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Therapeutic effect of a multi-targeted imidazolium compound in hepatocellular carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most commonly diagnosed lethal cancers in the world. We previously showed two imidazolium salts (IBN-1 and IBN-9) with a moderate efficacy for HCC. Here we report a more potent imidazolium compound IBN-65 (1-benzyl-2-phenyl-3-(4-isopropyl)-benzyl-imidazolium chloride) and the associated mechanisms of action in a mouse model of HCC. The IC_{50} of this compound in various liver cancer cell lines was around 5 μM. IBN-65 dose-dependently arrested cell cycle at G1 phase and was associated with the down-regulation of the cyclin-dependent kinase-4, -6, cyclin D1, and cyclin E. In addition, IBN-65 induced apoptosis by down-regulating Survivin, Bcl-2 and up-regulating Bax, leading to sequential activation of Caspase-3, Caspase-9 and the cleavage of poly(ADP-ribose) polymerase (PARP). Dysregulation of the epidermal growth factor receptor (EGFR) signaling network has been frequently reported in HCC. We found that IBN-65 displayed a profound inhibitory effect on the EGFR/Raf/MEK/ERK signaling at the phosphorylation level. In Huh7 or Hep3B cells, pretreatment with IBN-65 attenuated EGF-induced phosphorylation of both EGFR and the downstream p44/42 MAPK. A siRNA knockdown of EGFR also proved that IBN-65 induced apoptosis mostly through inhibiting downstream EGFR pathway signaling, much less at the receptor level. Infrequent administration of IBN-65 (i.p., 5 mg/kg once weekly for four weeks) to mice bearing the Huh7 cells significantly reduced the tumor volume by 65% without affecting the body weight. Critically, many of the anti-tumor signaling features observed in the HCC cell lines were recaptured in the xenografted tissues. Thus, the metal-free imidazolium compound IBN-65 could be a potential candidate towards therapeutic development for HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and third most frequent cause of malignant deaths worldwide, with more than 600,000 new cases diagnosed each year [1]. It has a poor prognosis due to its resistance to chemo and radiation therapies [2]. Hepatic resection remains the most effective treatment for early HCC, but patients with advanced HCC would not have a chance to undergo surgery. Post-operative reoccurrence and metastasis of the cancer also contribute to the poor prognosis.

The epidermal growth factor receptor (EGFR) signaling pathway plays a critical role in the control of cellular proliferation, differentiation, oncogenesis, and is often associated with aggressive disease, metastasis, and drug resistance in a wide range of human cancers [3–5]. EGFR over-expression and mal-signaling is common in HCC. Immunohistochemical analysis showed that EGFR was over-expressed in 66% of the HCCs [6]. In addition, EGFR-driven cell signaling contributes to the disease progression and cancer malignancy [7]. The downstream effectors of EGFR, including RAF-p44/p42 MAPK, were known to involve in cell growth, cell proliferation and survival. In response to growth factor stimulation or oncogene activation, the RAF → MEK → ERK pathway can elicit effects on gene transcription, mRNA translation, or post-translational modification on the D- and E-type cyclins, and cyclin-dependent kinases (cdk), to regulate $G_0 \rightarrow G_1 \rightarrow S$ phase progression during cell cycle [8].

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Currently several EGFR-targeting drugs, including two monoclonal antibodies (mAbs), Cetuximab and Panitumumab and small-molecule inhibitors of EGFR tyrosine kinases such as Gefitinib, Erlotinib are being evaluated for the treatment of HCC [9,10]. Lapatinib is a dual inhibitor of EGFR and HER-2/neu, and inhibits tumor growth by down-regulating MAPK, AKT, and p70S6 kinase [11]. Sorafenib is a small multi-kinase inhibitor approved by FDA for the management of late stage HCC [12]. It merely prolongs the life of advanced HCC patients by approximately three months, associated with significant side effects. Hence there is a great need to develop new therapeutics that will be more efficacious or synergistic with the current ones.

Imidazolium salts (IMSs) are precursors to N-heterocyclic carbene (NHC), which are routinely use as ligands or organo-catalysts in synthetic chemistry. Recently, we have developed various imidazolium salts and its oligomers, and further demonstrated that these IMSs have shown antioxidative, antifibrotic [13–15], anticancer [16], antibacterial and antifungal agents [17–19]. In this study, we described the synthesis and mechanistic study of an IMS with more potent antitumor efficacy in HCC cell lines and a xenografted HCC mouse model.

2. Materials and methods

2.1. Compounds and growth factor

The synthesis of IBN-65 was described in the Supporting information. Stock solutions of IBN-65 (1 and 10 mM) were prepared in dimethyl sulfoxide (DMSO). Aliquots of the stock solution were stored at room temperature. Sorafenib was purchased from Symansis (Auckland, NZ), U0126 inhibitor was purchased from Calbiochem (Billerica, MA, USA). Recombinant EGF was purchased from R&D systems (Minneapolis, MN, USA).

2.2. Cell culture, MTS assay, cell cycle analysis and Western blot analysis

All the procedures were essentially performed as previously described [16]. Antibodies used for Western blotting or immunohistochemistry were listed below. Antibodies against Survivin, Cdk2, Cdk4, Cdk6, Cyclin E and Cyclin D1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against pEGFR, EGFR, pMEK 1/2, MEK 1/2, P-p44/4 MAPK, p44/42 MAPK, B-Raf, C-Raf, pSTAT3 (T727), pSTAT3 (t705), STAT3, XIAP, Caspase-9, Caspase-3, cleaved Caspase-3, PARP, Bad, Bax, Bcl-$_{xL}$ and Bcl-2 were from Cell Signaling (Danvers, MA). Antibody against β-actin was from Sigma (St. Louis, MO). Horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgG was from GE Healthcare Biosciences (Piscataway, NJ).

2.3. STAT3 reporter assay

Huh7 cells cultured to 30% confluence in a 96 well plate were used for the STAT3 reporter (firefly luciferase, LumF) assay using Cignal™ STAT3 reporter assay kit (SABiosciences, Frederick, MD, USA). After 30 h of transfection, cells were treated with IBN-65 (1 μM or 10 μM) for 8 h. A dual luciferase assay was performed and promoter activity was expressed as arbitrary units. Renilla luciferase (LumR) vector was used as an internal control for transfections in the study. The relative reduction of STAT3 is calculated by (LumF IBN-65/LumR IBN-65)/(LumF control/LumR control) × 100%. Transfections were carried out in triplicates.

2.4. Survivin reporter assay

Huh-7 cells were plated onto 12-well plates at a density of 10^5 cells/well and grown for 24 h. Cells were transfected with plasmids from the Survivin Gene Promoter Reporter vector kit from Panomics Inc. (Fremont, USA). After transfection, the cells were treated for 7 h or 24 h with IBN-65 or vehicle at either 1 μM or 10 μM. Cell lysates were collected for luciferase activity assay according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA).

2.5. Small interfering RNA (siRNA)

The siRNA duplexes used in this study were purchased from Invitrogen and had the following sequences: EGFR (siRNA ID: VHS41680), GCA GUC UUA UCU AAC UAU GAU GCA A; and Stealth RNAi negative control. Huh7 and Hep3B cells were seeded onto 6-well plates and transiently transfected with siRNA oligonucleotides in OPTIMEM with Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommended protocol. Western blot was used to examine the siRNA knockdown efficiency at 48 h and 72 h post-transfection.

2.6. HCC xenograft tumor assay

Animal experiments were carried out with prior approval from the Institutional Animal Care and Use Committee (IACUC). Balb/c nude mice, 5–6 weeks old, were inoculated with 5 × 10^6 Huh7 cells in 0.2 ml volume of Matrigel (BD Biosciences, San Jose, CA USA)/DMEM mix. The tumor appeared after two weeks and the tumor-bearing mice were randomly divided to a control and a treatment group ($N = 8$ for each group), respectively. For the treatment group, mice were i.p. injected with IBN-65 (5 mg/kg) once per week for four weeks. The control group was treated with PBS. The tumor size was measured weekly on the same day of treatment for four weeks with an additional measurement at the end of experiment. The tumor volume was calculated using the formula: 0.52 × width^2 × length. The body weight was monitored during the experiment for evaluating the general toxicity of IBN-65.

2.7. Immunohistochemistry

The explants were cryosectioned and fixed with ice-cold methanol. The sections were blocked with normal goat serum (5% in PBS). The sections were incubated over night with specific primary antibodies diluted in 5% normal goat serum. The sections were washed with PBS and exposed for 2 h to Alexa fluor 647 labeled secondary antibodies. The mounted sections were observed under confocal microscope. Image J software was used to edit the micrographs.

3. Results

3.1. Effect of IBN-65 on HCC cell proliferation

Treatment of Huh7, HepG2, and Hep3B cells with IBN-65 (Fig. 1A) resulted in a dose-dependent inhibition of cell proliferation and the IC_{50} value of HepG2, Huh7 and Hep3B was found to be 4, 5 and 5.5 μM, respectively (Fig. 1B).

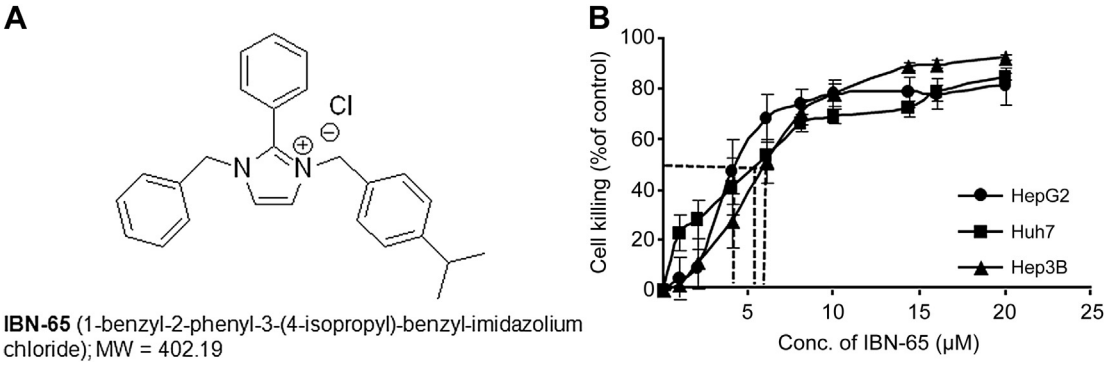

Fig. 1. IBN-65 structure and growth inhibition in HCC cells. (A) Chemical structure of IBN-65. (B) IBN-65 suppressed growth of Huh7, Hep3B and HepG2 cells. Cells were incubated with IBN-65 at the concentrations indicated for 48 h. Cell proliferation assays were performed using the MTS assay. A dose-dependent inhibition of growth in various hepatocarcinoma cells was observed. The growth inhibition was calculated as percentage with the control being taken as 100%.

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