription of encapsidated pgRNA into single-stranded DNA (ssDNA) or the maturation of ssDNA into double-stranded DNA (dsDNA). The results of this evaluation may imply a similarity of the antiviral response between DDB and/or amantadine and IFN-α. We determined that the

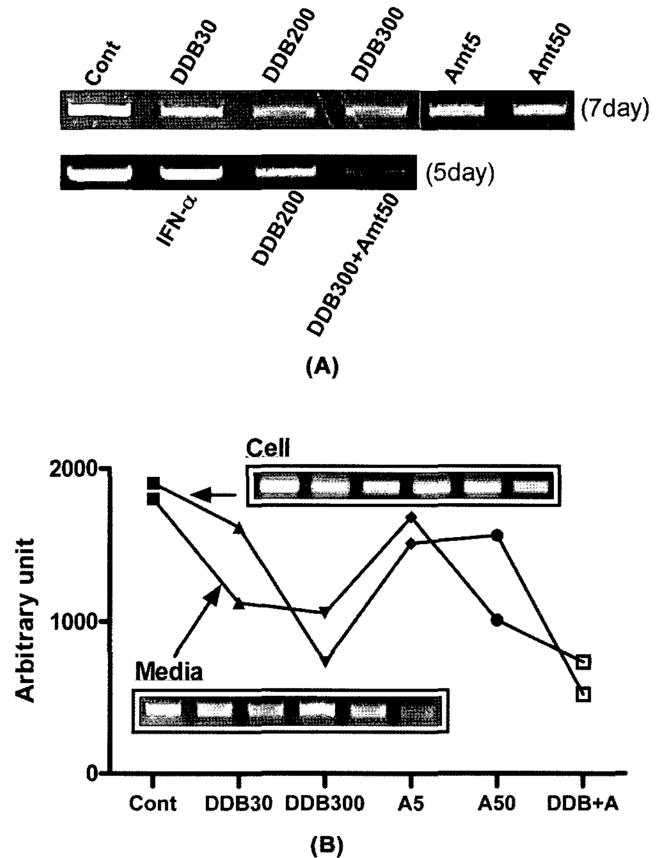


Fig. 5. The inhibition of pregenomic RNA and the suppression of HBV viral DNA. (A) pgRNA expression in the presence of DDB, amantadine, or DDB/amantadine. IFN-α was employed as a comparable positive control for the evaluation of antiviral effects. The cells were cultured at the indicated concentrations for 5 and 7 days, and the expression was detected *via* RT-PCR. (B) The suppression of the viral DNA isolated from cells and media. The cells were cultured for 7 days, and HBeAg DNA was evaluated *via* PCR at each concentration. The data represent the mean values of three different measurements.

transcription of pgRNA was dose-dependently inhibited by DDB (~250 μg/mL), and the degree to which this inhibition occurred was maximized under conditions including co-treatment with amantadine (Fig. 5). On day 7 of the DDB treatment, pgRNA was strongly inhibited at 300 μg/mL, whereas IFN-α did not effectively inhibit transcription.

CONCLUSION

The Hepatitis B virus (HBV) is a hepatotropic, noncytopathic DNA virus, which induces acute and chronic necroinflammatory liver disease, as well as hepatocellular carcinoma (HCC). For the last few decades, an effective vaccine has played a crucial role in the reduction of new infection rates. However, vaccination does not constitute a therapeutic modality for patients with type B hepatitis, and

rather is simply a tool for the prevention of new viral infections. Since the initial introduction of IFN monotherapy nearly 20 years ago, several therapeutic agents have been approved for the treatment of chronic hepatitis B, including lamivudine and adefovir. Regardless of their therapeutic efficacy, though these treatments are somewhat limited with regard to their general applications (Leung, 2002). IFN treatment is associated with severe flu-like symptoms in the majority of patients during the early stages of therapy, and nucleoside analogues have been significantly associated with viral resistance when applied over the long-term. Therefore, the primary objective of HBV therapy should be the reduction or amelioration of liver damage, either *via* the direct suppression of viral transcription, or indirectly, *via* the modulation of the host's immune responses through cytokines, possibly including IFN (Guidotti *et al.*, 1994). Thus, a truly viable treatment would be shorter, non-mutatory, effective for a longer time period, and non-complicative. Interestingly, IFN fulfills all of these criteria, although its expected efficacy is somewhat low, and it has been associated with some problematic side effects, as compared to other nucleoside analogues (Lim and Suh, 2004; Younger *et al.,* 2004). In a previous study, we uncovered a clue suggesting that DDB, an active compound obtained from *Schizandra fructus*, may share the IFN signaling pathway, and that its effects may be maximized when coupled with amantadine, an antiviral agent (Joo and Lee, 2005). As was determined in this study, we determined that DDB may interact with the IFN-α receptor, thus effecting the activation of the Jak/Stat pathway, which is indispensable to the induction of IFN-α signaling. In addition, we observed the expression of IFN-inducible genes, including 6-16 and ISG12, both of which are highly inducible by IFN-α (Gjermandsen *et al.*, 2000) and examined whether antiviral effectors such as PKR, OAS and MxA, played any significant antiviral roles. Over the entirety of the study, we discovered that DDB induced IFN-α signal transduction *via* its interactions with IFNAR (Fig. 2), and the regulation of IFNAR gene expression in the presence of DDB may constitute an additional antiviral potency, which has been significantly associated with the efficacy of IFN therapy (Morita *et al.*, 1998). Interestingly, both DDB alone and the combination of DDB and amantadine have been shown to stimulate Jak/Stat, a upstream signal pathway that induces ISG genes and antiviral effectors (Fig. 3,4). All of the signaling pathways associated with DDB contributed to an overall anti-HBV effect, thereby demonstrating that DDB inhibits the replication of pgRNA, which can be transcribed from covalently-closed-circular HBV DNA in the nuclei of hepatocytes, and is translated into core protein and polymerase, thus suppressing the formation of viral DNA (Fig. 5). Although the intensity of signaling responses, in both gene and protein analyses, were slightly weaker than those associated with IFN-α, we determined that DDB may exert an IFN-α-mimicking signaling effect, and may also exert an antiviral effect in the presence of DDB. These findings may provide us with some insight into the reasons that DDB is so profoundly associated with the removal of hepatitis indices, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Gao *et al.*, 2005), and may also provide us with the interesting conclusion that DDB constitutes an a novel anti-HBV therapeutic agent, without the mutation-inducing properties and severe side effects, but with an IFN-α-mimicking antiviral effects. Moreover, DDB, when applied in combination with amantadine, provided us with interesting information with regard to the theorized synergistic inhibitory effect against the replication of viral DNA (Fig. 5A). To date, no reports have been submitted to suggest that DDB induces mutations or flu-like symptoms after treatment, both of which would represent critically limiting factors for the general use of the agent, regardless of its evidenced antiviral prowess. This lack of contraindicatory evidence implies that DDB may, indeed, be a safe and effective antiviral agent, applicable in either short-term or longer-term therapeutic strategies. Also, the efficacy of DDB appears to increase in combination with amantadine. Therefore, in conclusion, DDB coupled with amantadine appears to effect a direct suppression of viral replication within infected hepatocytes, *via* the induced degradation of viral mRNA. This appears to constitute the principal mechanism underlying the observed antiviral effects of DDB, indicating that DDB can be used in the future in a clinical environment, as a safe and effective anti-HBV therapeutic agent. Regardless of the results generated in this study, however, more in-depth investigations into the action mechanism of this agent should be conducted, employing both *in vitro* and *in vivo* experimental designs.

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