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Article

ARTICLE

Sorafenib Action in Hepatitis B Virus X–Activated Oncogenic Androgen Pathway in Liver Through SHP-1

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Abstract

Background: Hepatitis B virus (HBV)–related hepatocellular carcinoma (HCC) shows a higher incidence in men, mainly because of hepatitis B X (HBx)–mediated enhancement of androgen receptor (AR) activity. We aimed to examine this pathway in hepatocarcinogenesis and to identify drug(s) specifically blocking this carcinogenic event in the liver.

Methods: HBx transgenic mice that spontaneously develop HCC (n = 28–34 per group) were used, either by knockout of hepatic AR or by castration. Efficacy of several HCC-targeted drugs in suppressing HBx-induced AR activity was evaluated, and cellular factors mediating suppression were investigated in cultured cells. Tissue specificity of the candidate drug was validated using mouse tissues. Data were analyzed with Chi-square and Student's *t* tests. All statistical tests were two-sided.

Results: The androgen pathway was shown to be important in early stage hepatocarcinogenesis of HBx transgenic mice. The tumor incidence was decreased from 80% to 32% by AR knockout ($P < .001$) and from 90% to 25% by early castration ($P < .001$). Sorafenib markedly inhibited the HBx-enhanced AR activity through activating the SHP-1 phosphatase, which antagonized the activation of Akt/GSK3β and c-Src by HBx. Moreover, SHP-1 protein level was much higher in the liver than in testis, which enabled sorafenib to inhibit aberrant AR activity in the HBx-expressing liver, while not affecting the physiological AR function in normal liver or testis.

Conclusions: The androgen pathway may be a druggable target for the chemoprevention of HBV-related HCC, and sorafenib might be used as a tissue- and disease-specific regimen for the chemoprevention of HBV-related HCC.

Hepatocellular carcinoma (HCC), the second leading cause of cancer death, accounts for approximately half a million deaths every year worldwide (1). Because half of the HCC cases are caused by chronic hepatitis B virus (HBV) infection, it is imperative to control HBV infection by universal vaccination and to utilize active antiviral therapies in chronic hepatitis B (CHB) patients. Despite these efforts, the incidence of HCC remains high, and therefore an understanding of HBV-related carcinogenesis is important for identifying novel drug target(s) to combat this disease.

One striking feature of HCC is its higher incidence in men (2,3). Several cohort studies have indicated that higher androgen/AR pathway activity is one major risk factor for HBV-related HCC in men (4,5), suggesting that HBV may stimulate the development

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of HCC by increasing AR activity. Our recent studies supported this hypothesis by identifying an HBx-AR–positive regulatory loop. In HBV-infected hepatocytes, the HBx viral protein increases AR transcriptional activity, predominantly by inhibiting GSK3β and activating c-Src to reinforce both AR dimerization and AR phosphorylation (6). Ligand-activated AR then binds to the androgen response elements in the HBV genome and stimulates the transcription of HBV mRNAs, including that encoding the HBx protein. A positive regulatory loop between HBx and the androgen/AR pathway thus arises preferentially in HBV-infected male hepatocytes and leads to persistently elevated AR activity and increased viral RNA synthesis (7).

Our previous study demonstrated the carcinogenic potential of this HBx-AR regulatory loop using soft agar colony formation assays (8). This, however, has not yet been validated in animal models, although the AR pathway was noted important for male HCC in the chemically induced HCC mouse model (9,10). In this study, we will further examine the role of the androgen pathway in hepatocarcinogenesis in HBx-transgenic (HBx-Tg) mice that spontaneously develop HCC (11) by mating with a hepatic AR knockout strain or by castration.

Considering the hepatic AR as a potential target for HCC drug development, this study also aimed to evaluate the ability of small molecules with anti-HCC potency to specifically inhibit the aberrant AR activity in the liver. It might shed light on circumventing the major disadvantage of most currently available anti-androgen drugs, which suppress the AR pathway in all tissues and may cause systemic adverse effects in CHB patients.

Methods

Hepatocyte-Specific HBx Transgenic and AR Knockout Mice

The HBx transgenic (HBx-Tg) mice were established by the pronuclear microinjection of an albumin promoter–driven HBx expression plasmid, pAlb-In-pA-HS4, into the C57BL/6-fertilized eggs. The plasmid contains two direct repeats of chicken HS4 insulator downstream of poly(A) signal to avoid the positional effects, with the details as described previously (11). All the four transgenic lines showed similar characteristics, including the tumor incidence, the pathologic changes, and also the male preference pattern of HCC. The transgenic line A112 was selected for most of the experiments in this study, because the development of HCC in this line is earlier than the other three lines (11). Two other transgenic lines, A106 and A110, were also included for the castration experiments.

The hepatocyte-specific AR knockout mice (ARKO) were established by crossing of the mice carrying the albumin-driven Cre recombinase (Alb-Cre) transgene with the floxed AR mice (C57BL/6) carrying two loxP sites flanking the exon 1 of AR gene, which were established and kindly provided by professor Shigeaki Kato (Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan) (12). The specificity of the AR knockout in liver was examined by immunoblot, using the lysates collected from different organs in two-month-old ARKO and wild-type (WT) control mice. The HBx-Tg mice with a concomitant knockout of hepatic AR (HBx/ARKO) were generated by crossing the ARKO mice with the HBx-Tg mice. The genotypes of these mice were determined by polymerase chain reaction (PCR) with the tail DNA with details as described (11,12). All of the mice were housed in a specific pathogen-free facility with ad libitum access to food and water, in the animal center at National Taiwan University. All procedures for the mouse experiments were approved by the Institutional Animal Care and Use Committee (IAUAC) at the National Taiwan University College of Medicine and were carried out according to the Committee's guidelines

Tumor Incidence and Liver Tissue Analyses

The tumor incidence was compared among WT control, HBx-Tg, HBx/ARKO, ARKO, and castrated HBx-Tg male mice. Different group sizes (n = 28–34 per group) were included for this study, according to the mice available in the animal center at the time of experiments. We included five groups of HBx-Tg mice for the castration experiments, either sham operated (control) or castrated at different ages (at 3–4 weeks, 10 weeks, 12 weeks, and 16 weeks of age), for comparing the tumor incidence at the end when they reached the same age of 20 months. For the ARKO experiments, the end point was set at 16 months. At the end of follow-up, the mice were killed and the liver tissues were collected for histopathologic examination of HCC, and the tumor incidence in each group was calculated accordingly. Meanwhile, the liver weight and body weight were determined, with the liver/body weight ratio as an index for tumor mass in liver (13). The serum alanine aminotransferase (ALT) levels at the end of follow-up were determined by SPOTCHEM EZ chemical analyzer (ARKRAY, Edina, Minnesota, USA) using the specific strips (Cat No. 77250).

The liver tissues were collected and processed for hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) analysis, with details as provided in the Supplementary Methods section (available online).

Androgen Pathway Activity and SHP-1 Expression in Liver and Testis

We conducted the in vivo hepatic AR reporter assay to examine the activity of AR pathway in liver. A plasmid mixture containing 10 μg of 5xARE-Luc and 1 μg of pCMV-Renilla was delivered into the liver of WT or HBx-Tg mice by hydrodynamic injections (HDI), as described in our previous study (7). To examine the effect of drug treatment, the mice were fed daily with 10 mg/kg/day of SC-1 or sorafenib by oral gavage, which started one day before HDI and continued until death at 48 hours postinjection. The liver tissues were collected from these mice for the reporter assays by dual luciferase reporter assay kit (Promega, Madison, WI). The effect of drug treatment on the expression of putative androgen responsive target genes in liver and testis was examined by real-time quantitative polymerase chain reaction (RT-qPCR) and western blot, with details as provided in the Supplementary Methods section (available online).

To get rid of the effect from blood cells in liver, we purified the hepatocytes by hepatic perfusion. The expression of SHP-1 in hepatocytes and testis was compared by immunoblot and RT-qPCR analysis. The details are provided in the Supplementary Methods section (available online).

Cell Culture, Transfection, Reporter Assay, and Western Blot

Four hepatoma cell lines, including HepG2, Huh-7, PLC-5, and Hep3B were purchased from the Bioresource Collection and Research Center (BCRC, http://www.bcrc.firdi.org.tw, Taipei, Taiwan), with the genetic profiles confirmed to be identical with ATCC data using AmpFLSTR Identifiler PCR amplification

kit (Applied Biosystems, Life Technologies, Taipei, Taiwan) (data provided by BCRC). The cell lines were cultured at 37°C in DMEM (Gibco, Carlsbad, CA) medium supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Kibbutz Beit Haemek, Israel) in a 5% CO_2 incubator. The transfection experiments were conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) by following the manufacture's instruction. Twenty hours after transfection, R1881 (10 nM, Perkin-Elmer, Wellesley, MA) and the specific drugs at indicated concentration were added to the culture medium containing 5% charcoal-stripped FBS. The cell lysates were harvested 20 hours after treatment for subsequent analysis.

The 5xARE-Luc plasmid was used to assay the AR-responsive transcriptional activity in cells. The Gal4-TK-Luc construct was applied to examine the interaction between the N- and C-terminal domains of AR, namely the N/C interaction, by mammalian two hybrid reporter assay. The dual-luciferase reporter assay was used for analyzing the reporter activity (Promega, Madison, WI) by following the manufacturer's instruction. The pCMV-Renilla plasmid (Promega) was cotransfected as an internal standard for normalization of the transfection efficiency. The details for the reagents used for cell culture studies, including the plasmid constructs, the chemicals, the antibodies used for the western blot analysis, the sh-RNA-lentivirus production, and soft agar colony formation assay, are provided in the Supplementary Methods section (available online).

Statistical Analysis

HCC incidence in wild-type and transgenic male mice was analyzed by Chi-square (χ^2) test. The reporter assay results, as well as soft agar colony and RT-qPCR data, were collected from three independent experiments, represented as the means ± standard deviations and evaluated by Student's *t* test. Two-sided tests were used, and *P* values less than .05 were regarded as statistically significant. All analyses were performed by Stata statistical software (Version 12.0, Stata Corp., College Station, TX).

Results

Androgen Pathway Is Important for Early Stage Hepatocarcinogenesis in HBx-Tg Mice

The HBx-Tg mouse model that spontaneously develops HCC mainly in males was used to evaluate the role of the androgen/AR axis in HBx-induced hepatocarcinogenesis in vivo (11). We first abolished the androgen pathway by conditional knockout of hepatic AR via the albumin promoter-driven *Cre-LoxP* system. As revealed by western blot, AR was specifically depleted in the livers of the ARKO mice, but not in other tissues such as testis and kidney (Supplementary Figure 1, available online). Depleting hepatic AR in the HBx-Tg mice statistically significantly decreased the tumor incidence from 80% to 32% at 16 months of age ($P < .001$) (Figure 1A). The reduction in liver tumor mass was consistent with a significant decrease in liver weight/body weight ratio in HBx/ARKO mice, compared with that of HBx-Tg mice (HBx-Tg, mean ± SD = 0.061±0.024; HBx/ARKO, mean ± SD = 0.049±0.013; $P = .025$) (Supplementary Figure 2, available online).

All the liver tissues were processed for H&E staining for histological analysis and HCC determination, with representative results shown in Figure 1B. We conducted IHC staining to further characterize several parameters of these liver tissues, including proliferation, fibrosis, and inflammatory cell infiltration (Supplementary Figure 3, available online). By Ki-67 staining, we found a marked decrease of proliferation activity by depletion of hepatic AR in HBx-Tg mice. We also noted a decrease of the inflammatory cells in the liver of HBx/ARKO mice, which was accompanied by decreased serum ALT levels (serum ALT units/L: HBx, mean ± SD = 397.3±691.6; HBx/ARKO, mean ± SD = 113.2±163.1; $P = .039$) (Figure 1C), an indicator of hepatic inflammation and liver damage (14,15). As noted in previous studies, we found ALT levels to be further elevated at around the start of HCC formation (13–14 months and thereafter), which indicated increased inflammation and liver damage predisposing to hepatocarcinogenesis in this animal model (11). Our data thus implicated the androgen/AR pathway to be important for the aberrant hepatic proliferation and inflammation/damage induced by HBx.

We castrated HBx-Tg male mice (line A112) at different ages as a second approach to repress androgen signaling, which also helped to identify the specific stage of this pathway involved in carcinogenesis. Castration at 10 weeks of age or younger statistically showed a significant decrease in the incidence of HCC at 20 months of age from 90% to 25% ($P < .001$) (Figure 1D), similar to the tumor incidence in the HBx/ARKO mice (Figure 1A). Such a decrease of tumor incidence by castration was also found in two other lines of HBx-Tg mice, A106 and A110 (Supplementary Figure 4, available online). Interestingly, when the mice were castrated at 12 weeks of age or older, the tumor incidence remained high, and no overt changes in protein expression of AR and HBx were observed in these mice (Figure 1D). Again, the liver tissues were processed for IHC staining, showing a decrease of hepatic proliferation by castration before two months of age in HBx-Tg mice (Supplementary Figure 5, available online).

These results altogether suggested that the active androgen pathway was important but likely exerted its effects mainly in the early stages of HBx-induced carcinogenesis, which is thus a potential target for development of chemoprevention drugs.

Effect of Sorafenib on HBx-Stimulated AR Activity Through Active SHP-1 Phosphatase

Currently, there are numerous anti-androgen drugs that effectively inhibit the AR pathway, but these agents lack the specificity required to inhibit the HBV-related activation of AR and would cause undesirable systemic effects, especially on the reproductive organs. Therefore, we searched for new drugs with greater specificity for HBx-mediated AR activation that would spare canonical AR signaling in reproductive tissues.

We evaluated several molecular targeting agents that are used for HCC therapy or tested in clinical trials. Three kinase inhibitors that target numerous kinases, sorafenib, sunitinib, and brivanib, were tested for their activities on a luciferase reporter driven by 5xARE in the presence of HBx. Interestingly, we discovered that sorafenib, but not sunitinib or brivanib, markedly suppressed R1881 (an AR agonist)-stimulated AR activity in HepG2 (Figure 2A) and in Huh7 cells (Figure 2B) in a dose-dependent manner (Supplementary Figure 6, A-C, available online). Moreover, sorafenib statistically significantly decreased the HBx-enhanced AR activation in a dose-dependent manner (reduction %, 2.5 μM, mean ± SD = 69±2%, $P < .001$; 5 μM, mean ± SD = 72±0%, $P < .001$; 10 μM, mean ± SD = 98±0%, $P < .001$) (Figure 2C). This suppression was also confirmed in PLC5 and Hep3B cell lines, which contain HBx in the genome (Supplementary Figure 7A, available online). Sorafenib was thus considered an effective drug that inhibited the HBx/AR-positive circuit in hepatocytes.

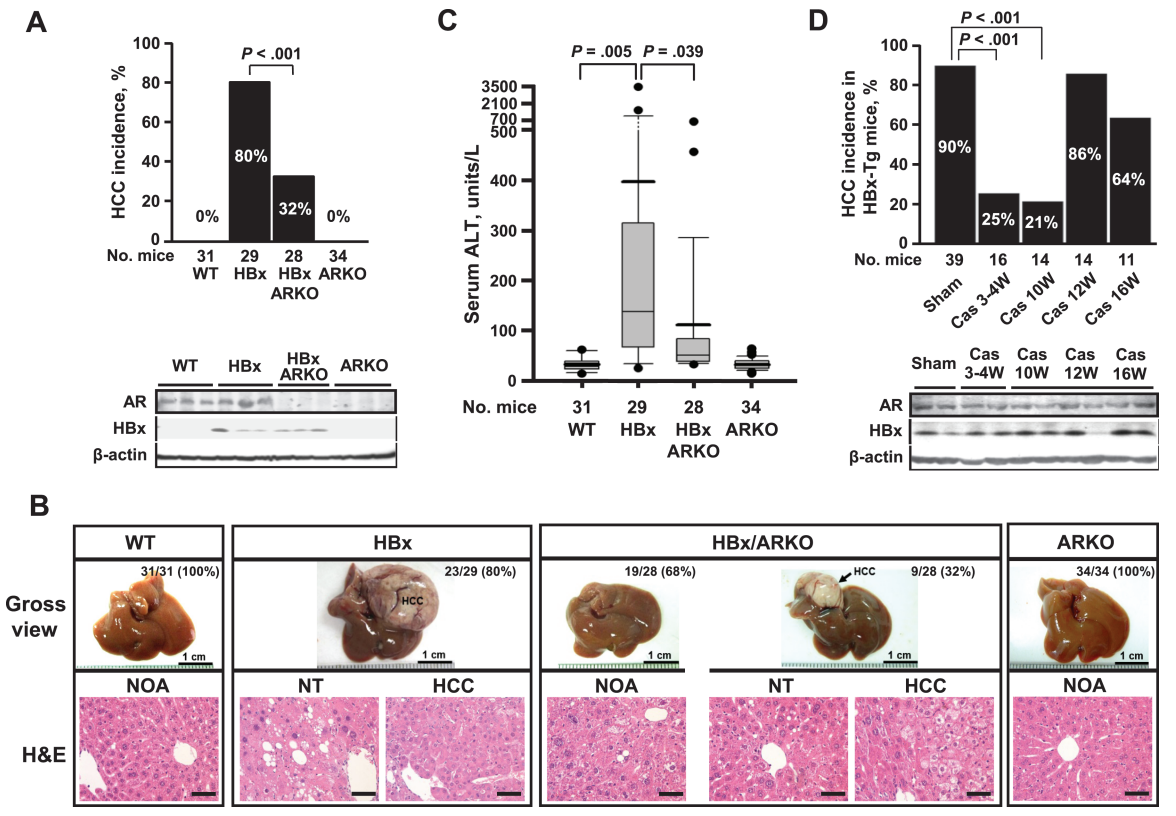

Figure 1. The androgen pathway in hepatocellular carcinoma (HCC) in male HBx transgenic (HBx-Tg) mice. **A) Upper panel**, the incidence of HCC was determined in wild-type and HBx-transgenic (HBx) male mice at 16 months of age with or without hepatic androgen receptor (AR) expression (ARKO or HBx/ARKO). *P* values by two-sided Chi-square test. **Lower panel**, the hepatic protein expression of AR and HBx was determined by western blotting. **B)** Representative gross appearance of the livers and hematoxylin and eosin staining of hepatic tissues (**scale bar** = 50 μm). The group of HBx/ARKO was further divided into those with no overt abnormality and those with HCC, with the percentage of mice with the same characteristic as the representative case as indicated. **C)** The serum alanine aminotransferase levels in the four groups of mice listed in **(A)** were measured at 16 months of age and presented as **box plots**. The **solid horizontal line** within each box indicates the mean value, while the **rectangle** represents the interquartile range, with outliers depicted as individual **dots**, and the **whiskers** represent data range omitted in the plot. *P* values by two-sided Student's *t* test. **D) Upper panel**, the incidence of HCC was quantitated in 19- to 20-month-old HBx-Tg male mice that were either castrated at the indicated age or sham-operated as a control. *P* values by two-sided Chi-square test. **Lower panel**, AR and HBx expression in the castrated mice was determined by western blot analysis. ALT = alanine aminotransferase; AR = androgen receptor; ARKO = AR knockout mice; H&E = hematoxylin and eosin; HBx = HBx-transgenic; HCC = hepatocellular carcinoma; NOA = no overt abnormality; NT = nontumor section; WT = wild-type.

Because AR inhibition was only observed after treatment with sorafenib, not with the other two multikinase inhibitors, we hypothesized that the repressive effects of sorafenib were mediated by a kinase-independent mechanism. We recently reported that sorafenib exerts its anticancer effects through an alternative mechanism that involves activating the cellular phosphatase SHP-1 (16). A series of new sorafenib derivatives that lack the ability to inhibit kinases but retain the ability to activate SHP-1 has been synthesized. These compounds are known as SC compounds, and they lack both the amide group and the pyridine ring, which are critical for interacting with the ATP-binding pockets of target kinases (16,17). To test this possible mechanism for blocking the HBx-mediated activation of AR, we evaluated compound SC-1 using a 5xARE-Luc reporter assay. Treatment with SC-1 reduced R1881-stimulated AR activity (Figure 2, D and E; Supplementary Figure 6, D-F, available online) and antagonized HBx-stimulated AR activation (Figure 2F), similar to the effects elicited by sorafenib.

To further address whether sorafenib and SC-1 suppressed the androgen pathway through SHP-1, we knocked down the expression of SHP-1 and observed that the basal level of R1881-stimulated AR activity was elevated (Figure 2G, set 4 vs set 1, DMSO treatment, 1.83-fold increase, $P = .002$). Moreover, the inhibitory effects of sorafenib and SC-1 were compromised when SHP-1 expression was downregulated (Figure 2G, reduction %: for sorafenib vs DMSO, si-LacZ set: mean ± SD = 71 ± 4%, $P < .001$; si-SHP-1 set: mean ± SD = 32 ± 4%, $P = .01$; for SC-1 vs DMSO, si-LacZ set: mean ± SD = 56 ± 4%, $P < .001$; si-SHP-1 set: mean ± SD = 0 ± 8%, $P = .959$). These results suggested that the inhibition of AR by sorafenib and SC-1 was predominantly mediated by SHP-1. As confirmation, we transfected Huh7 cells with different SHP-1 expression plasmids, including wild-type, constitutively active (dN1), and enzyme-dead mutant (C453S) constructs for evaluating their effects on AR activity (Figure 2H, upper panel) (18). The reporter activity was suppressed by overexpression of WT or dN1 SHP-1 but not by C453S mutant (Figure 2H, lower panel, reduction %: WT, mean ± SD = 55 ± 1%, $P = .015$; dN1, mean ± SD = 71 ± 1%, $P = .008$; C453S, mean ± SD = 6 ± 3%, $P = .466$), suggesting SHP-1 suppressed AR activity depending on its enzymatic activity.

SHP-1 Interfered With AR Dimerization and Reduced AR Phosphorylation at Serine 81

To dissect the mechanism by which sorafenib and SC-1 repressed AR activity through SHP-1, we focused on two major mechanisms for the HBx-mediated activation of the AR pathway identified in our previous study, the enhanced N/C dimerization

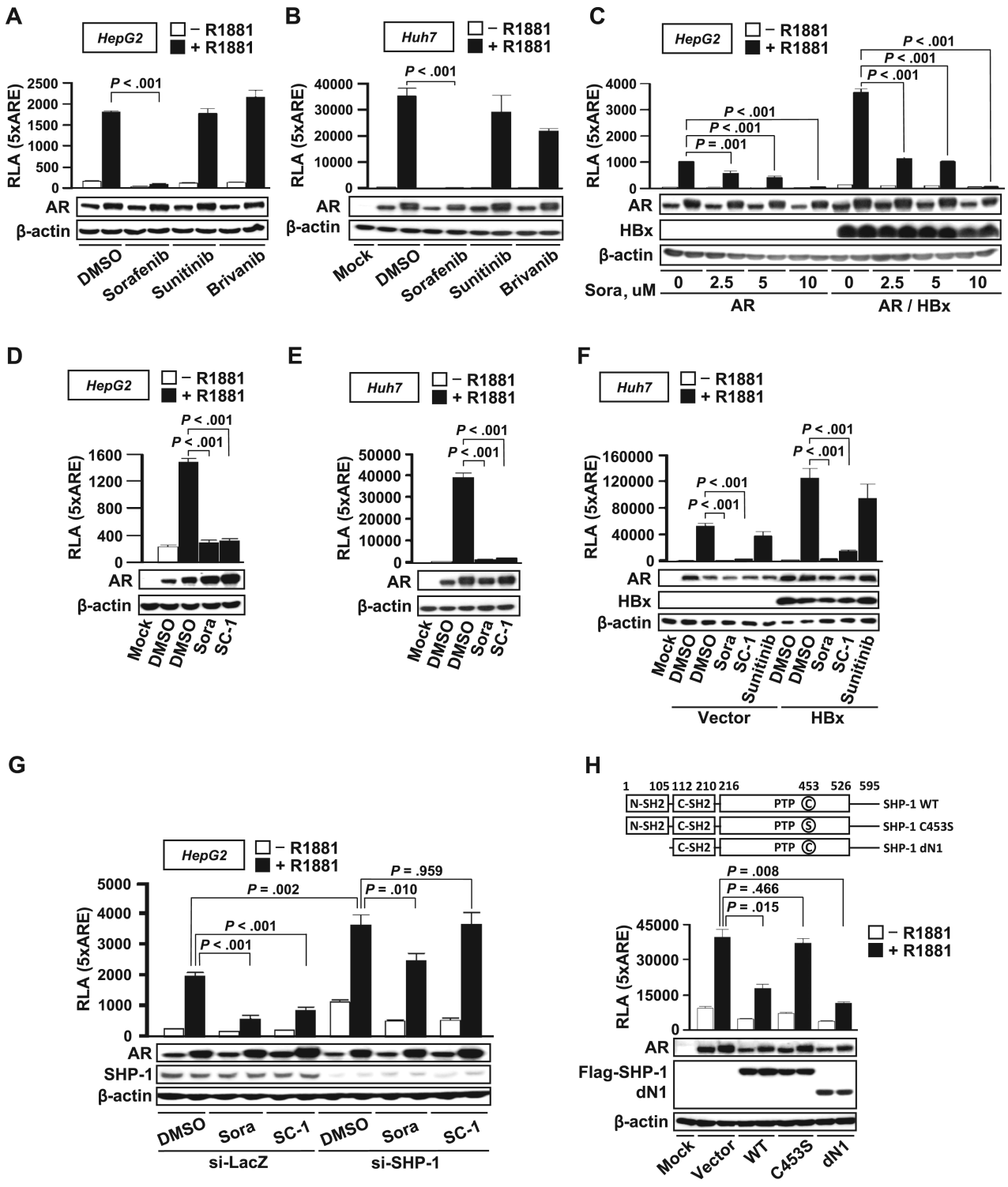

Figure 2. Effect of sorafenib on HBx-enhanced androgen receptor (AR) activity through SHP-1. **A and B)** AR activity was determined in HepG2 and Huh7 cell lines under the treatment of multiple kinase inhibitors. These cells were cotransfected with AR expression plasmids and the 5xARE-Luc reporter construct, simultaneously treated with androgen analog R1881 (10 nM) and indicated drugs (10 μM) for subsequent reporter assay. **C)** The dose-response of sorafenib for inhibiting HBx-enhanced AR activity was determined in HepG2 cells cotransfected with an HBx expression construct and assayed as described above. **D and E)** The ability of SC-1 (10 μM) in comparison with sorafenib to inhibit AR activity was evaluated in hepatic cell lines using the 5xARE-Luc reporter assay as described in **(A and B)**. **F)** As mentioned in **(C)**, SC-1 was examined for its targeting effect on HBx-enhanced AR activity in Huh7 cells. **G)** The repressive effects of SC-1 and sorafenib on AR activity were evaluated in HepG2 cells infected with lentivirus expressing either siRNA targeting SHP-1 (si-SHP-1) or control siRNA (si-LacZ). **H)** The FLAG-tagged wild-type (WT), phosphatase-dead (C453S), or constitutively active (N-terminal SH2 domain deleted, dN1) SHP-1 constructs are illustrated in the **upper panel**. **Lower panel**, the involvement of SHP-1 as a negative regulator of the androgen/AR axis was analyzed using 5xARE-Luc reporter assays in Huh7 cells that were cotransfected with AR plasmids and indicated SHP-1 expression constructs. AR, HBx, and SHP-1 protein expression were verified by western blot; a nontransfected cell lysate (Mock) served as a negative control. In all results, data are means of three independent experiments and **error bars** represent standard deviations. *P* values by two-sided Student's *t* test. 5xARE = the luciferase reporter with promoter containing five repeats of AR responsive element; AR = androgen receptor; RLA = relative luciferase activity; Sora = sorafenib; WT = wild-type.

and increased phosphorylation at serine 81 (S81), which are critical events in the AR signaling pathway (6).

We used a mammalian two-hybrid assay to analyze the dimerization step (6). The results showed the ligand-stimulated N/C interaction to be statistically significantly impaired by sorafenib and SC-1 (reduction %: HepG2, mean ± SD = 79 ± 2% by sorafenib, $P < .001$; mean ± SD = 72 ± 4% by SC-1, both $P < .001$; Huh7, mean ± SD = 96 ± 0% reduction by sorafenib; mean ± SD = 74 ± 4% by SC-1, both P values < .001) (Figure 3A). The drugs also interfered with the HBx-enhanced N/C interaction, in both Huh7 cells transfected with CMV-HBx plasmid (Figure 3B) and in PLC5 and Hep3B cell lines with HBx contained in their genomes (Supplementary Figure 7B, available online). Therefore, inhibition of the dimerization step was one mechanism by which these drugs suppressed androgen/AR activity. The SC-1-induced inhibition of AR dimerization could be completely restored by knocking down SHP-1 expression (reduction %: si-LacZ set, mean ± SD = 59 ± 3% by SC-1, $P = .003$; si-SHP-1 set, mean ± SD = 2 ± 3% by SC-1, $P = .759$) (Figure 3C), confirming the role of SHP-1 in mediating this suppressive effect.

At the same time, we found that phosphorylation of AR at S81, the other HBx-enhancing event for the androgen/AR pathway, was decreased by the treatment with sorafenib and SC-1 (Figure 3D). Overexpression of the WT or dN1 form of SHP-1, but not the C453S mutant, compromised both the dimerization (reduction %: WT, mean ± SD = 79 ± 3%, $P = .027$; dN1, mean ± SD = 76 ± 3%, $P = .029$; C453S, mean ± SD = 16 ± 5%, $P = .516$) (Figure 3E) and AR phosphorylation at S81 (Figure 3F). These results suggested that sorafenib and SC-1 could counteract the HBx-mediated enhancement of AR dimerization and S81 phosphorylation through active SHP-1.

SHP-1 Interfered With AR Dimerization by Activating GSK3β and Reduced S81 Phosphorylation by Antagonizing c-Src Activity

We next sought to elucidate the mechanism by which SHP-1 repressed AR dimerization and S81 phosphorylation. As the first candidate mediator, we selected signal transducer and activator of transcription 3 (STAT3), a well-documented target of SHP-1 that mediates its tumor suppressor function (16,18,19). Overexpression of constitutively active STAT3 construct (STAT3-C) failed to rescue the repressive effects of sorafenib and SC-1 on 5xARE-Luc reporter activity, S81 phosphorylation, and N/C dimerization (Supplementary Figure 8, A-C, available online). Furthermore, knockdown of STAT3 expression did not affect these particular events (Supplementary Figure 8, D-F, available online). Therefore, SHP-1 did not inhibit AR via STAT3; instead, some other novel targets, which are worthy of investigation, must mediate this inhibition.

Our previous study provided clues as to the identity of other potential targets; two kinase switches that are utilized by HBx to enhance AR activity were identified. HBx inhibits GSK3β to promote the N/C dimerization of AR and activates c-Src kinase to increase AR-S81 phosphorylation (6). Because SHP-1 effectively inhibited both AR dimerization and S81 phosphorylation (Figure 3), we tested the possibility that SHP-1 targeted these two key switch kinases to inhibit AR activity.

Both sorafenib and SC-1 substantially decreased the phosphorylation of GSK3β at S9, indicating that these drugs activated GSK3β (Figure 4A, Vector panel). The activity of Akt, which phosphorylates GSK3β at S9 (20), was simultaneously suppressed, as evidenced by reduced phosphorylation at S473 (Figure 4A, Vector panel). We further identified that both drugs inhibited the phosphorylation of c-Src at tyrosine (Y) 418 (but not at Y530) (Figure 4A, Vector panel), suggesting that they also inhibit c-Src kinase activity. The concomitant influences of drug treatment on these two kinase pathways were evident in the presence of HBx (Figure 4A, HBx panel). As a negative control, the ribosomal S6 kinase activity, as indicated by the phosphorylation at threonine (T) 389, remained unaffected by drug treatment (21). Moreover, the drug repressive effects for these two switch kinases were abolished by SHP-1 knockdown (Figure 4B), confirming their dependence on SHP-1. The SHP-1 effects on these two kinase switches were also validated by the SHP-1 mutant constructs (Figure 4C).

Finally, the roles of these two kinase switches in mediating the effects of SHP-1 on AR activity were evaluated using rescue experiments. We activated these two switches with LiCl (a GSK-3 inhibitor) and by expressing the constitutively active form of Src, a Y530F mutant (22), either alone or in combination, and examined whether the ability of SC-1 to inhibit 5xARE-Luc activity was restored. The SC-1–mediated inhibition of reporter activity was reversed by a combination of LiCl treatment and Src Y530F expression (Figure 4D, Vector panel). Both manipulations were also required to fully compromise the repressive effect of SC-1 in the presence of HBx (Figure 4D, HBx panel). Taken together, these data suggested that SC-1–activated SHP-1 suppressed androgen/AR activity mainly by inhibiting two key kinase switches, Akt/GSK3β and c-Src, leading to the decrement of AR dimerization and S81 phosphorylation.

Effect of Sorafenib on HBx-Induced Activation of Hepatic AR in Vivo

In addition to the cell culture–based assays, we validated the effects of sorafenib and SC-1 on AR activity in vivo. The 5xARE-Luc reporter plasmid was delivered into the livers of WT control and HBx-Tg mice via hydrodynamic injection (23). The mice were either castrated (as a control for androgen deprivation) or sorafenib or SC-1 were orally administered, with the dose similar to that in clinical use. The liver and testes were collected at 48 hours postinjection to evaluate the drug effect on AR reporter activity and the expression of specific hepatic and testicular AR-responsive genes.

The hepatic AR activity was approximately 15-fold higher in HBx-Tg mice than in WT control male mice, which exhibited a low basal level of AR activity (Figure 5A, lane 6 vs lane 2). This HBx-enhanced AR activity was decreased by castration and by oral gavage with sorafenib and SC-1 (Figure 5A, reduction %: castration, mean ± SD = 73 ± 2%, $P = .031$; sorafenib treatment, mean ± SD = 69 ± 16%, $P = .002$; SC-1 treatment, mean ± SD = 65 ± 13%, $P = .004$). In contrast, the repressive effects were minimal in WT control mice (Figure 5A, lane 1 for castration, lanes 3 and 4 for drug treatment), suggesting that the drugs preferentially targeted HBx-induced aberrant AR activation. This pattern was also verified by analyzing the mRNA level of the endogenous AR target gene *Tgfb1* in livers (Figure 5B) (24). Our western blot results confirmed the elevated phosphorylation of c-Src at Y418, Akt at S473, and GSK3β at S9 in HBx-Tg mice to be repressed by drug treatment (Figure 5C). These results provide in vivo evidence that the two kinase switches mediating the HBx-enhanced AR activity were elevated and targeted by sorafenib and SC-1 in the HBx-Tg mice; both switches, however, remained at low basal levels in WT mice and were not affected by the drug treatment.

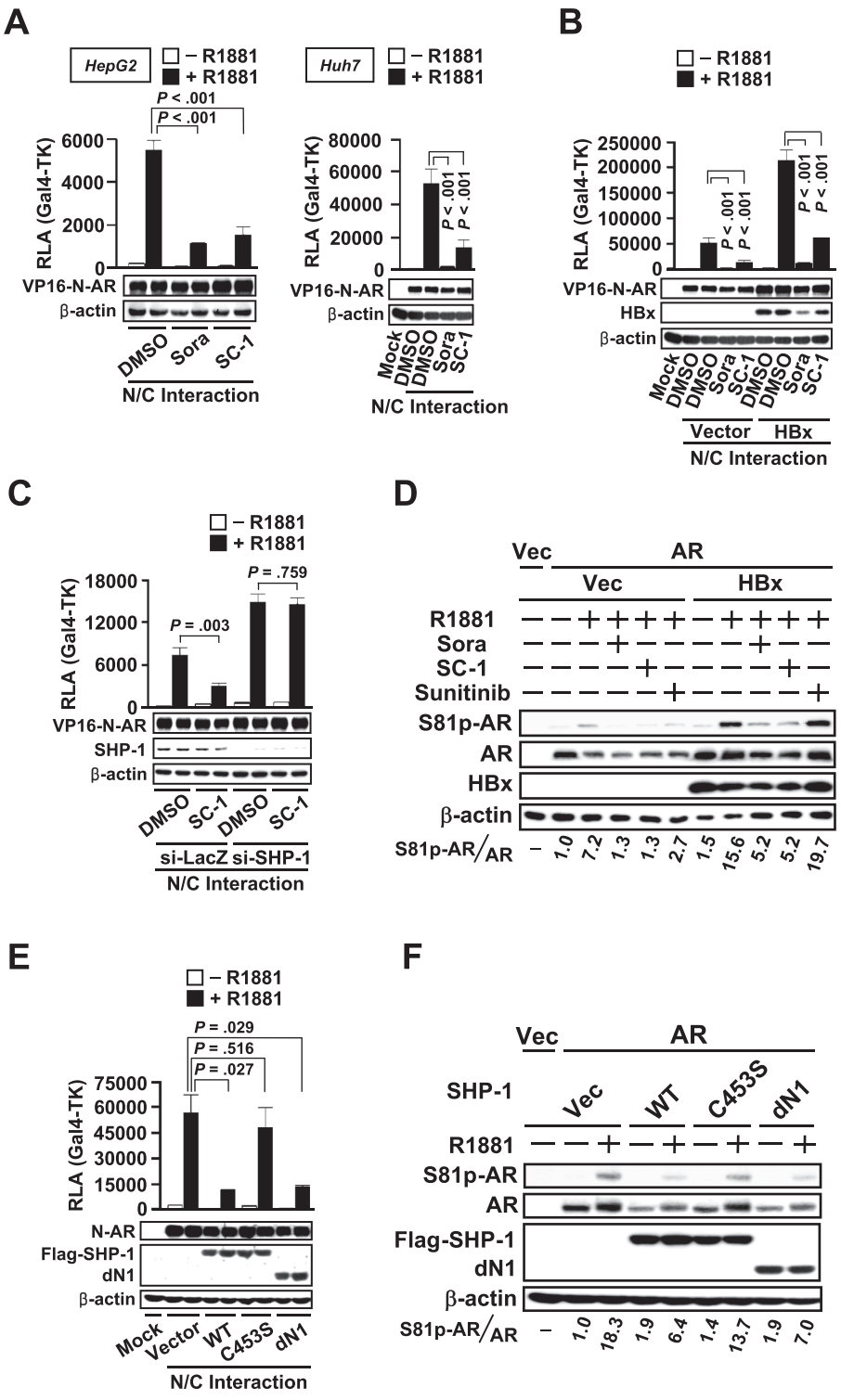

Figure 3. Effect of SHP-1 on androgen receptor (AR) dimerization and phosphorylation at serine 81 (S81). **A)** The R1881-induced AR amino/carboxy terminal (N/C) interaction was determined by mammalian two-hybrid reporter assay in HepG2 and Huh7 cells treated with sorafenib, SC-1, or vehicle control (10 μM). **B)** The ability of sorafenib and SC-1 to interfere with the N/C interaction of AR was verified in Huh7 cells with exogenous expression of HBx. **C)** The SC-1 effect on the AR N/C interaction was assayed in HepG2 cells infected with lentiviruses expressing either siRNA targeting SHP-1 (si-SHP-1) or control siRNA (si-LacZ). **D)** The S81 phosphorylation level of AR was analyzed by western blotting in Huh7 cells treated with indicated compounds. **E)** By hybrid reporter assay, the R1881-stimulated N/C interaction of AR was evaluated in Huh7 cells cotransfected with the indicated SHP-1 expression plasmids. **F)** By western blot, the AR S81 phosphorylation (S81p-AR) levels were compared in Huh7 cells cotransfected with various SHP-1 expression constructs. The **number at the bottom of each lane** in western blotting data indicates the relative signal strength of phospho-S81. The protein expression of HBx, SHP-1, and the N-terminal AR fragment in these cells were also confirmed by western blot. In all results, data are means of three independent experiments and **error bars** represent standard deviation. *P* values by two-sided Student's *t* test. AR = androgen receptor; Gal4-TK = the luciferase reporter with promoter containing Gal4-binding domain; RLA = relative luciferase activity; Sora = sorafenib.

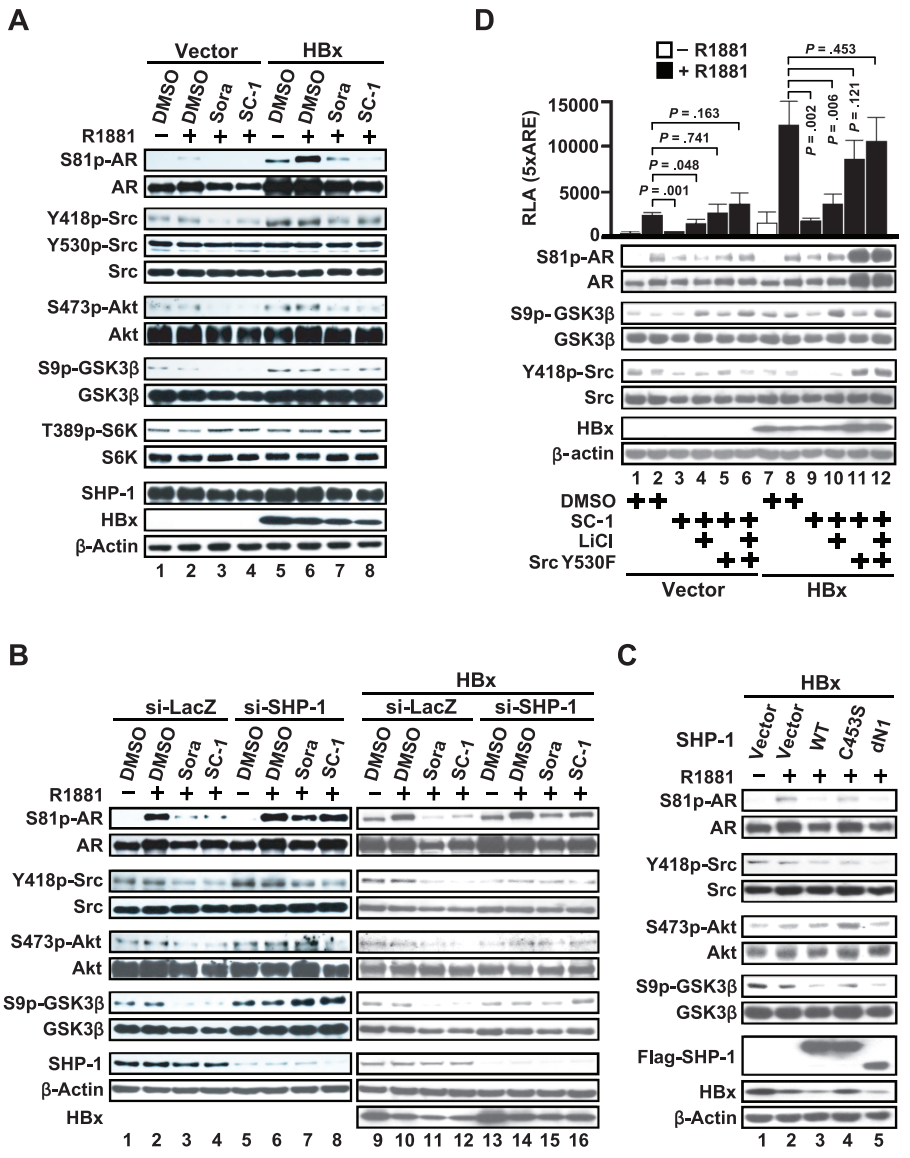

Figure 4. Effect of SHP-1 on androgen receptor (AR) dimerization by activating GSK3β and on serine 81 phosphorylation. **A)** The kinase activities of Akt, GSK3β, c-Src, and S6K, as evidenced by their phosphorylation at serine 473 (S473p), serine 9 (S9p), tyrosine 418 (Y418p) / tyrosine 530 (Y530p), and threonine 389 (T389p), respectively, were determined by western blot analysis in Huh7 cells treated with R1881 (10 nM) and the indicated drugs (10 μM) in the presence or absence of HBx expression. **B)** As mentioned in **(A)**, the effects of these drugs on Akt, GSK3β, and c-Src kinases were evaluated in SHP-1-knockdown cells (si-SHP-1) and in control cells (si-LacZ) with or without exogenous HBx expression. **C)** The Huh7 cells were cotransfected with HBx and various SHP-1 constructs as indicated, and the effects on the phosphorylation of c-Src, Akt, and GSK3β at the indicated sites were analyzed by western blot analysis. **D)** The AR-repressive effect of SC-1 was examined using the 5xARE-Luc reporter assay in HepG2 cells, in which GSK3β was inactivated by LiCl (10 μM) and/or a constitutively active form of c-Src (Src Y530F) was overexpressed. The expression of these proteins was verified by western blot analysis. Data are means of three independent experiments, and **error bars** represent standard deviations. *P* values were calculated by two-sided Student's *t* test. 5xARE = the luciferase reporter with promoter containing five repeats of AR responsive element; RLA = relative luciferase activity; Sora = sorafenib; WT = wild-type.

Interestingly, the mRNA and protein level of two well-documented testicular AR responsive genes, *Rhox5* and *Spinlw1* (25–27), were not affected by sorafenib or SC-1 in testes either from WT or HBx-Tg male mice (Figure 5D for mRNA and 5E for protein). Therefore, these two drugs appeared to affect AR activity only in HBx-expressing liver tissues but not in the testes. To understand this difference, the expression of SHP-1 protein in the mouse liver and testis was examined. We observed a markedly higher SHP-1 protein level in liver than in testis (Figure 5E), even in hepatocytes purified after hepatic perfusion (Supplementary Figure 9, A and B, available online). The SHP-1 mRNA level was not concomitantly elevated in hepatocytes, suggesting the difference occurred at the post-transcriptional level (Supplementary Figure 9C, available online). This liver-enriched expression of SHP-1 has also been validated in the human specimens, with an approximately three- to nine-fold higher level in liver than in testis (Figure 5F). In addition, we found the target kinases of SHP-1, Akt, and c-Src, which were aberrantly activated in HBx-expressing livers (Figure 5, C and G), remained at very low basal levels in testes (Figure 5G). These results may explain why sorafenib and SC-1 preferentially block HBx-induced AR activity specifically in the liver of HBx-Tg mice but scarcely affect the normal AR activity in WT livers and testes.

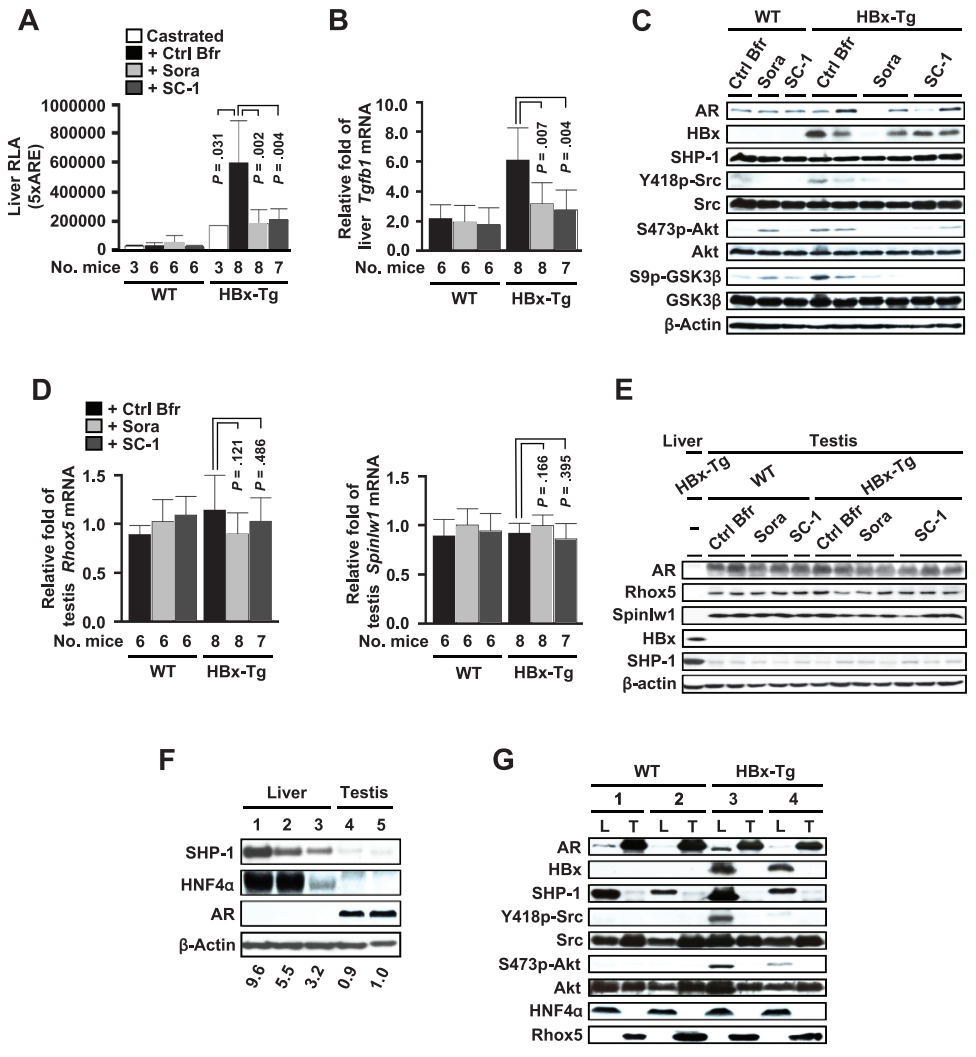

Figure 5. SC-1 and sorafenib specifically repressed HBx-induced hepatic androgen receptor (AR) activity in vivo. **A)** The 5xARE-Luc reporter construct was delivered into the livers of wild-type (WT) or HBx-transgenic (HBx-Tg) male mice by hydrodynamic injection. The hepatic AR activity in mice treated with sorafenib, SC-1 (10 mg/kg/day), or control buffer was determined via reporter assay at 48 hours postinjection. The castrated mice were included as a basal control. **B)** The expression of endogenous AR target gene *Tgfb1* in the liver of each group was determined by real-time quantitative polymerase chain reaction (RT-qPCR). The group of WT mice treated with control buffer was set as 1 for relative quantification. **C)** The hepatic expression of AR, HBx and the phosphorylation levels of indicated protein kinases were confirmed by western blot. **D)** The testicular AR function in these mice after drug treatment was evaluated via the expression of two AR target genes, *Rhox5* and *Spinlw1*. The RNA expression of these targets was quantitated by RT-qPCR. The group of WT mice treated with control buffer was set as 1 for relative quantification. **E)** The protein amounts of *Rhox5*, *Spinlw1*, and AR were examined by western blot. **F)** Hepatic and testicular SHP-1 protein expression in clinical human specimens was analyzed by western blotting. The **number at the bottom of each lane** indicates the relative signal strength of SHP-1. **G)** The kinase activities of Akt and c-Src in livers (**L**) and testes (**T**), indicated by their phosphorylation at specific residues, were evaluated in WT and HBx-Tg male mice for comparison. The expression of HNF4α and Rhox5 in livers and testes, respectively, served as tissue-specific markers. **A, B, and D)** Data are means, and **error bars** represent standard deviation. *P* values were calculated by two-sided Student's *t* test. AR = androgen receptor; 5xARE = the luciferase reporter with promoter containing five repeats of AR responsive element; RLA = relative luciferase activity; Sora = sorafenib; WT = wild-type.

Finally, we tried to examine if sorafenib could function as a chemopreventive drug of HCC in HBx-Tg mice. The slow-releasing sorafenib and the placebo tablets were implanted in the male HBx-Tg mice at six weeks of age. At 11 months of follow-up, the mean ALT level in six placebo control mice was 89 ± 62 unit/L, which, however, was 17 ± 4 unit/L in the six mice implanted with the sorafenib tablets ($P = .017$, by two-sided Student's *t* test). The preliminary results indicated that sorafenib treatment can reduce the serum ALT level, a surrogate tumor marker in this animal model (11), supporting its potential for chemoprevention of HCC in HBx-Tg male mice. The follow-up studies are ongoing and will end when these mice reach 24 months of age.

Discussion

The androgen pathway has long been implicated in HBV-related carcinogenesis in epidemiological studies (4,5). It is now proven by our HBx-induced spontaneous HCC mouse model in the current study. This HBx/AR is thus proposed as a target for HCC intervention (7,28). Interestingly, we identified that sorafenib, the only FDA-approved drug for advanced HCC (29,30), effectively suppressed HBx-induced AR activation, both in vitro and in vivo. This AR-targeting ability of sorafenib was not mediated by its well-known kinase inhibitory activity, but was instead achieved by enhancing the activity of SHP-1 tyrosine phosphatase. Our anchorage-independent colony formation assays well demonstrated that blocking this pathway by sorafenib and

SC-1 statistically significantly decreased the growth of the HBx/AR-stable cells in soft agar (Supplementary Figure 10, available online), supporting their potential to inhibit the oncogenic AR activity induced by HBx.

Our study further revealed that a novel function of SHP-1 is to shut down two kinase switches critical in mediating the HBx effects on AR, the c-Src, and Akt/GSK3β (6), which thus makes the AR inhibitory function of SHP-1 specific to the HBx-induced liver diseases (schematically illustrated in Figure 6A). However, how SHP-1, a tyrosine phosphatase, functions to regulate the phosphorylation of serine residues on Akt (at S473), GSK3β (at S9), and AR (at S81) remains to be addressed. One priority candidate is the c-Src kinase, which has been shown to execute the tyrosine phosphorylation of Akt at Tyr315/326 to prime for subsequent phosphorylation of Akt at S473 (31–33), which in turn decreases the phosphorylation of GSK3β-S9 (20). However, direct action by c-Src on AR-S81 is not possible and requires another mediator molecule, for example the CDK1 as a candidate (Supplementary Figure 11, available online) (34–36). The true molecules mediating these activities still remain to be identified.

Although the animal studies and colony formation experiments suggested a potential to inhibit the tumorigenic activity induced by an overactivated AR pathway, several randomized controlled trials of anti-androgens for advanced and unresectable HCC in men did not exhibit any efficacy or survival benefit (37–39). Our HBx-Tg mouse experiments provided valuable information by demonstrating that tumor incidence was only statistically significantly decreased by early castration (before 3 months of age) and was unaffected by castration thereafter (Figure 1D). These results suggested that the androgen-AR axis acts as a promoter in the early carcinogenic process, not as a cancer-perpetrating or cancer-maintaining moiety. Notably, the ligand-stimulated AR have been recently reported to have imprinting-like effects for the transcription of specific target genes via epigenetic modifications (40,41), which were activated by exposure to androgen in early life (42,43). Therefore, it is reasonable to speculate that the oncogenic effect of AR may

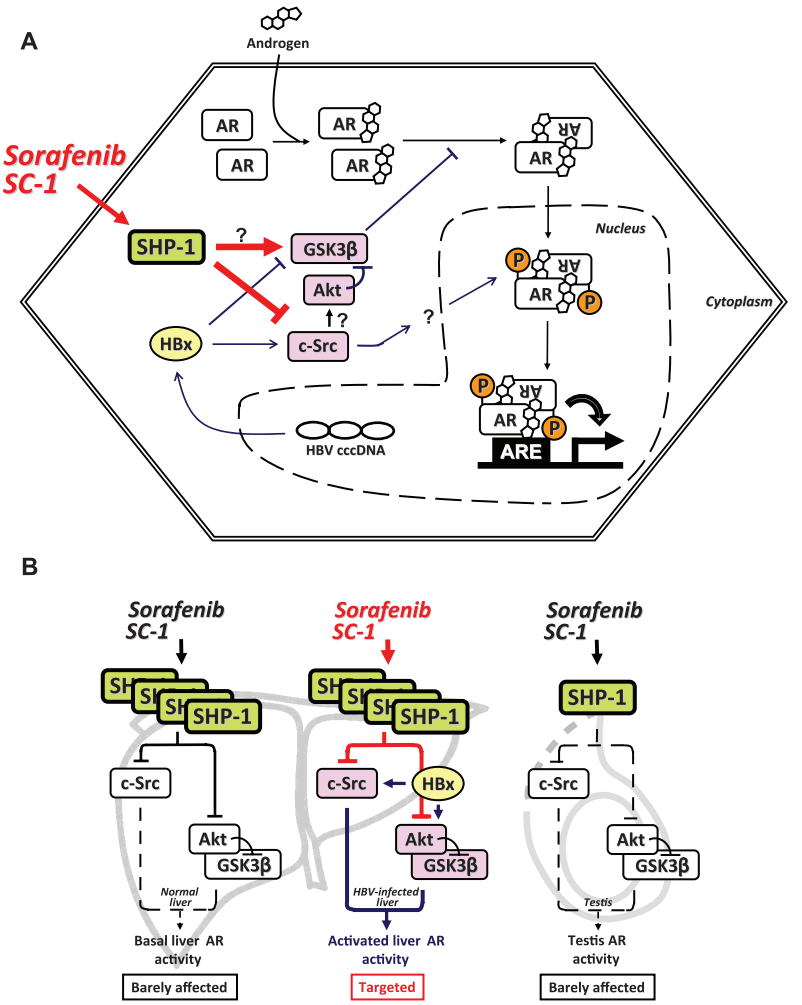

Figure 6. Effect of sorafenib and SC-1 on HBx-enhanced hepatic androgen receptor (AR) activity via SHP-1. **A)** In HBV-infected hepatocytes, sorafenib and SC-1 repressed the HBx-enhanced AR activity via SHP-1, leading to the shutdown of two kinase switches, Akt/GSK3β and c-Src, which are aberrantly activated by HBx. This SHP-1–mediated kinase targeting will interfere with the N/C interaction and AR phosphorylation at serine 81, counteracting the ability of HBx to promote the aberrant activation of the hepatic AR pathway. The steps to be conclusively addressed are indicated as **question marks**. **B)** A proposed model illustrating the tissue specificity of sorafenib and SC-1 for inhibiting AR activity between liver and testes. In HBx-expressing liver tissues (**middle panel**), both c-Src and Akt kinase switches are abnormally activated, leading to elevated hepatic AR activity. Sorafenib and SC-1 specifically antagonize HBx-evoked c-Src and Akt activity through SHP-1, thus turning down the AR aberrance in HBV-infected livers but barely affecting basal AR activity in normal livers (**left panel**). Based on the tissue difference of SHP-1 expression levels and kinase activities, the targeting effects of sorafenib and SC-1 on HBx-enhanced AR activity are barely detected in testes for reproductive AR functions (**right panel**). AR = androgen receptor; SHP-1 = Src homology region 2 domain-containing phosphatase-1.

be through the epigenetic modifications imprinted in the early stages of HBV-induced carcinogenesis. In previous clinical studies, the anti-androgens may have been administered too late during the advanced stages of HCC, in which androgen/AR is not critical anymore and thus were not effective. Instead, anti-androgens may be more effective when administered earlier as chemo-preventive agents.

Currently, most available anti-androgen drugs (as summarized in Supplementary Figure 12, available online), especially for treating prostate cancer, inhibit the canonical AR pathway in all tissues including the reproductive organs, and thus are not appropriate as chemopreventive agents for the long-term treatment of young CHB patients. Our current study may provide a solution for this dilemma. We established that sorafenib and its SC-1 derivative were able to specifically suppress the aberrant HBx-stimulated AR activity in liver, through activating the liver-enriched SHP-1.

Two tiers of control restrict the drug effects specifically in HBx-expressing liver but not in normal liver or testis. First, sorafenib and SC-1 inhibit the AR pathway in a disease-specific manner. In contrast to traditional anti-androgens, sorafenib and SC-1 block AR dimerization and S81 phosphorylation by dampening the activity of GSK3β and c-Src (Figure 6A). The aberrant activities of these two kinase switches were only observed in the HBx-expressing diseased liver tissues (Figure 5G). Our in vivo data did show that the drugs statistically significantly inhibited AR activity in the livers of HBx-Tg male mice but not in the livers of WT mice or in the testes of either mouse strain (Figure 5, A, B and D). Secondly, the higher expression level of hepatic SHP-1, acting as an essential mediator for the targeting mechanism of sorafenib and SC-1, provides a better drug responsiveness for activation of SHP-1 in the liver, but with less responsiveness in testis because of its very low expression (schematically illustrated in Figure 6B). Therefore, we expect these drugs will represent as a novel paradigm and provide a considerable advantage over the current systemic anti-androgen compounds for chemoprevention of HCC in CHB patients without causing chemical castration.

This work also had some limitations. First, whether this mechanism derived from the HBx-Tg mouse model can be applied to human cases remains to be examined. In fact, in the recent STORM trial, sorafenib adjuvant therapy has been given to postcurative HCC patients to prevent HCC recurrence (44). It may be informative to study the subgroup of male CHB patients enrolled in this trial for any efficacy. Secondly, it is noteworthy that male HCC could run through different pathways, depending upon the etiology. The heterogeneity of HCC might limit the generalizability of sorafenib, which warrants future identification of the proper biomarker for predicting its applicability in the clinic.

In addition to the tissue and disease specificity, we found that sorafenib and SC-1 were more effective in inhibiting the AR pathway than bicalutamide, a well-established drug that is commonly used in first-line treatment for prostate cancer (Supplementary Figure 13, available online). Currently, we have further developed a panel of novel SC compounds without kinase-inhibitory activity (18,19), which are assumed more suitable for chemopreventive purposes by showing higher efficacy in activating SHP-1. Their efficacy is currently being evaluated in our HBx-Tg mouse model. These sorafenib derivatives may hold a potential in preventing HBV-related HCC in high-risk groups, which can be used in conjunction with standard antiviral drugs as a chemopreventive agent in male CHB patients to further reduce the incidence of HCC (45,46).

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Notes

SHW: conducting experiments, acquisition of data, analysis and interpretation of data, drafting of the manuscript; SHY: designing study concept, analysis and interpretation of data, drafting of the manuscript, obtained funding, study supervision; CWS and KFC: technical and material support; WHL: help handling animal-related experiments; TFT and YCT: animal-related experiments and analysis; DSC: critical revision of the manuscript for important intellectual content; PJC: study concept and design, critical revision of the manuscript for important intellectual content.

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