A Novel Small Molecule Activator of Nuclear Receptor SHP Inhibits HCC Cell Migration via Suppressing Ccl2

Zhihong Yang1,2, Angela N. Koehler3,4, and Li Wang1,2,5,6

Abstract

Small heterodimer partner (SHP, NR0B2) is a nuclear orphan receptor without endogenous ligands. Due to its crucial inhibitory role in liver cancer, it is of importance to identify small molecule agonists of SHP. As such, we initiated a probe discovery effort to identify compounds capable of modulating SHP function. First, we performed binding assays using small molecule microarrays (SMM) and discovered 5-(diethylsulfamoyl)-3-hydroxynaphthalene-2-carboxylic acid (DSHN) as a novel activator of SHP. DSHN transcriptionally activated Shp mRNA, but also stabilized the SHP protein by preventing its ubiquitination and degradation. Second, we identified Ccl2 as a new SHP target gene by RNA-seq. We showed that activation of SHP by DSHN repressed Ccl2 expression and secretion by inhibiting p65 activation of CCL2 promoter activity, as demonstrated in vivo in Shp−/− mice and in vitro in HCC cells with SHP overexpression and knockdown. Third, we elucidated a strong inhibitory effect of SHP and DSHN on HCC cell migration and invasion by antagonizing the effect of CCL2. Lastly, by interrogating a publicly available database to retrieve SHP expression profiles from multiple types of human cancers, we established a negative association of SHP expression with human cancer metastasis and patient survival. In summary, the discovery of a novel small molecule activator of SHP provides a therapeutic perspective for future translational and preclinical studies to inhibit HCC metastasis by blocking Ccl2 signaling.

Introduction

Chemokines have been implicated to play an important role in many aspects of tumor cell biology, including regulation of tumor cell growth, metastasis, and host immune response (1). Chemokine (C-C motif) ligand 2 (CCL2), also known as monocyte chemoattractant protein-1 (MCP-1), recruits and activates monocytes during the inflammatory response. Recent studies showed that patients with high levels of tumor-associated CCL2 expression had a significantly shorter survival (2). CCL2 association with the epithelial–mesenchymal transition (EMT) and metastases was also reported (3). Targeting tumor-infiltrating macrophages via CCL2/CCR2 signaling was examined as a therapeutic strategy against hepatocellular carcinoma (HCC; ref. 4). Using a small molecule inhibitor to modulate CCL2 production may serve as a means to suppress HCC progression (5).

Small heterodimer partner (SHP, NR0B2) is a unique orphan nuclear receptor that acts as a transcriptional repressor (6) and inhibits transcription of its downstream target genes (7). SHP plays a crucial role in various hepatic metabolic pathways to control bile acid and lipid metabolism, homocysteine homeostasis, and circadian rhythm (8–15). Although the SHP protein contains a ligand-like binding domain (16), it remains unsolved whether SHP has endogenous ligands (17).

Our previous studies revealed a role of SHP in the negative regulation of HCC cell proliferation (18). The expression of SHP was markedly diminished in human HCC due to epigenetic silencing (19) and SHP repressed DNA methylation via suppressing DNA methyltransferase (Dnmt) expression (20, 21). Activation of SHP by its agonists retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN) and 4-[3-(1-adamantyl)-4-hydroxyphenyl]-3-chloroanilinic acid (3-Cl-AHPC) induced HCC cell apoptosis by modulating Bcl2 function (22, 23). SHP interacted with p53 and Mdm2 proteins to control their ubiquitination and stability (24, 25). These studies suggest strongly that SHP functions as a tumor suppressor in HCC.

The above findings prompted us to further explore whether there is a connection between the role of SHP and HCC metastasis. In the present study, we investigated the effect of SHP in HCC cell invasion and migration using a novel chemical probe. First, we discovered a small molecule compound 5-(diethylsulfamoyl)-3-hydroxynaphthalene-2-carboxylic acid (namely, DSHN) that functions as a transcriptional activator of SHP using small molecule microarrays (SMM; ref. 26). Second, we identified Ccl2 as a downstream target gene of SHP. Third, we showed that activation of SHP by its novel agonist inhibited HCC cell invasion and

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migration by suppressing Ccl2 expression. Lastly, we established a negative correlation between SHP expression and human cancer metastasis and patient survival. Because extrahepatic metastasis of HCC is a significant clinical problem, our studies shed lights on the perspective of using small molecule activator of SHP to inhibit HCC metastasis by blocking Ccl2 signaling.

Materials and Methods

Animals, cell lines, plasmids, and reagents

Wild-type (wt) and Shp−/− (sko), non-transgenic control (nc), and hepatocyte specific Shp transgenic (sgx) mice were described previously (9, 22). Protocols for animal use were approved by the Institutional Animal Care Committee at the University of Utah and University of Connecticut. Nmuli, Hepa1, HEK293T, MHCC97H, MHCC97L, Hep3B, and Huh7 cells were purchased from ATCC in 2010 and were made aliquots and stored in liquid nitrogen until use. Mouse serum was centrifuged and stored at −80°C until use. For mouse samples, liver tissues were homogenized in lysis buffer (50 mmol/L Tris-HCl, 2 mmol/L EDTA, pH 7.4 and 1× protease inhibitors; Thermo Scientific) on ice. Samples were centrifuged at 13,000 × g for 10 minutes and stored at −80°C until use. Mouse serum was centrifuged and stored at −80°C. Experiments were performed in triplicate wells, and the data were averaged.

Small molecule microarray screens

SMMs were manufactured as described previously using isocyanate-mediated capture (27). In total, 21,600 printed features were screened, including 9,152 products of diversity-oriented synthesis, 9,152 commercial compounds that included natural products and drug-like compounds, 772 known bioactive compounds, 144 compound controls, and 2,380 DMSO solvent controls. Printed microarrays were screened using three replicates and incubated with 1 μg/mL of purified SHP N-terminally tagged with His6 in TBST buffer for 60 minutes at 4°C with gentle agitation. The protein was purchased from Panomics (#RP1030) and supplied at 0.1 mg/mL in a storage buffer of 50 mmol/L Tris-HCl (pH 8.0), 138 mmol/L NaCl, 27 mmol/L KCl, and 1 mmol/L DTT. Slides were briefly rinsed in TBST buffer and then incubated with an Alexa Fluor-647-labeled anti-His5 antibody (Qiagen #34600) for 60 minutes at 4°C with gentle agitation. The slides were washed in TBST buffer for 1 minute followed by a wash in TBS buffer 1 minute and dried by centrifugation. Dried slides were scanned for fluorescence at 635 nm using a Genepix 4000B microarray scanner. Control arrays were probed with antibody only. GenePix Pro 6.0 software was used to identify fluorescence intensity values for a set 300-μm diameter centered over each feature. Each SMM spot intensity was scored by its deviation from a population of vehicle-control spots on the same slide as described previously (28). Three replicate measurements were combined as weighted averages of deviations, normalized by the variance of corresponding vehicle-control distributions and measurement uncertainties. We called a positive “hit” any compound whose normalized score for protein binding exceeded the expected score for the most extreme acceptable vehicle-control outlier at a fixed statistical significance (family-wise error rate P < 0.05 by the Holm–Bonferroni method; ref. 28), indicating a greater likelihood that the compound was a member of a putative “hit” distribution than of the vehicle-control distribution.

Cell migration and invasion assays

The cell invasion and migration assays were described previously (30, 31). Briefly, cells were infected with the indicated adenovirus for 48 hours. The cells were then serum starved for 24 hours and 5 × 10^5 cells were seeded on Transwell inserts (8-μm pore size; Cultrex 96-well cell migration assay; cat# 3465-095-K). Cells were allowed to migrate for 16 hours, and the nonmigratory cells were removed from the insert with a cotton swab. The migrated cells were fixed for 10 minutes (3.7% v/v formaldehyde in PBS) before staining with 0.1% crystal violet for 15 minutes, followed by washing with PBS. Pictures were taken with Microfire/Qam CCD Olympus 1 × 81 microscope. Crystal violet–stained cells were counted and then lysed with 1% SDS for 30 minutes, and absorbance was measured at 595 nm. Cell invasion assays were performed similarly as migration assays, except that cells were seeded on precoated inserts with BME solution (Cultrex 96 well BME cell invasion Assay; cat# 3455-096-K). Invaded cells were treated with Cell Dissociation Solution/Calcein-AM for 1 hour, and fluorescence was measured at 485 nm excitation and 520 nm emission.

qPCR, transient transfection, and luciferase assay

In brief, cells were transfected with the plasmids as indicated in the figure legends. Transfection was carried out using Lipofectamine 2000 (Invitrogen). Luciferase activities were measured and normalized against Renilla activities (Promega). Experiments were done in three independent triplicate transfection assays. Detailed methods were described previously (8, 9). The mammalian two-hybrid assay was described previously (16).

Statistical analysis

All the experiments were performed in triplicate and repeated at least two times. The data are presented as the mean values ± standard error of the mean (SE). Statistical analysis was carried out using the Student t test for unpaired data to compare the values between the two groups. P < 0.01 was considered statistically significant.
**Results**

Discovering a small molecule that functions as a transcriptional activator of SHP

We used SMMs for ligand discovery (32), which generated 20 hits (Supplementary Fig. S1). We tested the ability of the positive compounds to alter SHP expression and identified compound #29, i.e., DSHN as an assay positive that bound SHP protein applied to the microarray. The chemical structure and detailed information of DSHN is shown in Fig. 1A; Supplementary Fig. S2. Our initial test observed that treatment of HCC cells (MHCC97H, Hepa1, and Huh7) with high concentrations of DSHN did not affect cell viability as determined by the MIT assay (Supplementary Fig. S3). Further analysis showed that DSHN markedly reactivated Shp expression in both mouse (Nmul and Hepa-1) and human (MHCC97H) hepatic cells in which Shp expression was lost (Fig. 1B). In addition, DSHN activated Shp promoter luciferase reporter (Fig. 1C, 1st panel) and augmented the transcription of several known Shp activators dose dependently, including live receptor homolog 1 (LRH-1), hepatocyte nuclear factor 4α (HNF4α), and retinoid X receptor/retinoic acid receptor α (RXR/RARα; Fig. 1C, 2nd to 4th panels). DSHN also increased SHP protein half-life (30 minutes in the DMSO group vs. 90 minutes in the DSHN group) as determined by inhibiting protein synthesis using cycloheximide (CHX; Fig. 1D). The increased stability of SHP protein by DSHN was associated with the ability of DSHN to prevent SHP protein ubiquitination and degradation (Fig. 1E). AHPN and AHPC were reported to bind to SHP (33) and inhibit SHP protein ubiquitination (22), thus both served as positive controls. On the other hand, DSHN did not alter the interaction of SHP with LRH-1 and HNF4α (Supplementary Fig. S4A–S4B), as determined by the mammalian two-hybrid approach.

To test whether DSHN induces Shp expression in vivo in mouse liver, we treated mice with DSHN using two methods. Because Shp is an integral part of the liver circadian clock network and its expression shows a rhythmic variation (9), we collected samples at multiple time points. First, DSHN was given to mice via i.v. injection and livers were collected before injection and 2 hours, 4 hours, and 8 hours after injection. Shp mRNA exhibited a peak induction 2 hours after DSHN treatment (Fig. 1F, top). To further confirm a direct effect of DSHN on Shp expression, we conducted serum shock experiment to synchronize Huh7 cells (29). The expression of Shp mRNA exhibited two peaks; both were highly induced upon DSHN treatment (Supplementary Fig. S4C). Overall, compound DSHN is a potent activator of SHP through both transcriptional and post-transcriptional regulation.

Ccl2 is a direct transcriptional target of SHP

A recent study established a role of SHP in TLR-dependent inflammatory response (34). From our RNA-sequencing (RNA-seq) datasets (GEO accession no: GSE43893; ref. 13), we found an upregulation in the expression of Ccl family members in Shp+/− (sko) versus wt liver (Fig. 2A). In particular, Ccl2 showed the highest induction. Due to the importance of CCL2 in both inflammation and cancer metastasis, we examined Ccl2 expression regulation by SHP. The upregulation of Ccl2 and its receptor Ccr2 mRNA in sko versus wt liver was confirmed by RT-PCR (Fig. 2B, left). This was accompanied by the elevated levels of CCL2 production in sko liver and serum (Fig. 2B, middle and right). In contrast, Ccl2 mRNA was reduced in hepatocyte Shp-overexpressed transgenics (tg; ref. 20) as compared with the non-transgenic control (nc; Supplementary Fig. S5A).

To determine a direct cell-autonomous inhibition of Ccl2 by SHP, we used an in vitro cell culture system. Hep3B cells have a high basal level of SHP and were used to examine the effect of SHP knockdown on CCL2 expression. CCL2 mRNA (Fig. 2C) and CCL2 production (Fig. 2D) was markedly increased by SHP knockdown (siSHP) but decreased by Shp overexpression (Ade-Shp). CCL2 secretion was also largely diminished by Ade-Shp in Huh7 cells (Supplementary Fig. S5B).

We analyzed Ccl2 promoter and identified NF-κB binding sites (Supplementary Fig. S5C). SHP was reported to interact with NF-κB subunit p65 (RelA) to mediate cell apoptosis (35). Thus, we examined the effect of SHP on p65-mediated Ccl2 promoter activity. Promoter luciferase reporter assays revealed that p65 dose dependently activated Ccl2 promoter (both ENH and LNG), which was markedly suppressed by Shp (Fig. 2E; Supplementary Fig. S5D). In addition, DSHN treatment diminished CCL2 mRNA expression that correlated with SHP activation (Fig. 2F). Furthermore, CCL2 protein was dose dependently diminished by DSHN in multiple HCC cells (Fig. 2G). Interestingly, a stronger inhibition was observed in MHCC97H and Huh7 (high migration potential) as compared with HepG2 (low migration potential), suggesting that DSHN may be more effective in invasive cells. Taken together, our results demonstrate that Ccl2 is a direct transcriptional target of Shp and its expression can be inhibited by DSHN.

Activation of SHP inhibits Ccl2-mediated HCC cell migration and invasion

SHP served as a tumor suppressor in HCC by inhibiting cell proliferation and activating apoptosis (18, 22); however, the role of SHP in cell migration remains unknown. By comparing different HCC cell lines, MHCC97H exhibited the highest migration potential as compared with Hep3B, Huh7, and MHCC97L (Fig. 3A). Interestingly, CCL2 production and its expression were positively correlated with the migration potential of HCC cells (Fig. 3B), whereas SHP expression showed an inverse correlation. We determined the effect of SHP in inhibiting HCC cell migration and invasion by examining multiple HCC cell lines. Cell migration assay revealed that overexpression of SHP markedly suppressed the potential of Huh7 and Hepa1 (Fig. 3C) and MHCC97H cells (Fig. 3D) to migrate through the Transwell membrane.

Next, we determined the direct effect of SHP on CCL2-mediated H79 cell invasion. Treatment of recombinant CCL2 protein dramatically stimulated H79 cell invasion, whereas overexpression of SHP antagonized the ability of CCL2 to promote cell invasion (Fig. 4A). Huh7 cells expressed higher basal level of SHP than H79 and thus these were used to examine the effect of SHP knockdown. As expected, siSHP markedly augmented the effect of CCL2 to stimulate Huh7 cell migration (Fig. 4B). In addition, treatment of H79 cells with DSHN significantly diminished CCL2-induced cell migration (Fig. 4C). The cell migration potential of Huh7 cells was also inhibited by DSHN (Fig. 4D). It was...
noted that the inhibitory effect of DSHN was less striking than that of Ade-SHP (Fig. 4A), which could be due to the fact that the level of SHP was much highly expressed and induced by Ade-SHP than by DSHN. Overall, the results suggest that SHP inhibition of HCC cell invasion and migration is at least in part mediated by its ability to suppress CCL2 expression and function.
SHP expression is negatively associated with cancer metastasis and patient survival

To further establish the clinical relevance of SHP in human cancer metastasis and survival, we interrogated a publicly available database to retrieve the SHP gene expression profile from multiple types of human cancers. The DNA copy number of SHP was noticeably reduced in HCC patients based on The Cancer Genome Atlas (TCGA) database (Supplementary Fig. S6). As

Figure 2.
SHP functions as a transcriptional suppressor of Ccl2. A, RNA-seq of relative expression levels of the Ccl family in sko versus wt liver. B, left, semi-quantitative PCR of hepatic Ccl2, Ccr2, and Shp mRNA in wt and Shp−/− (sko). Middle and right, ELISA of CCL2 levels in liver and serum of wt and sko. Data represent mean ± SE of triplicate assays (*, P < 0.01 vs. white bar). C, semi-quantitative PCR (left) and dPCR (middle and right) of CCL2 and SHP mRNA in Hep3B cells with Shp overexpression or knockdown (siSHP) using adenovirus (100 MOI). Lower band, mouse Shp (Ade-Shp); higher band, endogenous human SHP. Data represent mean ± SE of triplicate assays (*, P < 0.01 vs. white bar). D, ELISA of CCL2 protein levels in culture medium of Hep3B cells infected with indicated adenovirus (Ad, 100 MOI). Data represent mean ± SE of triplicate assays (*, P < 0.01 vs. white bar; ¥, P < 0.01 vs. siSHP, black bar). E, promoter luciferase report assay. Hepa1 cells were cotransfected with the indicated plasmids for 48 hours. Data represent mean ± SE of triplicate assays (*, P < 0.01 vs. white bar; ¥, P < 0.01 vs. 2nd bar). F, semi-quantitative PCR of SHP (left) and CCL2 (right) mRNA in Huh7 cells treated with DSHN for 24 hours (10 μm). G, Western blot of the CCL2 protein in HCC cells treated with different doses of DSHN for 24 hours (MHCC97H, Huh7) or 48 hours (HepG2). Data are representative of two or more experiments.

Figure 3.
Overexpression of SHP inhibits HCC cell invasion and migration. A, comparing the migration potential in Hep3B, Huh7, MHCC97L, and MHCC97H cells. Migrated cells were visualized by the staining of crystal violet. B, left, the levels of secreted CCL2 protein in culture medium as examined by ELISA. Right, semi-quantitative PCR of CCL2 mRNA in various HCC cells. C, HCC cell migration assay. Huh7 (left) and Hepa1 (middle) cells were overexpressed Ade-ShP (adenovirus, 50 MOI) for 48 hours. Data represent mean ± SE of triplicate assays (*, P < 0.01 vs. white bar). Right, images of invaded cells stained with crystal violet. D, HCC cell migration assay. MHCC97H cells were transduced with Ade-ShP (50 MOI) for 48 hours and migrated cells were visualized by the staining of crystal violet. Data are representative of two or more experiments.
expected, SHP expression was further decreased in human metastatic HCC versus HCC (Fig. 5A). SHP expression was also markedly decreased in human gastric cancer relative to normal (Fig. 5B; ref. 36).

The Kaplan–Meier plotter is capable to assess the effect of 54,675/22,277 genes on survival using 10,188 cancer samples. These include 4,142 breast, 1,648 ovarian, 2,437 lung, and 1,065 gastric cancer patients with a mean follow-up of 69/40/49/33 months (37). Kaplan–Meier plotter analysis revealed a significant association between SHP expression and patient’s survival time. The high expression of SHP were associated significantly with better patient survival in human gastric cancer (Fig. 5C) and breast cancer (Fig. 5D). SHP expression was also negatively associated with patients’ survival time in ovarian cancer and lung cancer. However, the $P$ value did not reach significance (Supplementary Fig. S7A–B). Nonetheless, SHP expression was significantly decreased in lung cancer compared with normal epithelium (Supplementary Fig. S7C). Together, these clinical data analyses clearly indicate that SHP may function as an important survival factor and its expression may serve as a negative prognostic factor associated with high rates of metastasis and decreased disease-free survival in a wide range of human cancers.

Discussion

Small molecule microarrays (SMM) have been used previously to identify small molecules that bind and modulate specific protein targets in a cellular context, including various transcription factors (26, 28, 38). Using the SMM-based approach, we identified DSHN as a putative binder and novel agonist of SHP. Our results demonstrate a dual-function of DSHN; it not only activates Shp promoter to induce Shp gene transcription but also prevents proteasome-mediated SHP degradation to increase SHP protein stability. More importantly, DSHN may serve as a
potential therapeutic agent to inhibit HCC metastasis via SHP-mediated repression of Ccl2 signaling.

SHP possesses the essential characteristics as a tumor suppressor gene, based on its ability to inhibit HCC cell proliferation (18), activate apoptosis (22, 23), repress DNA methylation (20, 21), suppress HCC cell migration and invasion (the present study), its downregulation in HCC (19), and its inhibition of oncogenic non-coding RNAs (39, 40). In particular, SHP expression is negatively correlated with patient survival in several types of aggressive and highly metastatic human cancers, including gastric cancer and breast cancer, suggesting it may be an attractive target for cancer therapy that is not limited to HCC. It was of considerable interest to note that SHP was identified as a single gene prognostic lung cancer biomarker (41). The regulatory function of SHP in other cancers remain to be investigated.

One major obstacle to explore the possibility of targeting SHP in HCC therapeutics is that SHP is an orphan receptor and endogenous ligands for SHP have not yet been identified. Significant efforts were made in the past, which identified AHPN and 3-Cl-AHPC as the first exogenous SHP agonists (33) that were potent apoptosis inducer (22). Our study opens possible new avenues for treatment of liver cancer by restoring the lost SHP expression in HCC using DSHN. Of equal importance, identifying Ccl2 as a SHP target gene allows future mechanistic studies on the action of DSHN beyond liver cancer. DSHN might be tested in preclinical models to see whether it can induce SHP expression and inhibit tumorigenesis or malignant behavior. In summary, our study highlights the potential use of SHP as a therapeutically tractable gene in future translational and preclinical studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: Z. Yang, A.N. Koehler, Li Wang
Development of methodology: Z. Yang, A.N. Koehler, Li Wang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Yang, A.N. Koehler, Li Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Yang, A.N. Koehler, Li Wang
Writing, review, and/or revision of the manuscript: Z. Yang, A.N. Koehler, Li Wang

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Li Wang
Study supervision: Li Wang

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