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A novel obatoclax derivative, SC-2001, induces apoptosis in hepatocellular carcinoma cells through SHP-1-dependent STAT3 inactivation

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ABSTRACT

We investigated the effects of a novel compound, SC-2001, on hepatocellular carcinoma (HCC). SC-2001, which is structurally related to the Mcl-1 inhibitor obatoclax, showed better antitumor effects than obatoclax in HCC cell lines, including HepG2, PLC5 and Huh-7. Like obatoclax, SC-2001 inhibited the protein–protein interactions between Mcl-1 and Bak. However, SC-2001 downregulated the protein levels of Mcl-1 by reducing its transcription whereas obatoclax had no significant effect on Mcl-1 expression. As Mcl-1 is regulated by signal transducers and activators of transcription 3 (STAT3), we found that SC-2001 downregulated the phosphorylation of STAT3 (Tyr 705) and subsequently inhibited transcriptional activities of STAT3 in a dose-dependent manner. In addition to Mcl-1, STAT3-regulated proteins, including survivin and cyclin D1, were also repressed by SC-2001. Notably, SC-2001 reduced IL-6-induced STAT3 activation in HepG2 and PLC5 cells. Ectopic expression of STAT3 abolished the prominent apoptotic death in SC-2001-treated PLC5 cells, indicating that STAT3 is indispensable in mediating the effects of SC-2001. Importantly, SC-2001 enhanced the expression of SHP1, a negative regulator of STAT3. Inhibition of SHP-1 by either specific inhibitor or small interference RNA reduced the apoptotic effects of SC-2001, indicating that SHP-1 plays a key role in mediating SC2001-induced cell death. SC-2001 enhanced the activity of SHP-1 in all tested HCC cells including HepG2, PLC5 and Huh-7. Finally, SC-2001 reduced PLC5 tumor growth, downregulated p-STAT3 and upregulated SHP-1 expression and activity *in vivo*. In conclusion, our results suggest that SC-2001 induces apoptosis in HCC, and that this effect is mediated through SHP-1-dependent STAT3 inactivation.

1. Introduction

Advanced or recurrent hepatocellular carcinoma (HCC) is frequently resistant to conventional chemotherapeutic agents and radiation and thus remains one of the most difficult cancers to treat [1,2]. The discovery of targeted agents with tolerable toxicity is, therefore, mandatory to advance anti-HCC therapy [2]. The success of sorafenib, a multi-targeted receptor tyrosine kinase (RTK) inhibitor, in two randomized controlled phase III trials for advanced HCC [3,4] supports the use of molecularly targeted therapies in the treatment of advanced HCC.

One of the chief reasons that HCC cells are resistant to chemotherapy is their ability to resist apoptosis [5]. One important mechanism of apoptosis-resistance involves the dominant apoptosis regulatory protein family, B-cell lymphoma 2 (Bcl-2). Bcl-2

Abbreviations: HCC, hepatocellular carcinoma; STAT3, signal transducers and activators of transcription 3; SOCS, suppressor of cytokine signaling; SHP-1, Src homology region 2 domain-containing phosphatase 1; PARP, poly ADP-ribose polymerase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; NMR, nuclear magnetic resonance; HRMS, high-resolution mass spectrometry; LC/MS, liquid chromatography/mass spectrometry; ESI, electrospray ionization.

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family members are key regulators of the intrinsic apoptotic pathway and include both antiapoptotic (Bcl-2, Mcl-1 and Mcl-xL, *etc.*) and proapoptotic (such as BH3-only BIM and the apoptotic effectors BAX and BAK, *etc.*) proteins [6]. The antiapoptotic proteins of the BCL-2 family are overexpressed and dysregulated in various cancers, including HCC [5]. Bcl-xL and Mcl-1 in particular play significant cytoprotective roles in HCC [7–10], therefore, Bcl-2 proteins have emerged as attractive targets for novel anticancer drugs [6]. A common strategy in the design of Bcl-2 protein inhibitors is based on mimicking the actions of endogenous inhibitors that bind antiapoptotic Bcl-2 proteins via the Bcl-2 homology 3 (BH3) domains (BH3 mimetics) [11]. Obatoclax (GX15–070, Gemin X Pharmaceuticals) is a small molecule pan-Bcl-2 inhibitor that acts as a BH3-mimetic to disrupt the interactions of anti-apoptotic and proapoptotic proteins, such as Mcl-1 and Bak, and has shown early evidence of efficacy and safety in several phase I trials [12–15]. Obatoclax is currently being tested both alone and in combination with other chemotherapeutics in phase II trials in hematological malignancies and solid tumors [6]. Therefore, obatoclax is considered a promising agent for hepatobilliary cancers [5].

It is notable that some important antiapoptotic members of the Bcl-2 members family, such as Bcl-xL and Mcl-1, can be regulated by oncogenic transcription factors, such as STAT3. STAT3 is crucial in the regulation of genes involved in cell proliferation and survival, and is constitutively activated in common human cancers, including HCC [16]. Upon stimulation by cytokines, growth factors or hormones, STAT3 is phosphorylated (activated) and homodimerizes or heterodimerizes with STAT1 in the cytoplasm and then translocates to the nucleus. In cancer cells, constitutively activated STAT3 directly contributes to tumorigenesis, invasion, and metastasis [16]. Downregulation of STAT3 activity has been shown to be a promising anti-HCC strategy [16–18]. Recently, we also found that sorafenib inhibited p-STAT3, which contributed to its anti-HCC efficacy [19,20]. Interestingly, a number of protein tyrosine phosphatases have been shown to negatively regulate STAT3 signaling through direct dephosphorylation of p-STAT3 (Tyr 705); these include members of the SH2-domain containing tyrosine phosphatase family (SHP-1 and SHP-2), and protein tyrosine phosphatase 1B (PTP-1B). Loss of SHP-1 has been shown to enhance JAK3/STAT3 signaling in ALK-positive anaplastic large-cell lymphoma and in cutaneous T-cell lymphoma [21,22]. In addition, several agents such as betulinic acid [23], boswellic acid [24], and 5-hydroxy-2-methyl-1,4-naphthoquinone (a vit-K3 analogue) [25] that can enhance the SHP-1 pathway have shown anti-cancer potential. Therefore, activity of protein tyrosine phosphatases may be critical for the regulation of STAT3 phosphorylation in cancer cells.

In this study, we found that SC-2001, a novel Mcl-1 inhibiting compound which is structurally related to obatoclax, has more potent antitumor activity than obatoclax and has a novel drug mechanism (SHP-1-dependent STAT3 inactivation) that is distinct from obatoclax in HCC cells. We showed that SC-2001 not only inhibits the protein–protein interactions between Mcl-1 and Bak, but also downregulates the protein levels of Mcl-1 by reducing its transcription. We further discovered that SC-2001 downregulated the phosphorylation of STAT3 (Tyr 705) and subsequently inhibited the transcriptional activities of STAT3. Furthermore, we noticed that SC-2001 enhanced the expression of SHP-1, which played a key role in p-STAT3 downregulation and in mediating SC2001-induced apoptosis. Importantly, this SHP-1-dependent STAT3 inhibitory mechanism that mediates the efficacy of SC-2001 was confirmed in an *in vivo* nude mouse model. Our results suggest that SC-2001 is a novel STAT3 inhibitor that acts through enhancing SHP-1 activity and has promising anti-HCC efficacy.

2. Materials and methods

2.1. Synthesis, purification, and characterization of SC-2001

$PdCl_2$ (0.1 equiv, 59%) and PPh_3 (0.45 equiv) were added to a solution of toluene (1 mL). The mixture was stirred at 70 °C under nitrogen for 20 min and transferred into a flask with (Z)-2-((1H-pyrrol-2-yl)methylene)-3-methoxy-2H-pyrrol-5-yl trifluoromethanesulfonate (1.0 equiv), 5-bromo-1H-indol-2-ylboronic acid (1.2 equiv), solid sodium carbonate (1.0 equiv) and 10% water/dioxane (5 mL). The reaction mixture was stirred at 100 °C for 90 min, then poured into 10 mL water and extracted with ethyl acetate (20 mL) three times. The organic layer was collected, washed with brine, dried over $MgSO_4$ and concentrated. The crude product was collected by silica gel with the eluent ethyl acetate:hexane (1:20–1:5). SC-2001 compound was determined by nuclear magnetic resonance (NMR) and mass spectrometry (MS). ^{1}H NMR (400 MHz, $CDCl_3$) 7.65 (d, J = 1.6 Hz, 1H), 7.15 (dd, J = 8.8 Hz, J = 2.0 Hz, 1H), 7.07 (s, 1H), 6.88 (s, 1H), 6.83 (d, J = 8.4 Hz, 1H), 6.73 (s, 1H), 6.60 (d, J = 3.6 Hz, 1H), 6.22 (s, 1H), 6.12 (t, J = 3.2 Hz, 1H), 4.04 (s, 3H); LCMS (ESI): m/z 368.2 (100, M^+H^+); high-resolution mass spectrometry (HRMS) calculated for $C_{18}H_{14}BrN_3O$ (M^+H^+): 368.0393. Found 368.0345. Yield: 93%.

2.2. Reagents and antibodies

SHP-1 inhibitor was purchased from Cayman Chemical (Ann Arbor, MI). Antibodies for immunoblotting such as cyclin D1, and PARP were purchased from Santa Cruz Biotechnology (San Diego, CA). Other antibodies such as survivin, phospho-STAT3 (Tyr705), STAT3, SHP-1, SHP-2, survivin, and PTP-1B were from Cell Signaling (Danvers, MA).

2.3. Cell culture

The Huh-7 HCC cell line was obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan; JCRB0403). The PLC/PRF/5 (PLC5) and HepG2 cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). All cells obtained from HSRRB or ATCC were immediately expanded and frozen such that all cell lines could be restarted every 3 months from a frozen vial of the same batch of cells. No further authentication was conducted in our laboratory. Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G, 100 μg/mL streptomycin sulfate and 25 μg/mL amphotericin B in a humidified incubator at 37 °C in an atmosphere of 5% CO_2 in air.

2.4. Apoptosis analysis

The following methods were used to assess drug-induced apoptotic cell death: cell death detection ELISA for cytoplasmic histone associated DNA fragments (Roche, Indianapolis, IN), and western blot for PARP cleavage. Further, HCC cells were treated with DMSO or obatoclax or SC-2001 at the indicated dose for 12 h, and the specific enrichment of oligonucleosomes released into cytoplasm was quantified by cell death ELISA.

2.5. Reverse transcriptase PCR

Total RNA was isolated from cell lines with TRIzol (Invitrogen) and cDNA was prepared from 2 mg of RNA using a First-Strand Cdna Synthesis Kit according to the manufacturer's instructions (Amersham Biosciences, Amersham, UK). Oligonucleotide sequences were as follows: SHP-1, 5′-GCC CAG TTC ATT GAA ACC AC- 3′ (sense) and 5′-GAG GGA ACC CTT GCT CTT CT-3′ (antisense); GAPDH, 5′-CGA CCA CTT TGT CAA GCT CA-3′(sense) and 5′-AGG GGT CTA CAT GGC AAC TG-3′ (antisense); Mcl-1: 5′-CTT GCC ACT TGC TTT TCT GG-3′ (sense) and 5′-CAA GGC ATG CTT CGG AAA CT-3′ (antisense). The following PCR conditions were used: denaturation at 95 °C for 10 min followed by 35 cycles of 94 °C for 1 min, annealing for 1 min at 57 °C, and elongation for 1 min at 72 °C, and a final elongation step at 72 °C for 10 min.

2.6. Gene knockdown using siRNA

Smart-pool small interfering RNAs (siRNAs), including the control (D-001810-10) and SHP-1 were purchased from Dharmacon (Chicago, IL). The knockdown procedure was as described previously [26]. Briefly, PLC5 cells were transfected with siRNAs against the phosphatases given above or the control sequence for 48 h and then treated with SC-2001 at the indicated concentrations. The cell extracts were analyzed by western blot.

2.7. PLC5 cells with ectopic expression of STAT3

STAT3 cDNA (KIAA1524) was purchased from Addgene plasmid repository (http://www.addgene.org/). STAT3-overexpresed PLC5 cells derived from a single stable clone were prepared for evaluating the major target of SC-2001. Briefly, following transfection, cells were cultured in the presence of G418 (0.8 mg/mL)