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 IC50 of this compound in various liver cancer cell lines was around 5 \( \mu \)M. 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.

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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 recurrence 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 \( G_0 \) → \( G_1 \) → \( S \) 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-molecule 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 used 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 antioxidant, 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 (Billericia, 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-p44/42 MAPK, p44/42 MAPK, B-Raf, C-Raf, pSTAT3 (T272), 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 10³ cells/well and grown for 24 h. Cells were transfected with plasmids from the Survivin Gene 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 × 10⁶ 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 × width² × 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 overnight 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 IC₅₀ 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%.
3.2. Effect of IBN-65 on HCC cell cycle

As shown in Fig. 2A and B, IBN-65 treatment (10 μM for 48 h) increased the percentage of cells accumulated in G1 phase from 58% to 80%, and the same time lowered the percentage of cells in the S and G2-M phase. Furthermore, IBN-65 decreased the expression of cell cycle regulatory molecules such as Cyclin D1, Cdk4, and Cdk6 (Fig. 2C). We observed that prolonged exposure to 10 μM of IBN-65 led to an increase in apoptotic cells. A two-day treatment with 10 μM of IBN-65 induced 8% of the cells going through apoptosis (Fig. 2A and B), and a five-day treatment resulted in a 10-fold increase (80%) in apoptotic cell population (Fig. 2D and E). To further investigate whether IBN-65 induced apoptosis in Huh7 cells through Caspase-dependent mechanism, cell lysates from IBN-65 treated or untreated Huh7 cells were subjected to Western blot analysis. As shown in Fig. 2F, significant activation of Caspase-3, Caspase-9 and PARP cleavage through apoptosis (Fig. 2A and B), and a greater percentage (80%) of cell killing (Fig. 4A). Cell cycle analysis showed an increasing number of cells (20% in subG0 cell cycle phase) in the case of combination of IBN-65 (10 μM) and U0126 (20 μM), as compared to 10 μM of IBN-65 alone (9% in subG0 cell cycle phase) (Fig. 4B). The significantly potentiating effect with the combination of IBN-65 and U0126 at the level of apoptosis was shown by activation of Caspase-3, Caspase-9 and PARP cleavage (Fig. 4C). These results concluded that the IBN-65 induced cell death indeed through the MEK/ERK pathway.

3.5. Effects of IBN-65 on STAT3 and Survivin expression

ERK is a major MAP kinase downstream of the EGFR pathway. Phosphorylation of ERK leads to the activation of its downstream transcription factor STAT3 and Survivin, which are responsible for cell proliferation and survival. We studied the effect of IBN-65 on STAT3 and Survivin. Western blot analysis (Fig. 5A) showed that IBN-65 dose- and time-dependently downregulated the phosphorylation of STAT3 at Ser727 and the total STAT3 protein. We also found that IBN-65 reduced the expression of Survivin in Huh7 cells (Fig. 5B). To determine if IBN-65 can regulate the transcription of STAT3 and Survivin genes, we transiently transfected the Huh7 cells with the STAT3 and the Survivin promoter- luciferase constructs separately. Twenty-four hours after transfection, IBN-65 was added to the culture media, and the cells were incubated for additional 7 h. Assay data showed that 10 μM of IBN-65 significantly suppressed the Survivin and STAT3 transgene promoter activity by 80% (Fig. 5C) and 50% (Fig. 5D), respectively. Our additional data indicated that the inhibitory effects by IBN-65 on the transcription of the transgenes can occur as early as 6 h after the compound addition and last at least to up to 24 h (data not shown).

3.6. Effects of IBN-65 on EGFR phosphorylation

EGFR is an upstream receptor of the Raf/MEK1/2/ERK pathway. Binding of EGF to EGFR activates EGFR, which further signals to molecules such as PI3K/Akt, Ras, and MAPK. In this experiment, we used small molecule inhibitor U0126 to inhibit ERK signaling. EGFR at 2.5 ng/ml or 10 ng/ml was noted to enhance phosphorylation of EGFR and p44/42 MAPKs without changing the expression of either EGFR or p44/42 MAPKs (Fig. 6A and B). We observed a maximum phosphorylation of EGFR and p44/42 MAPKs after 10 min with either 2.5 or 10 ng/ml of EGF treatment, whereas phosphorylation of EGFR and ERK1/2 decreased after 30 min. Hence we next chose the 10 min time point to examine whether pre-treatment with IBN-65 could prevent EGFR phosphorylation and p44/42 MAPKs. Pre-incubation of Huh7 and Hep3B cells for 2 h with 10 μM of IBN-65 diminished the EGF-dependent phosphorylation of EGFR and p44/42 MAPKs proteins (Fig. 6C and D).

3.7. Minimal role of ERK on apoptosis

To investigate the possible role of EGFR in IBN-65 mediated cell death, we knocked down the expression of EGFR using siEGFR in Huh7 and Hep3B cells. EGFR expression in the cell lines transfected with EGFR-specific siRNAs was severely reduced, compared to the negative control siRNA (Fig. 7A and B). The downstream target of EGFR, P-p44/42, also significantly decreased in the EGFR knockdown cells (Fig. 7C, lane 4). Knocking down EGFR alone (Fig. 7C, lane 4) resulted in a 25% increase in cell killing, when compared to the negative control siRNA (Fig. 7C, lane 1). Treatment of EGFR knockdown group with IBN-65 (0, 1, and 10 μM) caused an increase in cell killing in a dose-dependent manner (Fig. 7C, lanes 4–6). Similarly, IBN-65 treatment also resulted in a dose-dependent cell killing in the scramble siRNA group with (Fig. 7C, lanes 1–3). Western blotting results revealed several lines of information: 1) the EGFR knockdown by siRNA was not complete, since a faint residual EGFR signal was visible in Fig. 7D, lanes 5 and 6 (the band in lane 5 may be too faint to been seen on the final print); 2) a very low level of residual EGFR was dramatically induced by EGF to yield a very high level of pEGFR, alone with a
Fig. 2. IBN-65 treatment significantly induces a G1 phase cell cycle arrest and apoptosis. The cells were serum starved for 24 h prior and treated with different doses of IBN-65 for 2 days and 5 days. Treatment with 0.05% DMSO was used as solvent control. The cells were fixed with 70% ice-cold ethanol, stained with PI and analyzed with flow cytometry. Representative results of the cell cycle profile of Huh7 at Day 2 (A) and Day 5 (D). A summary of cell cycle distribution in Huh7 cells after treatment with different doses of IBN-65 (B–E). Data represents the mean of three independent experiments and error bars indicate standard deviation. (C) Western blot analysis of cyclin E1, cyclin E2, CDK4, CDK6 and pRB. (F) Western blot analysis of activation of Caspases and the degradation of the PARP in Huh7 cells treated with DMSO, 1 and 10 μM of IBN-65 for the indicated times. The relative levels of protein expression were normalized with β-actin.

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Fig. 3. IBN-65 inhibits Raf/MEK/p44/42 signaling proteins. (A) Huh7 cells were treated with DMSO (vehicle control) and IBN-65 (1 µM & 10 mM) for 2 h, 4 h and 14 h. Western blot analysis was performed using antibodies specific to the phosphorylated forms of MEK 1/2 and p44/42. Antibodies against total B-Raf, C-Raf, MEK 1/2 and p44/42 were also used in Huh7 (A) and Hep3B (B).

Fig. 4. MEK inhibitor U0126 sensitizes Huh7 cells to IBN-65-induced apoptosis. Huh7 cells were exposed to 1 or 10 mM of IBN-65 for 48 h either directly or after pre-incubation with 10 µM or 20 µM U0126 for 1 h and then the old medium was replaced with fresh medium containing IBN-65 only. A represents the percentage of cell killing counted by hemocytometer. B represents the percentage of cells undergoing apoptosis as detected by PI staining using flow cytometry. C represents the immunoblot analysis on cell lysates with anti-Caspase-3, anti-Caspase-9, anti-PARP, and anti-β-actin antibodies.
Fig. 5. IBN-65 inhibits STAT3 and Survivin expression. Huh7 cells were treated either with DMSO (vehicle control) or 1 μM or 10 μM of IBN-65 for 2 h, 4 h and 14 h. Cell lysates were subjected to Western blotting to analyze the expression levels of pSTAT3 and total STAT3 (A), dose-dependent effect of IBN-65 on Survivin (B), Survivin promoter activity (C) and STAT3 promoter activity in Huh7 cells (D). Twenty-four hours after transfection, the indicated doses of IBN-65 were added to the culture medium and incubated for an additional 24 h. Luciferase assay was carried out as described in the methods. Values are expressed as percentage increase in relative luciferase activity over control. Data are expressed as mean with SD. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001.

Fig. 6. IBN-65 inhibits EGF-induced phosphorylation of EGFR and p44/42 proteins in hepatocellular carcinoma cells. Serum-starved Huh7 (A) and Hep3B (B) cells were treated with either 2.5 ng or 10 ng EGF for the indicated period of time and cell lysates were analyzed for phosphorylation status of EGFR and p44/42 by Western blot. The same blot was re-probed with β-actin or antibodies against total EGFR or total p44/42. Huh7 (C) and Hep3B (D) cells were treated with EGF (2.5 ng or 10 ng) followed by treatment with IBN-65 (10 μM). Cells were harvested and analyzed the expression of phosphorylation of EGFR and p44/42 by Western blot. The same blot was re-probed with β-actin or antibodies against total EGFR or total p44/42.

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3.8. Effects of IBN-65 on mouse HCC model

We further investigated of the effect of IBN-65 in a xenograft mouse model bearing Huh7 tumors. Results showed that IBN-65 treatment (5 mg/kg, i.p. injection once weekly for four weeks) reduced the average tumor volume by more than 65% as compared with the control group (N = 8) (Fig. 8A). No significant change in body weight was noted between the control and IBN-65 groups (Fig. 8B), suggesting that IBN-65 at this dosing scheme had minimal general toxicity. The explants were further evaluated by histology and immunohistochemistry. Overall the tumor size in the mice treated with IBN-65 was significantly reduced, when compared to the control group. H&E staining of the tumor explant sections revealed less fibrotic tissue in the animals treated with IBN-65. Immunohistochemistry with specific antibody against cleaved Caspase-3 showed substantial positive staining in the IBN-65 treated samples indicating that the cells have undergone apoptosis in this group (Fig. 8C). Control samples did not show significant staining for Caspase-3 antibody. Staining with proliferation marker Ki67 indicated that IBN-65 treated group displayed lower expression of Ki67 when compared to the control group (Supplementary Fig. S4A). The Ki67 staining further supports the finding that IBN-65 inhibits the cell proliferation. The extent of vascularization was analyzed in the sections from the explants using an antibody against CD31. Immunostaining results revealed an overall reduction in the CD31 expression in the IBN-65 treated group, when compared to the control group (Fig. 8C). This result suggested that IBN65 could potentially inhibit angiogenesis. Furthermore, immunostaining with antibodies to phospho EGFR and phospho p44/42 revealed a substantial reduction in the phosphorylation of EGFR and p44/42 (Fig. 8D). Additionally we did not observe any change in the expression p44/42 between control group and IBN-65 treated group (Supplementary Fig. S4B). The immunohistochemical data were further confirmed by Western blotting of protein lysates prepared from the explants. In particular, P-p44/42 was almost completely abolished by IBN-65 treatment (Fig. 8D). Taken together, these in vivo results were in agreement with our in vitro results.

Fig. 7. Down-regulation of EGFR signaling by IBN-65. Huh7 (A) and Hep3B (B) cells were transfected with EGFR siRNA or scrambled siRNA. Knockdown of EGFR was checked by Western blotting. After 48 h transfection with siControl and siEGFR, transfected cells were treated with IBN-65 for 48 h. Cell viability was measured after treatment by hemocytometer (C). The cells were harvested and analyzed the expression of total and phosphorylation of EGFR and p44/42 by Western blot (D). Lanes 1, 3, 4, 5, 6 and 7: serum-free medium; Lane 2: serum-containing medium.
4. Discussion

In this study, we investigated the potential of IBN-65 to inhibit the growth of HCC. We showed that IBN-65 exhibited dose- and time-dependent cytotoxic activity against liver cancer cell lines with different p53 status, we demonstrated that IBN-65 possessed an IC<sub>50</sub> value (~5 μM), comparable to Sorafenib, inhibiting the proliferation and inducing apoptosis in various HCC cells. We showed that IBN-65 decreased the expression of Cdk4, Cdk6 and cyclin D1, resulting in a prominent G1/S arrest of Huh7 cells. These data suggested that cell cycle arrest is mediated by limiting the supply of Cdk4, Cdk6, and cyclin D, which are essential executors in regulating G1 to S phase transition. Overexpression of Survivin in Huh7 has also been demonstrated to decrease G<sub>0</sub>/G<sub>1</sub> populations and increase S phase populations [20]. This suggests that IBN-65 regulates the cell cycle leading to cell death through Survivin downregulation. This data correlated with our previous report that IBN-1 and IBN-9 inhibited Survivin and Cdk4, and induced apoptosis [16]. It is known that activation of ERK can lead to transcription of several genes thereby promoting cell survival and cell cycle progression [21]. Our present results are consistent with other tumor models and suggest that blockade of EGFR signaling pathway by IBN-65 results in cell cycle arrest at G1 phase in Huh7 cells [22].

Huh7 cells treated with IBN-65 for 5 days underwent apoptosis as demonstrated by FACS analysis. Apoptosis took place through the intrinsic pathway involving the modulation of Bcl-2 family proteins and subsequent activation of Caspase-3 and Caspase-9 and cleavage of PARP in a p53 independent manner. This apoptotic network was detected in both p53 mutant cell line Huh7 and p53 null cell line Hep3B. In the Hep3B cells, the activation of Caspase-3, Caspase-9 and cleavage of PARP were comparable with Sorafenib. Interestingly, the action of IBN-65 in the Huh7 cells seemed to be independent of p53 at 1 μM or 10 μM, when examined for a time course from 12 h to 120 h (Supplementary Fig. S5). The action of IBN-65 in Huh7 (p53 mutant) and Hep3B (p53 null) remained similar, suggesting that the mode of action of IBN-65 is independent of p53 pathway (Supplementary Fig. S5).

Abnormal activation of EGFR signaling is a signature for many cancers, including liver cancer. The activation of EGFR and ERK1/2 MAPK signaling pathways by diverse range of extracellular stimuli including mitogens, growth factors, and cytokines makes it a target in the diagnosis and treatment of cancer [3–7]. Suppression of EGFR,
more specifically the activated EGFR and ERK1/2 MAPKs, as well as STAT3 signaling pathways could inhibit the proliferation of HCC cells [23–25]. An earlier study found that Sorafenib, a multi-kinase inhibitor for several RTKs and the Raf/MEK/ERK cascade, synergistically inhibits the growth of HCC cells [12]. Results presented here have confirmed that IBN-65 remarkably suppressed the phosphorylation of EGFR and their downstream targets such as p44/42, STAT3 and Survivin in HCC cells, which could contribute to inhibition of HCC (Huh7 and Hep3B) cell proliferation. Furthermore, pretreatment of cells with IBN-65 decreased the EGFR-mediated phosphorylation of EGFR and p44/42 in Huh7 and Hep3B cells. Blocking the EGFR expression by siRNA alone induced cell killing by 25%, when compared with the control siRNA (Fig. 7C, lanes 1 and 4). The combined treatment of EGFR siRNA and IBN-65 marginally increased tumor cell killing by roughly about 10%, when compared with control siRNA plus IBN-65 in the Huh7 cells (Fig. 7C, lanes 3 and 6). This critical observation prompted us to speculate that most of the IBN-65 cell killing effects were mediated through the suppression of the EGFR pathway downstream molecules, particularly P-p44/42 (Fig. 7D, lane 7), but not the EGFR receptor itself. Nevertheless, another line of evidence also affirmed that the IBN-65 was a moderate inhibitor for receptor tyrosine kinase, since it reduced the EGFR-induced phosphorylation of EGFR (Fig. 6C and D). It is interesting to note that the mode of action for IBN-65 is partly overlapped with that of AG1478 and Gefitinib, which was demonstrated to induce apoptosis in liver and lung cancer cells respectively through the inhibition of phosphorylation of EGFR and cell cycle arrest [26,27].

The anti-tumor property of IBN-65 was investigated in a xenograft animal model with Huh7 cells. Suppression of tumor growth in nude mice was evident without alteration in body weight. The inhibition was further evident in treated group by the increased apoptotic cells, decreased phosphorylation of EGFR and p44/42. The decreased phosphorylation status of EGFR and p44/42 was similar to the in vitro effects of IBN-65. In addition, we noticed a drastic loss of CD31 staining in IBN-65 treated explants, which hints that IBN-65 may possess some anti-angiogenic property. However, whether this property is a direct or bystander effect of the IBN-65 requires further studies.

5. Conclusions

We have demonstrated the feasibility of using IBN-65 as a potent anti-tumor agent against HCC. We have shown that IBN-65 suppressed the HCC cell proliferation by arresting cells at G1 phase and inducing apoptosis possibly via targeting the EGFR pathways, particularly the downstream signaling molecules such as p44/42. IBN-65 also induced the apoptosis independent of p53 status of the HCC cells. All these findings indicate that IBN-65 may represent a class of imidazolium salts with unique anti-cancer properties that may deserve our additional attention for their translational potential.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2014.05.022.

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