

Deactivation of Akt and STAT3 signaling promotes apoptosis, inhibits proliferation, and enhances the sensitivity of hepatocellular carcinoma cells to an anticancer agent, Atiprimod

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Abstract

Atiprimod is a novel anticancer and antiangiogenic drug candidate which is currently being evaluated in patients with liver carcinoid and multiple myeloma. In this study, we report that atiprimod selectively inhibited proliferation and induced apoptosis in HCC cells that expressed either hepatitis B virus (HBV) or hepatitis C virus, through deactivation of protein kinase B (Akt) and signal transducers and activators of transcription 3 (STAT3) signaling. In HepG2 AD38 cells, which express HBV genome under the control of a tetracycline-off promoter, both Akt and STAT3 were constitutively activated in response to HBV expression. However, this constitutive activation was not sensitive to lamivudine, a drug that inhibits HBV replication without affecting its gene expression, suggesting that HBV replication per se might not be responsible for the activation. Interestingly, the electrophoretic mobility of p-STAT3 protein bands on immunoblot was slower when AD38 cells were cultured in the absence of tetracycline, suggesting a differential phosphorylation in response to HBV expression. In HCC cells, interleukin 6 stimulates the phosphorylation of STAT3 both at serine 727 and at tyrosine 705 positions. The interleukin 6-stimulated activation of STAT3 and Akt was inhibited not only by atiprimod but also by LY294002, a phosphoinositide-3-kinase-specific inhibitor, and by NS398, a cyclooxygenase-2-selective inhibitor. The combination of these

compounds did not produce any additive effect, implying that the mechanisms by which HBV activates Akt and STAT3 might also involve phosphoinositide-3-kinase and cyclooxygenase-2. Collectively, these results suggest that atiprimod could be useful as a multifunctional drug candidate for the treatment of HCC in humans. [Mol Cancer Ther 2007;6(1):112–21]

Introduction

Hepatocellular carcinoma (HCC), which accounts for >90% of all primary liver cancers, is one of the most common causes of cancer mortality, with a median survival time from the date of diagnosis of 7 to 8 months (1, 2). The potentially curative therapies are liver resection, transplantation, and a few pharmaceutical interventions. However, hepatic resection and liver transplantation in HCC are limited by the fact that most patients have underlying cirrhosis (1), which makes these therapies inappropriate options. Chronic infections of hepatitis B virus (HBV) and hepatitis C virus (HCV) are closely associated with liver diseases and with HCC in humans (3, 4). Thus, there is an intense interest to understand the roles of viral proteins in causing liver cirrhosis and HCC. Among the viral proteins, the HBx protein of HBV and the NS5A protein of HCV have drawn considerable attention because of their activating effects on cellular signaling pathways, such as mitogen-activated protein kinase, *c-Jun* NH₂-terminal kinase, and Src tyrosine kinase (5–7). Because these signaling pathways are known to be ubiquitously involved in the processes leading to carcinogenesis and tissue inflammation, it is possible that HBV/HCV might mediate their pro-HCC activities via one or more of these pathways.

The phosphoinositide-3-kinase (PI-3K)/protein kinase B (Akt) pathway is a crucial regulator of a number of cellular processes including proliferation, differentiation, and metastasis, and up-regulation of this pathway through the phosphorylation of Akt has been documented as a frequent occurrence in several human cancers (8). Several studies have shown that activation of PI-3K/Akt signaling can inhibit apoptosis via the disruption of p53-mediated apoptosis, inactivation of caspase-9 and Bad, and also via the suppression of the death receptor-mediated apoptosis (9). Moreover, the activation of Akt also correlated well with the loss of the tumor suppressor gene, *PTEN*, a negative regulator of the PI-3K/Akt pathway in many types of cancers (10). In addition, both the NS5A protein of HCV and the HBx protein of HBV have also been shown to activate a variety of cellular kinases and transcriptional factors that are known to associate with oncogenic

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transformation (11–14). Chronic infections of HBV and HCV are also associated with the increased production of reactive oxygen species, which is commonly linked with activations of a wide range of protumorigenic kinases and transcriptional activators (15, 16). Collectively, these findings implicate a potential role of hepatitis virus-induced oxidative stress in HCC development.

Atiprimod [*N,N'*-diethyl-8,8-dipropyl-2-azaspiro(4,5)-decane-2-propanamine; dimaleate salt] is a novel orally bioavailable cationic amphiphilic agent which has been studied for its antiinflammatory and anticancer properties. Previously, we reported that atiprimod induces apoptosis via the activation of caspase-3 and caspase-9, inhibits the proliferation of a wide range of human cancers, and retards angiogenesis (17, 18), and the compound was found to be efficacious in animal models for human xenografts of multiple myeloma (19–21). Atiprimod is currently being evaluated in patients with multiple myeloma and in liver carcinoid. Early clinical observations revealed a clear-cut efficacy response in patients with advanced liver carcinoid cancer (22). Despite these extensive preclinical and clinical studies, the mechanism of action of atiprimod still remains to be determined. Here, we report that atiprimod selectively inhibits proliferation and induces apoptosis in HCC cells via inhibiting constitutive activation of Akt and signal transducers and activators of transcription 3 (STAT3) signaling pathways.

Materials and Methods

Materials

HCC cell lines (Huh-7, HepG2, HepG2.2.15, and HepG2) were obtained from American Type Culture Collection (Rockville, MD) and routinely cultured in our laboratories. AD38, a variant of HepG2 cells (23), T cells (a variant clone of Huh-7 cells), and G54 cells expressing HCV replicon, were generous gifts from Dr. Pamela Norton and Dr. Xuanyang Lu (Drexel Institute of Biotechnology and Viral Research), respectively. Atiprimod was obtained from Callisto Pharmaceuticals, Inc. (New York, NY). Antibodies (Akt, pS473Akt, p-T308-Akt, and α -tubulin) were purchased from Cell Signaling Technology (Beverly, MA) and antibodies for STAT proteins were purchased from Upstate Biotechnology (Lake Placid, NY). Akt and STAT3 CASE assay kits were obtained from SuperArray Biosciences, Corp. (Frederick, MD). Culture media and the heat-inactivated fetal bovine serum were obtained from Invitrogen (Carlsbad, CA).

Cell Cultures

HCC cells (HepG2, HepG2.2.15, AD38, T, and G54) were cultured in a 1:1 mixture of Ham's F-12 medium and DMEM supplemented with 10% fetal bovine serum. Cells were fed fresh medium every 3rd day and split by trypsinization at a confluence of ~80%. Culture media for HepG2.2.15 and G54 also contained 200 μ g/mL of G418 for maintenance of the viral load. The AD38 cell culture medium also contained tetracycline (1 μ g/mL) when requiring the expression of HBV genes. Penicillin and streptomycin (100 units/mL each) were added in all culture media.

Proliferation Assay

The effect of atiprimod on the proliferation of HCC cells was measured by the conversion of WST-1 dye to Formazon by using a proliferation kit from Roche Diagnostics (Indianapolis, IN). The procedure used was essentially the same as that described in ref. 17.

DNA Fragmentation Assay

The DNA fragmentation assay to measure the effect of atiprimod on the induction of apoptosis in HepG2 and HepG2.2.15 cells was carried out using the procedure described in ref. 17. The apoptotic DNA fragments were separated on 1.5% agarose gel electrophoresis followed by staining with ethidium bromide for visualization.

Colony Formation on Soft Agar

The colony-forming ability of HepG2 and HepG2.2.15 cells was evaluated by using the soft agar culture assay. Briefly, 3 mL of 0.6% ultrapure agarose in culture medium containing the indicated concentration of test compounds was poured in 35 mm six-well plates. After the bottom agar was solidified, ~20,000 cells in 1 mL of agar (0.3%) in culture medium containing the indicated concentrations of test compound were layered on top of the bottom layer. Cells were fed every 4 to 5 days by adding a new layer of top agar and plates were incubated for 2 weeks for cell colonies to appear. Colonies containing >50 cells were scored under the microscope.

Caspase-3/7 Assay

The activities of caspase-3/7 were measured using the Caspase-Glo 3/7 kit (Promega Corp., Madison, WI). Briefly, cells were grown in 96-well plates until the semiconfluency stage and then treated with the indicated concentrations of atiprimod or vehicle for 16 h in culture medium containing 2% fetal bovine serum. Plates were removed from the incubator and kept at room temperature for 30 min and the Caspase-Glo 3/7 reagent (100 μ L) was added. Plates were further incubated on a shaker at room temperature for 2 h and read using a luminometer.

Cell-Based Assays for Akt and STAT3 Phosphorylation

The procedure used was essentially the same as the manufacturer's instructions (SuperArray Biosciences, Frederick, MD) with some minor modifications. Briefly, 10,000 to 20,000 cells were seeded in 96-well plates and cultured for 2 days, with a period of serum-deprivation of 16 h, and then treated with test compounds for 24 to 48 h in a cell culture incubator. In some experiments, cells were stimulated with interleukin 6 (IL-6; 50 ng/mL) or hepatocyte growth factor (100 ng/mL) to induce Akt and STAT3 phosphorylation. Following the treatments, cells were fixed with 8% formaldehyde for 30 min and processed for ELISA measurements with anti-pS473-Akt or anti-Akt antibodies. The bound antibodies were then detected with anti-mouse acetylcholine esterase conjugate, followed by color development. Plates were read in an ELISA reader (Molecular Dynamics, Sunnyvale, CA). After washing thrice with PBS to remove immunocomplexes, the plates were stained with crystal violet, and readings were taken to assess the total number of cells per well. Data were normalized for the

number of cells in each well. Essentially, the same cell-based assay was used for STAT3 phosphorylation, except that ELISA was developed using either the anti-pY705-STAT3 or the anti-STAT3 antibodies that were provided in the kit.

Preparation of Cell Lysates

Cells were washed with fresh serum-free medium and starved for 16 h and treated with fresh serum-free medium containing the test compounds for 24 to 48 h (37°C/5% CO₂). In some experiments, cells were treated with IL-6 or growth factors 60 min prior to harvesting. Cells were scraped and suspended in chilled PBS and centrifuged (3,000 × *g*/3 min). Pellets were resuspended in 200 to 300 μL of lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 10% glycerol, 1% NP40, 10 mmol/L NaF, 2.5 mmol/L sodium PPI, 1 mmol/L sodium orthovanadate, and 1 mmol/L EGTA]. A final concentration of leupeptin (5 μg/mL), pepstatin (5 μg/mL), 1 mmol/L each of DL-norleucine and phenylmethylsulfonyl fluoride was added to the lysis buffer just prior to its use. After an incubation of 30 min on ice, the cell suspension was centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatants were collected and immediately stored in aliquots at -80°C for immunoblotting analyses. The protein contents in cell lysates were determined using BCA protein assay (Pierce Chemical Co., Rockford, IL).

Immunoblotting

For immunoblotting, cell lysate samples (50–100 μg total proteins) were mixed with equal volumes of SDS-PAGE buffer (2×) containing β-mercaptoethanol, boiled for 2 min, and loaded on 7.5% or 10% SDS-PAGE gels. The conditions for protein electrophoresis and for transfer of protein bands on polyvinylidene difluoride membranes were the same as described in ref. 24. The procedures used for immunoblotting were the same as described in ref. 25. Antibodies used for immunoblotting were diluted as follows: Akt, 1:2,000; pS473-Akt, 1:3,000; pT308-Akt, 1:2,000; STAT3, 1:2,500; pY-STAT3, 1:2,500; and α-tubulin, 1:1,000. The bound primary antibody was detected by either anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase, followed by detection with an enhanced chemiluminescence reagent (GE Healthcare Biosciences, Corp., Piscataway, NJ). Polyvinylidene difluoride membranes were often reprobated a couple of times with other antibodies according to the instructions of the manufacturer.

Results

Atiprimod Inhibits the Proliferation of HCC Cells

The antiproliferative activity of atiprimod was evaluated against three different HCC cell lines: HepG2, HepG2.2.15, and Huh-7 cells with an assay that used the conversion of WST-1 dye to Formazon (17). As shown in Fig. 1A, atiprimod inhibited the proliferation of Huh-7, HepG2, and HepG2.2.15 cells with an IC₅₀ value ranging between 0.5 and 1.5 μmol/L. Interestingly, HepG2.2.15 cells that consistently produce HBV virus seemed to be considerably more sensitive to atiprimod as compared with the non-viral-producing HepG2 and Huh-7 cells. Similarly, atiprimod inhibited colony formation by HepG2.2.15 more effectively compared with HepG2 (Fig. 1B).

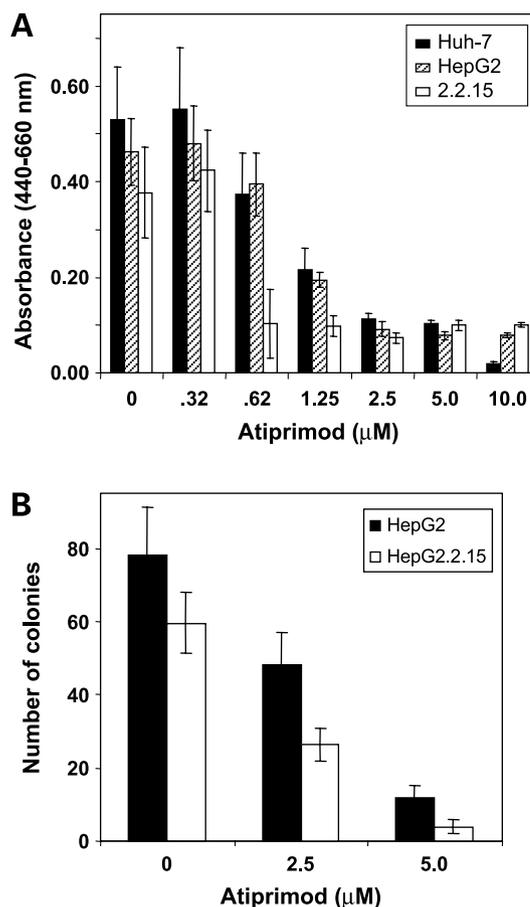


Figure 1. Atiprimod preferentially inhibits the proliferation of HBV-expressing HCC cells. **A**, HCC cells (Huh-7, HepG2, and HepG2.2.15) were cultured overnight in 96-well plates in their respective media. After the cells were attached to the bottom of wells, the indicated concentrations of atiprimod were added and cells were cultured for 72 h. Ten microliters of WST-1 dye was added and readings were recorded at 440 to 660 nm. Columns, mean average of three determinations; bars, SE. **B**, atiprimod inhibits colony formation on soft agar. Approximately 20,000 cells were mixed with 1 mL of 0.3% ultrapure agar in culture media and plated gently on top of the solidified media (ultrapure agar 0.6%). The indicated concentrations of atiprimod were added in both the top and the bottom media and cells were fed every 4 to 5 d by adding a new layer of the top agar. After 2 wks, colonies containing >50 cells were scored using a microscope. Columns, mean average three determinations; bars, SE.

To address the possibility that the expression of HBV might be responsible for the increased sensitivity of HepG2.2.15 cells towards atiprimod, we evaluated the effect of atiprimod on the proliferation of AD38 cells (Fig. 2A), which express complete HBV genome under the control of a tetracycline (Tc)-off promoter (23). In this cell line, the expression of HBV genes is suppressed in the presence of Tc. However, in the absence of Tc, these cells produce 3.5 kb pregenomic RNA of HBV that serves as the mRNA for translation to produce the viral proteins. Atiprimod inhibited the proliferation of AD38 cells irrespective of the presence or absence of Tc, albeit the

inhibition in proliferation was more pronounced in the Tc- culture. Similarly, atiprimod inhibited the proliferation of G54 cells, which express HCV replicon, more effectively than the parental T cells. Taken together, these results suggested that the preferential antiproliferative activity of atiprimod might be due to the expression of HCV/HBV viruses.

Atiprimod Induces Apoptosis

The proapoptotic activity of atiprimod was evaluated using a DNA fragmentation assay and also by measuring the activation of caspase-3/7 (Fig. 3), two hallmarks for the induction of apoptosis. As shown, treatment of (a) HepG2 and (b) HepG2.2.15 cells with atiprimod resulted in the formation of distinct DNA ladders, indicative of the induction of apoptosis. The induction of apoptosis was further supported by measuring the activation of caspases-3/7 in cells treated with atiprimod. During the preliminary standardization of the assay, we found that the optimum treatment time for atiprimod to induce caspase-3/7 in

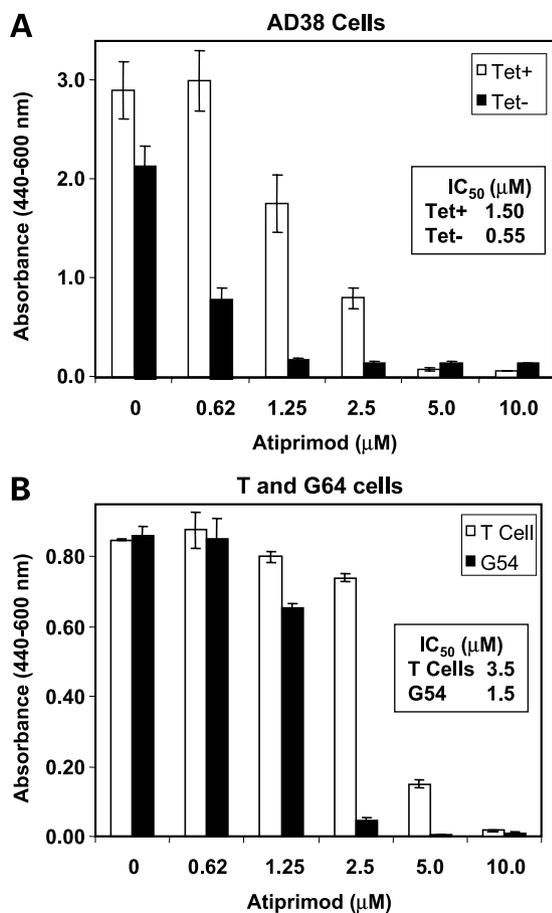


Figure 2. Atiprimod preferentially inhibits proliferation of HBV- and HCV-expressing cells. The proliferations of HBV- and HCV-expressing cells were measured using WST-1 conversion assay. **A**, AD38 cells were cultured with (Tet+) or without (Tet-) tetracycline. **B**, the parent Huh-7 T cells and the G54 cells that expressed the HCV replicon were cultured in their respective media. Columns, mean average of three determinations; bars, SE.

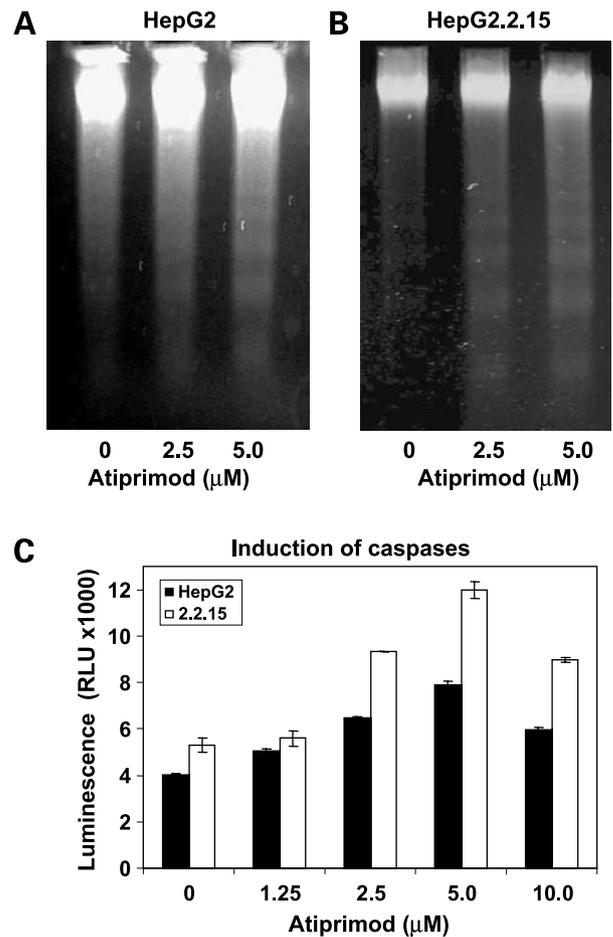


Figure 3. Atiprimod-induced apoptosis in HepG2 and HepG2.2.15 cells. **A** and **B**, cells were cultured in 100 mm dishes for 3 d and then treated with atiprimod for 48 h with the indicated concentrations of atiprimod in culture medium containing 2% fetal bovine serum. The nucleosomal DNA was isolated and DNA fragments were separated by 1.5% agarose gel electrophoresis. **C**, atiprimod activates caspases-3/7 in HCC cells. Cells were cultured in 96-well plates until they achieved semiconfluency and then treated with the indicated concentrations of atiprimod for 16 h. The Caspase-Glo 3/7 reagent (100 μL) was added directly to the wells and plates were incubated for 1 h before recording the luminescence reading. Each value represents the average of four wells. The "no cell" blank control value was subtracted from each reading. Columns, mean average of four determinations; bars, SE.

HepG2 and HepG2.2.15 was between 16 and 21 h (data not shown). Thus, the semiconfluent monolayer of cells was treated with atiprimod for 16 h to measure the induction of caspase-3/7. Although atiprimod increased the activities of caspase-3/7 in both cell lines, the activation of caspase-3/7 was more prominent in HepG2.2.15. The caspase-3/7 activity in cells treated with the 10 $\mu\text{mol/L}$ concentration was lower, probably due to the cytotoxic effect of atiprimod at this concentration.

Activation of Akt and STAT3 Is Constitutive and PI-3K – Dependent

The phosphorylation of Akt represents one of the key events in the survival of cells exposed to different apoptotic stimuli such as serum deprivation, DNA damage, and viral

infections, etc. (8). Hence, we determined the levels of Akt and phosphorylated-Akt by immunoblotting. The semi-confluent monolayers of HepG2 (Fig. 4) and HepG2.2.15 (Fig. 5) were serum-deprived for 16 h, a condition in which the expression of phosphorylated-Akt is often diminished. After serum-deprivation, cells were treated with atiprimod or vehicle for 24 h in serum-free medium, and the levels of Akt and phosphorylated-Akt in cell lysates were determined. Atiprimod inhibited the phosphorylation of Akt at S473 in a dose-dependent manner, but not significantly at the T308 position (Figs. 4A and 5A). These results were further confirmed by a quantitative ELISA that only measures Akt phosphorylation at the S473 position (Figs. 4B and 5B). Atiprimod inhibited the phosphorylation of Akt in HepG2 and HepG2.2.15 cells.

Because PI-3K is known to be involved in Akt activation, we evaluated the effect of LY294002, a widely used specific inhibitor of PI-3K, on Akt phosphorylation. In this set of

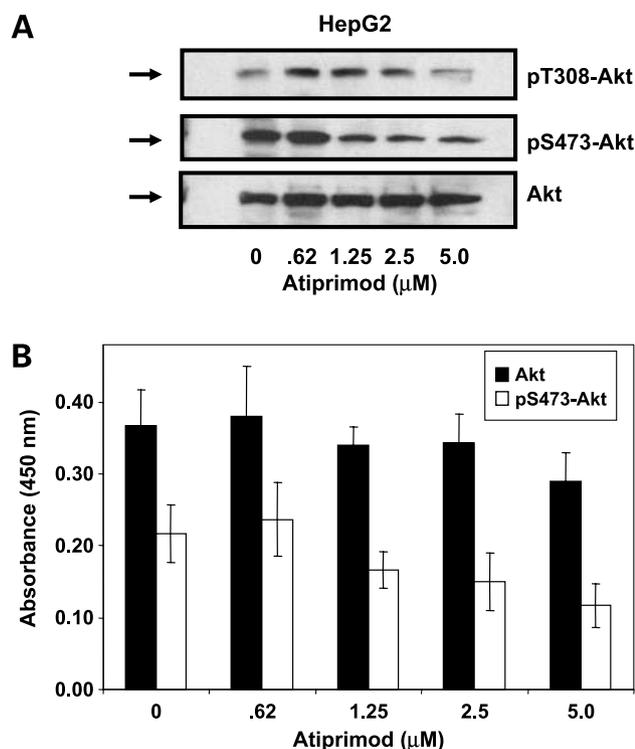


Figure 4. Atiprimod inhibited Akt phosphorylation in HepG2 cells. **A**, immunoblotting analysis: HepG2 cells were cultured in 100 mm plates until they reached semiconfluency. After serum-deprivation for 16 h, cells were treated with the indicated concentrations of atiprimod for 24 h and the cell lysates were subjected to reducing SDS-PAGE followed by electrophoretic transfer to Immobilon polyvinylidene difluoride membranes. The membranes were used for immunoblotting with antibodies specific to either Akt or p-S473-Akt or pT308-Akt. Membranes were reprobbed with other antibodies for at least a couple of times. **B**, approximately 15,000 cells were seeded in each well of a 96-well plate and cultured for 24 h. Following serum-deprivation for 16 h, the cells were treated with the indicated concentrations of atiprimod for 24 h. Levels of Akt and p-S473-Akt were determined using the CASE assay kit (Super-Array Biosciences). Crystal violet staining was used to assess the number of cells in each well and the data were normalized. *Columns*, mean average of three determinations; *bars*, SE.

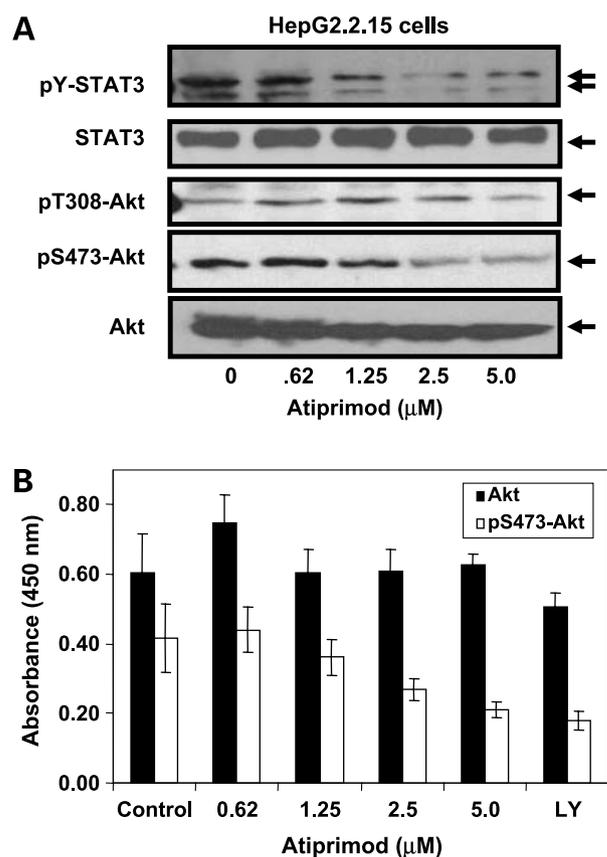


Figure 5. Atiprimod inhibited the phosphorylation of Akt and STAT3 in HepG2.2.15 cells. **A**, immunoblotting analysis: cells were grown to semiconfluency, followed by serum starvation (16 h) and treated with atiprimod for 24 h. The cell lysates were used for immunoblotting with antibodies specific to Akt, pS473-Akt, pT308-Akt, STAT3, and pY705-STAT3. **B**, approximately 15,000 cells were cultured in each well of a 96-well plate and cultured for 24 h, followed by a serum-starvation period of 16 h. Cells were treated with the indicated concentrations of atiprimod and used in ELISA using antibodies specific for STAT3 and pY705-STAT3. *Columns*, mean average of three determinations (results were normalized for cell numbers); *bars*, SE.

experiments, we used 50 μmol/L of LY294002, a concentration known to completely inhibit PI-3K activity in HCC cells (13). As expected, the treatment of HepG2.2.15 cells with LY294002 inhibited the phosphorylation of Akt at the S473 residue (Fig. 5B). However, neither LY294002 nor atiprimod significantly affected the levels of native Akt, indicating that the inhibition in phosphorylation at S473-Akt residue was not due to a nonspecific effect.

The Janus-activated kinase (JAK)-STAT signaling pathway is known to be a major cascade associated with signal transduction for many cytokines and growth factors (26). Thus, we evaluated the effect of atiprimod treatment on the phosphorylation of STAT3 in HepG2.2.15 cells. The semi-confluent monolayer of HepG2.2.15 was serum-deprived, treated with the indicated concentrations of atiprimod, and cell lysates were used for immunoblotting (Fig. 5A). The expression of pY705-STAT, a doublet of protein bands, was detected even in serum-deprived conditions, suggesting its

constitutive activation in HepG2.2.15 cells. Atiprimod inhibited the phosphorylation of STAT3 in a dose-dependent manner without affecting the levels of the native STAT3, suggesting that the constitutive activation might be due to either HBV replication and/or due to viral gene expression.

HBV Replication Is Not Involved in the Constitutive Activation of Akt

To examine if HBV replication was associated with the activation of Akt, we used lamivudine, a drug that inhibits HBV replication without affecting the translation of the 3.5 kb pregenomic RNA into viral proteins, to inhibit HBV replication in HepG2.2.15 cells. However, treatment with lamivudine did not show any effect on Akt phosphorylation (Fig. 6). In fact, cells seemed to grow slightly faster when lamivudine (2 $\mu\text{g}/\text{mL}$) was added in the culture medium. Similarly, lamivudine treatment did not affect the activation of STAT3 (data not shown). These results are consistent with a previous report that the expression of viral proteins, and not the HCV/HBV replication, is involved in Akt phosphorylation (27, 28).

HBV Gene Expression Activates Akt and STAT3

This set of experiments were done in AD38 cells in which HBV gene expression could be modulated by the presence or absence of Tc in the culture medium. In vehicle-treated cells, the ratio of pS473-Akt/Akt was markedly higher in Tc- (0.55), as compared with that in Tc+ (0.38) culture of vehicle-treated cells (Fig. 7). Atiprimod inhibited the phosphorylation of Akt at S473 residue irrespective of the presence or absence of Tc in the culture medium. However, the inhibition was more distinct when cells were cultured in the absence of Tc (Fig. 7A and B). Similarly, immunoblotting of cell lysates also showed more pronounced

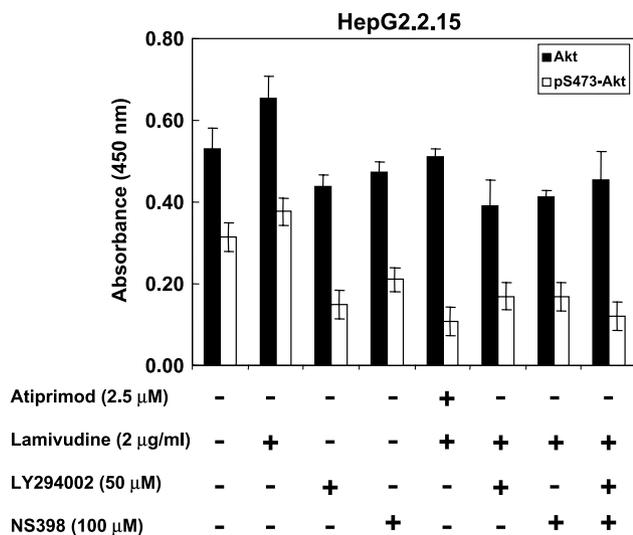


Figure 6. Phosphorylation of Akt is not dependent on HBV replication in HepG2.2.15 cells. Approximately 15,000 cells were cultured, serum-starved, and treated with test agents, as indicated, and used for ELISA. The procedure used was the same as described in Fig. 4. Columns, mean average of three determinations (data were normalized for cell numbers); bars, SE.

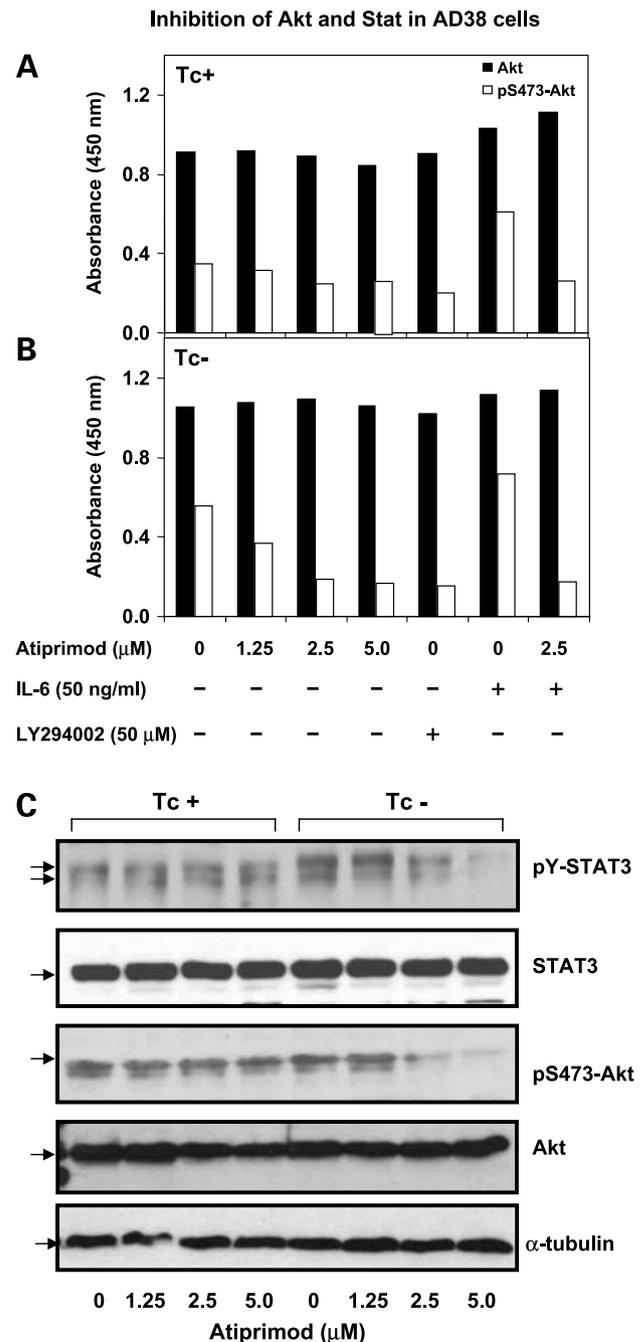


Figure 7. Atiprimod inhibits phosphorylation of Akt and STAT3 in AD38 cells. The AD38 cells were cultured for 4 d in the presence (A) or absence (B) of Tc. After the culture period, cells were serum-starved for 16 h and then incubated with serum-free medium containing the indicated concentrations of atiprimod or LY294002 (50 $\mu\text{mol}/\text{L}$) for 24 h. In some wells, IL-6 (50 ng/ml) was added 60 min prior to the ELISA. Results were normalized for cell numbers and are expressed as an average of duplicates. C, for immunoblotting, cells were cultured in 100 mm dishes with Tc- or Tc+ containing media for 4 d, serum-starved for 16 h, and then treated with the indicated concentrations of atiprimod for 24 h. Cell lysates were used for 10% SDS-PAGE under reducing conditions, followed by immunoblotting with the respective antibodies, as indicated. Membranes were reprobbed with α -tubulin and other antibodies.

inhibition of Akt phosphorylation by atiprimod in the Tc- culture (Fig. 7C). Furthermore, the levels of pS473-Akt increased ~2-fold upon stimulation with IL-6, and this IL-6-stimulated activation of Akt was completely abolished when cells were pretreated with atiprimod (Fig. 7B).

Next, we examined the levels of STAT3 and p-Y705-STAT3 by immunoblotting of cell lysates (Fig. 7C). In the Tc+ culture condition, AD38 cells showed basal levels of pY705-STAT3 protein bands. However, this doublet of protein bands increased markedly when cells were cultured in the absence of Tc, and this up-regulation was completely abolished upon treatment with atiprimod. Interestingly, the electrophoretic mobility of the protein doublet seemed to be slightly reduced in Tc- cultures, suggesting differential phosphorylation in response to HBV expression.

The serum levels of IL-6 and hepatocyte growth factor were higher in various liver disease states, such as in hepatitis, cirrhosis, and HCC (29, 30). In addition, IL-6 is also known to activate the JAK-STAT pathway via Y705 phosphorylation of STAT3. Hence, we examined the ability of IL-6 and hepatocyte growth factor to stimulate STAT3 phosphorylation in HepG2 and HepG2.2.15 cells (Fig. 8). As expected, the level of pY705-STAT3 was increased in HepG2.2.15 cells, as compared with that in HepG2 cells. However, IL-6 stimulated STAT3 phosphorylation equally in both cell lines. Nevertheless, the IL-6-stimulated phosphorylation of STAT3 was completely abolished when cells were pretreated with either atiprimod or with LY294002. Treatment with hepatocyte growth factor did not show any statistically significant effect on the phosphorylation of STAT3 in either of the cell lines.

Role of Cyclooxygenase-2 in the Activation of Akt and STAT3

Overexpression of cyclooxygenase-2 (COX-2) has been documented in human HCC tissues (31), and this up-regulation correlated very well with Akt phosphorylation in HCC cells as well as in HCC tissues (32). In addition, HBx expression has also been shown to activate COX-2 in HCC cells (33). Hence, we examined the possibility that COX-2 might be involved in Akt activation in HepG2.2.15 cells. In this set of experiments, we used NS398 at a concentration (100 $\mu\text{mol/L}$) that inhibited ~60% of the total prostaglandin E₂ production in HepG2.2.15 cells (data not shown). As shown in Fig. 6, treatment with NS398 reduced the phosphorylation of Akt, indicating that phosphorylation might be dependent on COX-2 activity. This is consistent with the finding that celecoxib, a COX-2-selective inhibitor, blocked the activation of Akt in prostate cancer cells (34).

It is known that COX-2 is involved in IL-6-mediated activation of STAT3 (35). Thus, we examined the effect of NS398, atiprimod, and LY294002 on the phosphorylation of STAT3 in HepG2.2.15 cells (Fig. 9). As expected, IL-6 increased the phosphorylation of STAT3, which was completely abolished when cells were pretreated with atiprimod, LY294002, and NS398, either alone or in combination. The results, showing that the combination of

these compounds did not confer any additive effect, suggests a common mode of action for these compounds to inhibit STAT3 phosphorylation.

Discussion

HCC is one of the most common malignancies in Asia and its incidence is rapidly increasing in Europe and in the U.S. (1, 2), yet there are no effective drugs for prevention and/or for therapeutic measures. Earlier, we reported that atiprimod preferentially inhibited the proliferation of metastatic cells as compared with the nonmetastatic cells from the same tumor or tissue types (17). The antiproliferative activity of atiprimod is not due to a nonspecific cytotoxic effect because the compound did not affect the proliferation of freshly isolated lymphocytes at concentrations up to 10 $\mu\text{mol/L}$ (19). Atiprimod has also been shown to retard the growth of human multiple myeloma xenografts in nude mice (20, 21). In one phase I clinical trial, atiprimod showed a clear-cut efficacy response in patients with liver carcinoid tumors (22). In this study, we show for the first time that atiprimod preferentially inhibits proliferation and induces

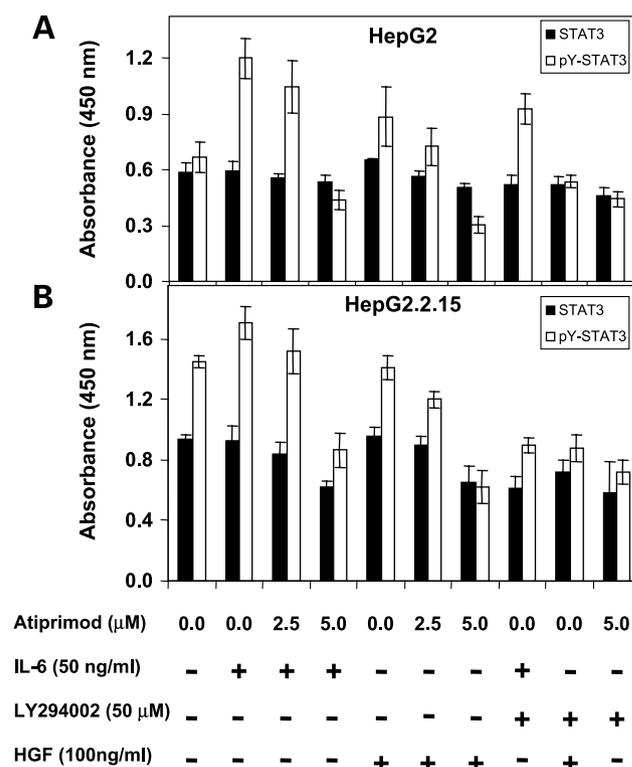


Figure 8. Atiprimod inhibits the IL-6-stimulated phosphorylation of STAT3 in HCC cells. Approximately 20,000 cells, (A) HepG2 and (B) HepG2.2.15, were seeded in each well and cultured for 3 d, followed by serum-deprivation for 16 h and then treated with either atiprimod or LY294002 for 24 h. IL-6 or hepatocyte growth factor were added 60 min prior to harvesting to stimulate STAT3 phosphorylation. Levels of total STAT3 and p-Y705-STAT3 were measured using the ELISA as described before. Columns, mean average of three determinations (data were normalized for cell numbers); bars, SE.

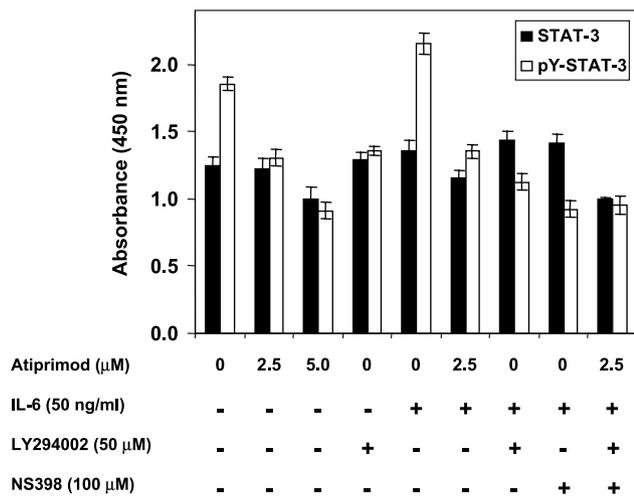


Figure 9. STAT3 activation is dependent on COX-2 and PI-3K in HepG2.2.15 cells. The procedure used was the same as that described in Fig. 8, except that cells were treated with LY294002, NS398, and atiprimod either alone or in combination. Some wells were stimulated with IL-6 for 60 min. The levels of total STAT3 and p-Y705-STAT3 were measured using the CASE assay as described previously. Columns, mean average of three determinations (data were normalized for cell numbers); bars, SE.

apoptosis in HCC cells that express HBVs or HCVs. Although the precise mechanism is still not clear, our results show that the preferential antiproliferative activity of atiprimod might be through the deactivation of PI-3K/Akt and JAK-STAT3 pathways.

One of the possible explanations for the preferential antiproliferative activity of atiprimod towards HBV-expressing cells could be that the intracellular accumulation of viral proteins might be sensitizing cells to atiprimod. For example, it is very well known that the level of HBx protein expression is crucial to determine the fate of cells. Low levels of HBx expression have been shown to sensitize cells to proapoptotic agents, including the chemotherapeutic drugs (36). By contrast, the overexpression of HBx triggers apoptotic death (37). In fact, expression of HBx protein is hardly detectable in the hepatocytes of infected patients (37), and the expression of HBx was found to be extremely low in the liver tissues from infected woodchuck (38). A low level of HBx expression, during replication of HBV in tissue cultures, has also been shown to cause proapoptotic effects (39). Thus, it is possible that the low level of HBx expression might sensitize the HBV-expressing cells to atiprimod. A similar phenomenon might also explain the preferential antiproliferative activity of atiprimod towards HCV-expressing cells. In this regard, the NS5A protein of HCV is similar in many ways to HBx in its cellular activities (6, 14).

Our results also show the constitutive activation of Akt in serum-starved HCC cells, and that this activation was inhibited by atiprimod. However, interestingly, atiprimod preferentially inhibited the phosphorylation of Akt at S473 but not significantly at T308. This discrepancy could be

attributed to the fact that two different kinases, PDK1 and PDK2, are responsible for the phosphorylation of Akt at T308 and S473, respectively (40). PDK1 has been cloned and sequenced (40). However, the mechanism by which S473 undergoes phosphorylation remains obscure. It has been proposed that S473 might be phosphorylated by PDK2, a kinase that has been characterized biochemically but its molecular identity still remains to be determined (41). We have not yet determined if atiprimod also inhibits any of these PDKs. Although our results clearly show that LY294002 inhibited the phosphorylation of Akt, presumably via inhibition of PI-3K, we have yet to determine if atiprimod directly inhibits PI-3K activity. On the other hand, it is also possible that when cells are preincubated with atiprimod, the expression of PI-3K is somehow reduced, resulting in the reduced phosphorylation of Akt. Alternatively, the possibility that the reduced phosphorylation of Akt at S473, following treatment with atiprimod, could be due to the activation of PTEN, a negative regulator of Akt phosphorylation, has not been completely excluded.

Chronic infections with HBV or HCV have been shown to produce oxidative stress, which is known to activate transcriptional activators such as nuclear factor κ B, activator protein 1, nuclear factor of activated T cells, STAT3, and others (6). In addition, the transgenic expressions of HBx or NS5A in HCC cells have also been shown to activate some of these transcriptional activators, possibly through oxidative stress (6, 27). This is further supported by recent findings that the glutathione level and activities of reduced glutathione-dependent enzymes in the liver tissue of patients with cirrhosis and HCC were severely impaired (42). In addition, an overwhelming number of reports suggest definitive roles for reactive oxygen species in viral pathogenesis, inflammatory diseases, and malignancies (6, 15). Previous studies have also shown that reactive oxygen species can stimulate the phosphorylation of several kinases that are also induced upon HCV infection (43). Thus, it is also possible that oxidative stress might be one of the mechanisms responsible for the constitutive activation of Akt and STAT3 in HBV/HCV-expressing

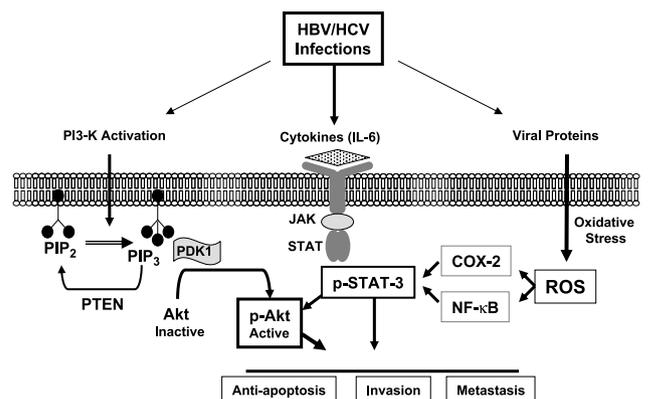


Figure 10. Model illustrating possible mechanisms involved in HBV/HCV-induced HCC.

HCC cells. Consistent with this notion, it has been shown that the HBx-mediated activation of STAT3 and other transcriptional factors were sensitive to antioxidants, implying the involvement of oxidative stress in their activations (43, 44).

The JAK-STAT signaling pathway is known to be a major cascade responsive to the stimulation of many growth factors and cytokines, and this pathway is also involved in promoting cell growth (26). Our results show that STAT3 was constitutively activated in HBV-expressing HepG2.2.15 and AD38 cells, and this activation was completely abolished when cells were treated with atiprimod. Interestingly, HBV expression in AD38 cells altered the electrophoretic mobility of pS473-STAT protein bands, suggesting a differential phosphorylation of STAT3. Phosphorylation of STAT3 has been shown to occur both at the tyrosine 705 (Y705) and at the serine 727 (S727) residues (45). Although phosphorylation at the Y705 position is essential for the receptor-associated Jak-mediated activation of STAT3, the phosphorylation at S727 is mediated by several converging kinases, including mitogen-activated protein kinase, p38, *c-Jun* NH₂-terminal kinase, and protein kinase C δ (46). More importantly, S727 phosphorylation has also been shown to be a negative modulator of Y705 phosphorylation (46), which is essential for the full activation of the JAK-STAT3 pathway. Furthermore, IL-6 is also known to stimulate STAT3 at the Y705 residue in HCC cells (46). Thus, it is possible that the phosphorylation at Y705 occurs preferentially in response to HBV gene expression.

Our results showing that the IL-6-stimulated phosphorylation of STAT3 was inhibited not only by atiprimod but also by NS398, suggests that the activation of Akt and STAT3 in HCC cells might be interlinked with a COX-2-mediated mechanism. This is consistent with the finding that celecoxib, a COX-2-selective inhibitor, also induced apoptosis in HCC cells through the inhibition of Akt phosphorylation (47). In addition, COX-2-selective inhibitors have also been shown to inhibit the IL-6-stimulated phosphorylations of STAT3 in non-small cell lung carcinoma cells (48). Previous studies have also shown that elevated levels of COX-2 are linked with the production of prostaglandin E₂, which serves as a second messenger to activate cellular processes leading to tissue inflammation and carcinogenesis (49). Thus, the preceding discussion suggests that reactive oxygen species, COX-2, and PI-3K pathways collectively mediate the activation of Akt and STAT3 in HBV-expressing cells.

The model shown in Fig. 10 illustrates the possible biochemical mechanisms underlying HBV/HCV-induced HCC in human. The focal points of this model are the constitutive activation of PI-3K/Akt and JAK-STAT signaling pathways, which are activated in response to HBV/HCV infections. Thus, the fate of virus-infected cells is likely to be regulated by the activation of these pathways. From this perspective, the constitutive activation of Akt and STAT3 is of central importance as they are known to be involved not only in cell survival but also in the

modulation of invasive and metastatic potentials of cancer cells. An important aspect of this study that needs to be investigated in future work concerns the exact mechanism by which atiprimod inhibits constitutive activation of Akt and STAT3. Recently, it was shown that atiprimod reduced Jak2 protein levels in acute myeloid cells without affecting its transcript levels, presumably via the inhibition of the ubiquitin-proteasome pathway (18, 50). The proteasome is a ubiquitous enzyme complex that plays a critical role in the regulation of many proteins involved in cell cycle regulation, apoptosis, and angiogenesis. Because transformed cells, compared with normal cells, are more susceptible to apoptosis following proteasome inhibition, inhibition of the proteasome is an attractive approach in developing anticancer therapeutics.

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