

Metal-free imidazolium salts inhibit the growth of hepatocellular carcinoma in a mouse model

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Imidazolium salts (IMs) are precursors to N-heterocyclic carbenes (NHCs), which are routinely used as ligands or organo-catalysts in synthetic chemistry. We recently identified several IMs as anti-fibrotic agents in liver fibrosis, which often has a consequence in the oncogenesis of hepatocellular carcinoma (HCC). Here, we investigate the potential anti-tumor property of three IMs (named IBN-1, IBN-9, and DPIM) in HCC cell lines and in a xenograft mouse model. Our results showed that both IBN-1 and IBN-9 significantly inhibited the cell proliferation and arrested HCC cells in the G1-phase, whereas DPIM did not have any anti-tumor activity. When tested in a Huh7 HCC xenograft mouse model, IBN-1 reduced the tumor volume by 31% ($P < 0.05$), however accompanied by a 9% loss in body weight ($P < 0.005$), suggesting a general toxicity. In contrast, IBN-9 significantly reduced the tumor volume by 45% ($P < 0.05$) and 60% ($P < 0.01$) at doses of 0.6 and 1.5 g/l in drinking water, respectively, without any loss in body weight. Our *in vitro* and *in vivo* data suggested that IBN-1 and IBN-9 inhibited the growth of HCC by suppressing the expression of Survivin and Cyclin-dependent kinases. The current study provides a proof of concept for using the metal-free IMs to develop novel anti-cancer agents.

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Hepatocellular carcinoma (HCC) is the fifth most common cancer and third most frequent cause of death of malignant deaths worldwide, with > 600 000 new cases diagnosed each year.¹ HCC develops in a setting of liver cellular injury, often accompanied with inflammation, hepatocyte regeneration, extracellular matrix protein deposition, and fibrosis/cirrhosis.² After several decades of intense research and development, the only FDA-approved drug for the management of late stage HCC so far is a small molecule multi-kinase inhibitor (Sorafenib), which prolongs the life of advanced HCC patients by ~3 months, but with significant side effects including diarrhea, weight loss, hand-foot skin reaction, and hypophosphatemia.³

Survivin is a member of the IAP (inhibitors of apoptosis proteins) family and is generally considered to be an inhibitor of apoptosis. It expresses in a number of cell types during development, but not in healthy differentiated cells, therefore in general, it is considered to be a good molecular target for developing anti-cancer therapy.⁴ So far, a small molecule YM155 has been developed as a Survivin

suppressant in a preclinical model for prostate cancer⁵ and in a clinical setting for a number of advanced solid tumors, exclusive of HCC.⁶ Furthermore, anti-sense oligonucleotides for colorectal cancer⁷ and Survivin peptide vaccine for breast cancer patients⁸ have also been attempted. A decade ago, Survivin was initially found to overexpress in several HCC cell lines and in a small-sized clinical HCC samples,⁹ which was subsequently confirmed in an independent study.¹⁰ Mechanistic studies revealed that Survivin is not only important in regulating apoptosis, but also pivotal in initializing cell cycle by interacting with Cdk4 and p21 in HCC cells.⁹ Overexpressing Survivin in several HCC lines (HepG2, Huh7, SK-Hep1, and HLE) all led to an increase in cell cycling, accompanied by a decrease in the number of cells in the G1-phase.⁹ The observations suggest Survivin as an ideal target for developing anti-HCC agents.

In addition to Survivin, Cyclin-dependent kinases (Cdks) have been investigated as a target for anti-cancer agents for anti-HCC therapies. In particular, the Cyclin D1–Cdk4 complex, which is dispensable for normal development of

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mammary gland, but is required for the development of mammary malignancy and has become an attractive target for cancer therapy.¹¹ Recently, a small molecule inhibitor (BS-181) for the Cdk-activating kinase (CAK) has been identified with IC_{50} of 21 nM for the Cdk7 in a cell-free environment, and a cellular IC_{50} in the range of 15–30 μ M for multiple breast cancer cell lines and a HCC line HepG2. When tested in a breast cancer xenograft mouse model with MCF-7, the compound was found to inhibit the growth of tumor mass by 50% after 2 weeks of treatment (i.p., at 20 mg/kg/day).¹² To our knowledge, no success has been reported for targeting HCC based on Survivin or Cdk suppression alone, not to mention a strategy for simultaneously targeting both Survivin and Cdk in HCC.

Imidazolium salts (IMs) are precursors to N-heterocyclic carbenes (NHCs), which are routinely used as ligands or organo-catalysts in synthetic chemistry. We recently showed that a novel class of IMs are anti-oxidative *in vitro*¹³ and anti-fibrotic *in vivo* in hepatic stellate cells (HSCs),¹⁴ which is a major stromal cell type contributing to fibrosis¹⁵ and carcinogenesis¹⁶ during liver injury. In fact, an anti-fibrotic compound (Tranilast) has been explored for its potential as an anti-cancer agent.¹⁷ In this study, we evaluated the anti-HCC potential of three IMs (IBN-1, IBN-9, and DPIM) and investigated the possible mechanisms of action.

MATERIALS AND METHODS

Compounds and Cell Culture

The chemical structures, names, and molecular weights for IBN-1, IBN-9, and DPIM were depicted in Figure 1. IBN-1 and IBN-9 (both 99% purity) were synthesized as previously described,¹⁸ and DPIM (96% purity) was purchased from Sigma-Aldrich Chemicals (St Louis, MO, USA). All three compounds are soluble in water or DMSO. Liver cancer cell lines (HLE, Huh7, Hep3B, HepG2) were purchased from Health Science Research Resource Bank, Japan. Normal liver (THLE-2), lung (IMR90), and breast MCF-10 cell lines were

purchased from American Type Culture Collection (ATCC, VA, USA). All these cell lines were routinely cultured in DMEM (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum and 1% of mixture of penicillin and streptomycin in a humidified CO_2 incubator at 37 °C, and split at 1:4 ratio by trypsinization at confluence.

Cell Proliferation Assay, Immunocytochemistry, and High Content Screening

Cell proliferation assay and immunocytochemistry were performed as previously described.¹⁴ The high content screening (HCS) and imaging (Figure 4a and b) were acquired on the ArrayScan VTI HCS reader (Cellomics, Pittsburgh, PA, USA) using antibody against Survivin (Lab Vision, Fremont, CA, USA).

Cell-Cycle Analysis

Cells initially seeded in the regular medium on 10 cm dishes for 24 h were synchronized with the serum-free DMEM medium for additional 24 h. The cells were then treated with IBN-1 (10 or 100 μ M) or IBN-9 (120 μ M) for 24 h prior to harvest by trypsinization. After a fixation in 70% ethanol for 2 h, the cells were resuspended in propidium iodide (3 μ M) staining solution containing RNase A. The DNA content and the cell-cycle distribution of cancer cells were measured with a flow cytometer and quantified with the CellQuest software (FACSCalibur, BD Biosciences, NJ, USA). These experiments were repeated three times for statistical analysis.

TUNEL and Immunohistochemistry

The tumors were collected and fixed in 4% paraformaldehyde (in 1 × PBS, pH 7.4) for overnight. After fixation, the tumors were embedded in paraffin and sectioned in 5 μ m thickness. TUNEL staining was performed using TdT *In Situ* Apoptosis Detection Kit (TA4627, R&D System) according to its standard protocol. For immunohistochemistry, tissue sections were deparaffinized with xylene, rehydrated in a series of descending ethanol concentrations and microwaved in antigen retrieval buffer (Tris-EDTA, pH 9.0). The sections were then permeabilized with 0.5% Triton X-100 and blocked in PBS containing 10% horse serum. The slides were incubated with rabbit anti-human Ki67 antibody (1:250, AB833, Abcam) overnight at 4 °C. After rinsing with PBS three times, the slides were incubated with donkey anti-rabbit Alexa Fluor 488 (1:250, Molecular probes, Invitrogen) for 1 h at room temperature. DAPI was used for counterstaining of cell nuclei. The slides were rinsed with PBS three times and mounted with VECTASHIELD Fluorescent Mounting Media (H-1000, Vector Labs). The tissues were examined and photographed using Leica DM IRB inverted microscope.

Western Blot

Protein extraction and blotting were done similarly as previously reported.¹⁹ The membrane was then probed with primary antibodies against cIAP, xIAP (R&D Systems,

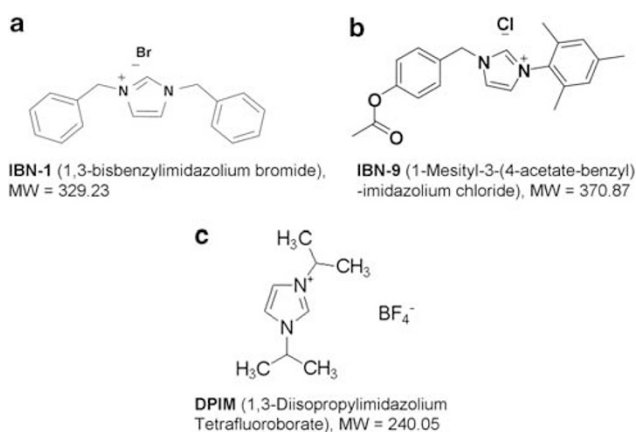


Figure 1 Chemical name and structure for IBN-1 (a), IBN-9 (b), and DPIM (c).

Minneapolis, MN, USA), Survivin, p53, Mdm2, Cdk2, Cdk4, Cdk6 (Santa Cruz Biotechnology, CA, USA), Cdc2 (Cdk1), Cyclin B, D, E, pRB, E2F1, Caspase-3, -9, PARP, Bcl-2, Bax (Cell Signaling Technology, MA, USA), and β -actin (Sigma, USA), and detected by horseradish peroxidase-conjugated secondary antibody (GE Healthcare, UK). Protein bands were recorded on X-ray films by reacting with ECL chemiluminescence reagents (Amersham Biosciences, NJ, USA).

HCC Xenograft Model

Balb/c nude mice, 5–6 weeks old, were inoculated with 1×10^7 Huh7 cells in 0.2 ml volume of Matrigel/DMEM mix. Since not every mouse developed tumor after the inoculation of HCC cells, only those with visible tumors ($\sim 50 \text{ mm}^3$ in volume; about 8 weeks after inoculation) were used for subsequent experiment. The tumor-bearing mice were randomly divided to the control and the treatment group ($N=8$), respectively. Mice in the treatment group had free access to drinking water containing IBN-1 (2 g/l) or IBN-9 (0.6 or 1.5 g/l), while mice in the control group had free access to water only. The compound treatment lasted for 3 weeks, with a weekly change of compound solution. On average, each mouse consumed about 4 ml of water or water containing IBN-1 or IBN-9 daily. The tumor size was measured weekly, and the tumor volume was calculated using the formula: $0.52 \times \text{width}^2 \times \text{length}$.

RESULTS

IBN-1 and IBN-9 Arrested HCC Cells in G1-Phase

The IC_{50} values for IBN-1 and IBN-9 in the HCC cell line HLE (day 3) were estimated to be 100 and 120 μM , respectively, which was about one fold higher than that of 5-fluorouracil (5-Fu). Similar values were obtained with the two compounds for three HCC lines (Huh7, Hep3B, and HepG2) regardless of their p53 status. Notably, IBN-1 and IBN-9 did not significantly inhibit the proliferation of non-cancerous liver (THLE-2), breast (MCF-10), and lung (IMR90) cell lines and, hence no reliable IC_{50} values could be obtained up to the mM range. DPIM did not inhibit any of the four HCC cell lines (HLE, Huh7, Hep3B, and HepG2), even when measured in the mM range. Our additional data also indicated that IBN-1 and IBN-9 lacked significant cytotoxicity, as assayed for LDH leakage in HLE and HepG2 cells using a concentration up to 2.5 mM for 24 h (data not shown). These preliminary observations suggested that the IBN serial compounds were relatively non-cytotoxic, and the anti-proliferative property of the compounds may be due to cell-cycle arrest. To investigate this possibility, HLE cells treated with IBN-1 (10 or 100 μM for 24 h) and Huh7 cells with IBN-9 (120 μM for 24 h) were harvested for cell-cycle analysis with flow cytometry. The results showed that IBN-1 (100 μM) significantly increased the percentage of cells in the G1-phase by approximately 14 percentage points

(Figure 2a). A similar G1-arresting efficiency was observed for IBN-9 (Figure 2b).

IBN-1 and IBN-9 Suppressed Expression of Survivin and Cdk5

Given the importance of Survivin as a novel regulator in multiple signaling pathways including cell cycle and apoptosis,⁴ we investigated whether the IMS compounds would have any influence on the expression of Survivin, Cdk5 and other proteins relevant to cell cycling. We treated four HCC cell lines with three compounds (IBN-1, IBN-9, and DPIM) at 10 or 100 μM for 24 h and immunoblotted the total cellular proteins to look for changes. In the HLE cells, both IBN-1 and IBN-9 dose dependently inhibited the expression levels of Survivin and Cdk4, respectively (Figure 3a). In addition, IBN-1 inhibited Cyclin D1, an interacting partner of Cdk4 and Cdk6 (Figure 3b). In a sharp contrast, DPIM did not alter the expression of Survivin nor any Cdk5. In the Huh7 cells, both IBN-1 and IBN-9 also consistently inhibited the expression of Survivin (Figure 3c). At a slightly higher concentration (160 μM , 24 h), IBN-9 inhibited the expression of Cdk2, Cdk4, Cyclin D1, Cyclin D3, and its downstream targets RB and E2F1 (Figure 3d). In contrast, DPIM had no effect on the expression of Survivin nor Cdk5/Cyclins. In the Hep3B and HepG2 cells, a variable susceptibility to the compounds was noted in terms of Survivin suppression, for example, from a very weak suppression in the Hep3B (Supplementary Figure S1A) to the very strong one in the HepG2 (Supplementary Figure S1B). To test the specificity of Survivin suppression, we treated the HLE (for 72 h) and HepG2 (24 h) cells with higher concentrations of IBN-1 (up to 1000 μM) and immunoblotted for members of the IAP family (Survivin, xIAP, and cIAP). The results showed no non-specific inhibition of xIAP or cIAP even at 1000 μM (Supplementary Figure S1C and D).

In addition to its expression level, the subcellular localization of Survivin may be critical to both mitosis and carcinogenesis,²⁰ particularly a majority of human HCC tissues displayed a predominantly nuclear staining for Survivin.^{9,10} To survey the Survivin cellular distribution in the HLE cells, an approximately 5000 unsynchronized cells stained with Survivin antibody were scanned on the HC ArrayScan VTI HCS reader, and the representative image (Figure 4a) indicated a mix of nuclear (arrow) and cytoplasmic (dotted arrows) staining for Survivin. When the cells were treated with IBN-1 (1000 μM for 24 h), the Survivin was found to translocate from the nucleus to the cytoplasm (Figure 4b). Similarly when the unsynchronized HLE cells were examined with immunocytochemistry, pan-cellular Survivin staining was noted. In addition, an intense Survivin assembly with α -tubulin in anaphase (Figure 4c, empty arrowheads) and with the midbody spindle fibers in late telophase (Figure 4c, arrowheads) was seen in late mitotic cells. Of a great interest, when the cells were treated with IBN-9 for 24 h, all the

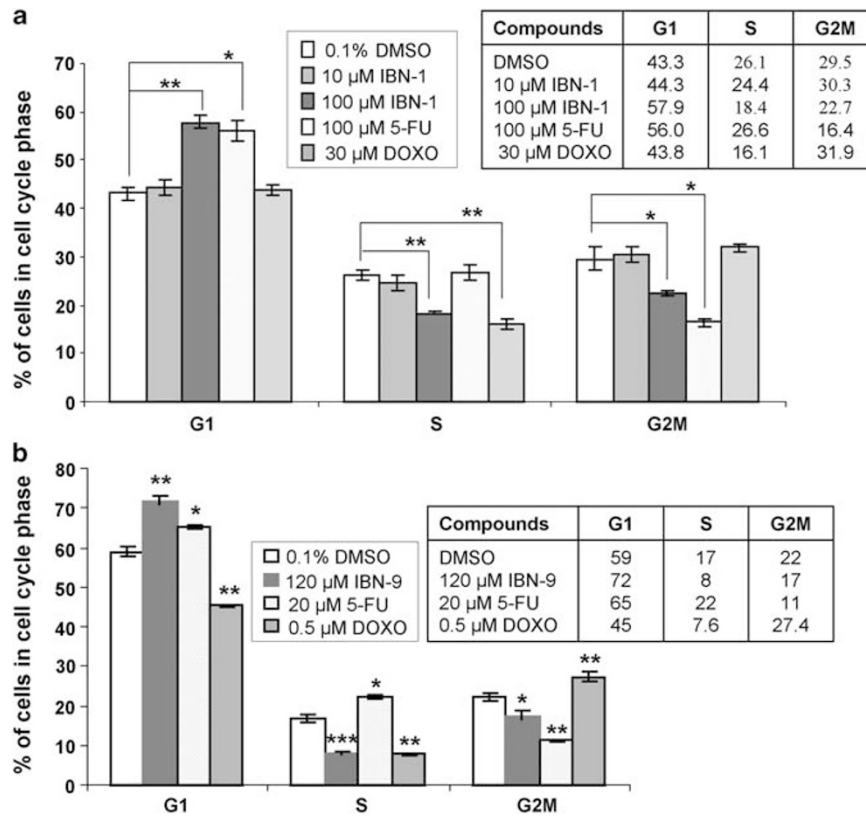


Figure 2 IBN-1 and IBN-9 arrested HCC cells in G1-phase. (a) Treatment of HLE cells with IBN-1 (10 or 100 μ M for 24 h) arrested cells in the G1-phase. Notably, the number of cells in the G1-phase increased from 43 to 58%, with a significant concurrent decrease in the number of cells in the S-phase. (b) Treatment of Huh7 cells with IBN-9 (120 μ M for 24 h) yielded similar results. All results were expressed as mean \pm s.e.m. of three experiments. * P <0.05, ** P <0.01 and *** P <0.001.

Survivin assemblies were found to be completely abolished (Figure 4d).

IBN-1 and IBN-9 Induced Caspases, but with Minimal Effect on p53 Signaling

In addition to its critical role in cell cycling (eg, inducing Cdk4), Survivin is also known to induce Bcl-2 and to inhibit Bax expression to resist apoptosis.⁴ To investigate whether the IMS compounds would have any effect on several key proteins relevant in apoptosis, we treated HLE and Huh7 cells with either IBN-1 or IBN-9 and immunoblotted total cellular protein extracts with various antibodies. The results showed that none of the two compounds had any significant influence on the protein level of p53, MDM2, and AIF (Supplementary Figure S2A–C). Similarly, the two compounds did not significantly alter the protein level for Bcl-2 and Bax families in HLE and HepG2 (Supplementary Figure S3A and B), except a slight induction of Bax by IBN-9 in the Huh7 cells (Supplementary Figure S3C). These results suggested that the two IMS compounds are not naive inducers or genotoxic agents that could trigger p53 signaling. A separate test also confirmed these IMS compounds to be free of genotoxicity (data not shown). Nevertheless, both com-

pounds can induce and cleave procaspases in Huh7 cells (Supplementary Figure S4A and B).

IBN-1 and IBN-9 Reduced the Growth of HCC in a Xenograft Mouse Model

We first tested five HCC cell lines (HepG2, Hep3B, Huh7, PLC, and HLE) for their ability to form HCC in Balb/c nude mice, and found that Huh7 line, which is enriched with cancer stem cells,^{21,22} was the most aggressive, and therefore chosen for the xenograft modeling. At the end of the treatment (third week), mice treated with either IBN-1 or IBN-9 had a reduced tumor mass (Figure 5a). Calculation from the measurement indicated a 31% (P <0.05) reduction in tumor volume in mice treated with IBN-1 (2 g/l) (Figure 5b), however, accompanied by a 9% decrease (P <0.005) in body weight (Supplementary Figure S5A). For tumor-bearing mice treated with IBN-9, a 45% (P <0.05) and a 60% (P <0.01) reduction in tumor volume was achieved at the 0.6 and 1.5 g/l dose, respectively (Figure 5c), without any body weight loss (Supplementary Figure S5B).

To assess the effects of IBN-9 on cell proliferation *in vivo*, immunohistochemistry with Ki67, Survivin and Cdk4 were performed on tumor tissues isolated from mice treated with water alone or with IBN-9 (1.5 g/l). The results showed that

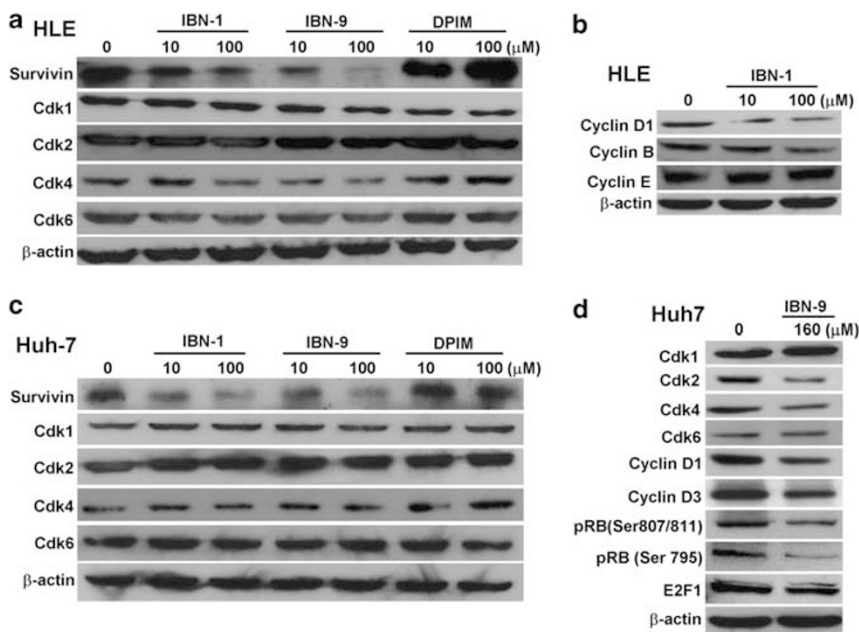


Figure 3 IBN-1 and IBN-9, but not DPIM, suppressed the expression of survivin and Cdk4 in HCC cell lines. Both IBN-1 and IBN-9 (10 and 100 μM , 24 h) inhibited the protein expression of survivin and Cdk4 (a) and Cyclin D1 (b) in the HLE cells. Similarly IBN-1 and IBN-9 (10 and 100 μM , 24 h) also inhibited the protein expression of survivin (c) in the Huh7 cells. At a slightly higher concentration (160 μM , 24 h), IBN-9 not only suppressed Cdk4, but also its binding partner Cyclin Ds and their downstream targets pRB and E2F1 (d), which would help to arrest cells in the G1-phase. Finally, DPIM did not suppress survivin nor any Cdks in both cell lines.

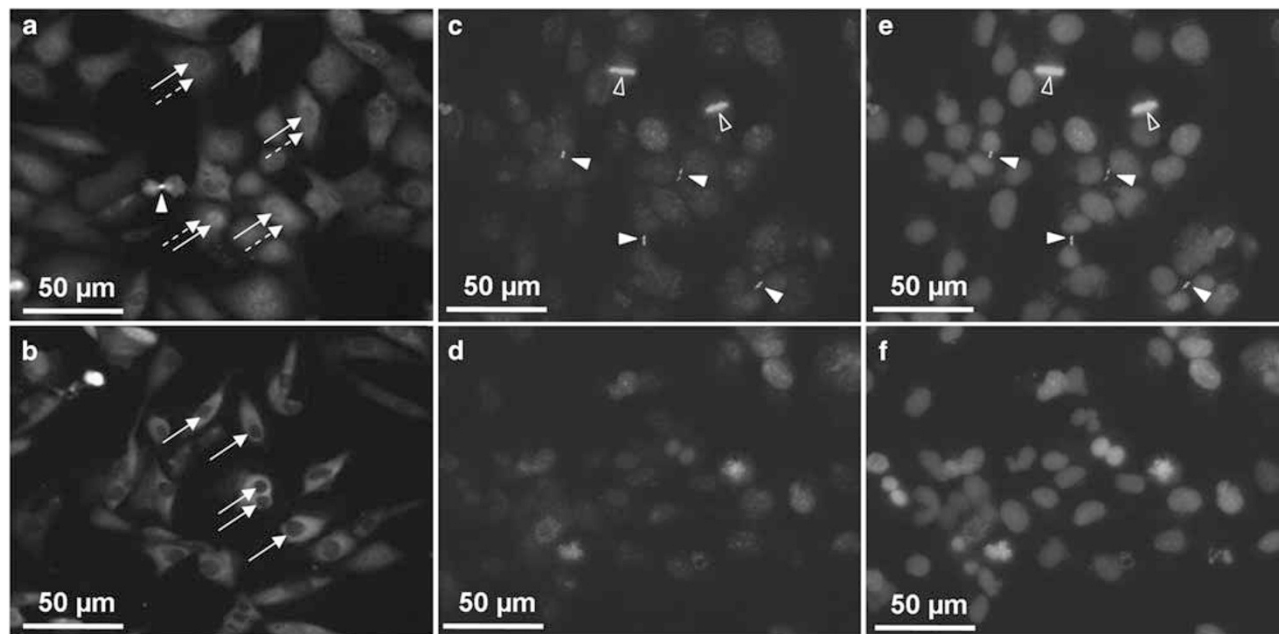


Figure 4 Effects of IMSs on survivin cellular translocation and assembly during mitosis. (a) Survivin locates in both nucleus (arrows) and cytoplasm (dotted arrows) in the HLE cells. An intense staining for survivin was seen in a cell undergoing through mitosis at the telophase (arrowhead). (b) Treatment with 1000 μM of IBN-1 for 24 h translocated survivin from the nucleus (arrows) to the cytoplasm, presumably de-coupling the survivin–Cdk4 complex and preventing cells from entering the cycling process. (c) Survivin associated with α -tubulin in the anaphase (empty arrowheads) and telophase (arrowheads) is visible in the proliferating Huh7 cells, as revealed by survivin antibody. (d) Treatment with 200 μM of IBN-9 for 24 h completely abolished the survivin assembly and interfered with cell mitosis. (e, f) Images of (c, d) co-stained with DAPI. Note in (e) that the survivin assembly at the telophase (arrowheads) was located in the midbody between two newly formed nucleus in the adjacent daughter cells.

IBN-9 treatment significantly suppressed the protein expression of Ki67, Survivin, and Cdk4 (Figure 6). By quantification, the number of Ki67⁺ cells in the treated tissue was

reduced by 66% ($P < 0.001$). In addition, the TUNEL assay results also revealed that IBN-9 triggered apoptosis in tumor tissues (Figure 7).

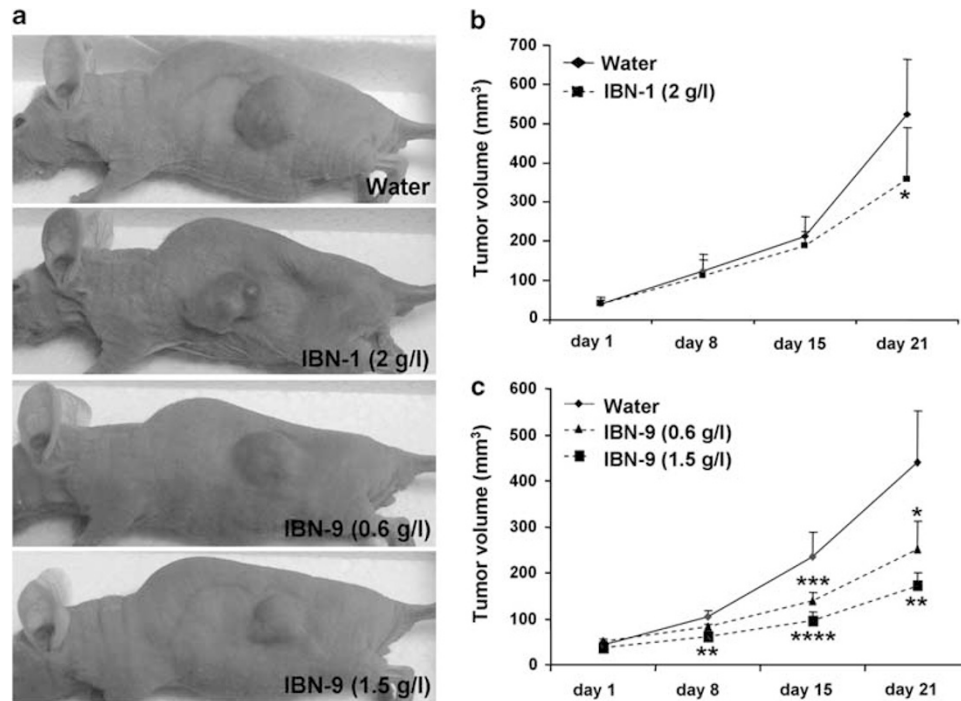


Figure 5 IBN-1 and IBN-9 inhibited Huh7 HCC xenograft in nude mice. (a) Treatment of tumor-bearing mice with IBN-1 (2 g/l) or IBN-9 (0.6 or 1.5 g/l) reduced the tumor volume by 31% (b), 45% and 60% (c), respectively. $N = 8$ for all groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ and **** $P < 0.001$.

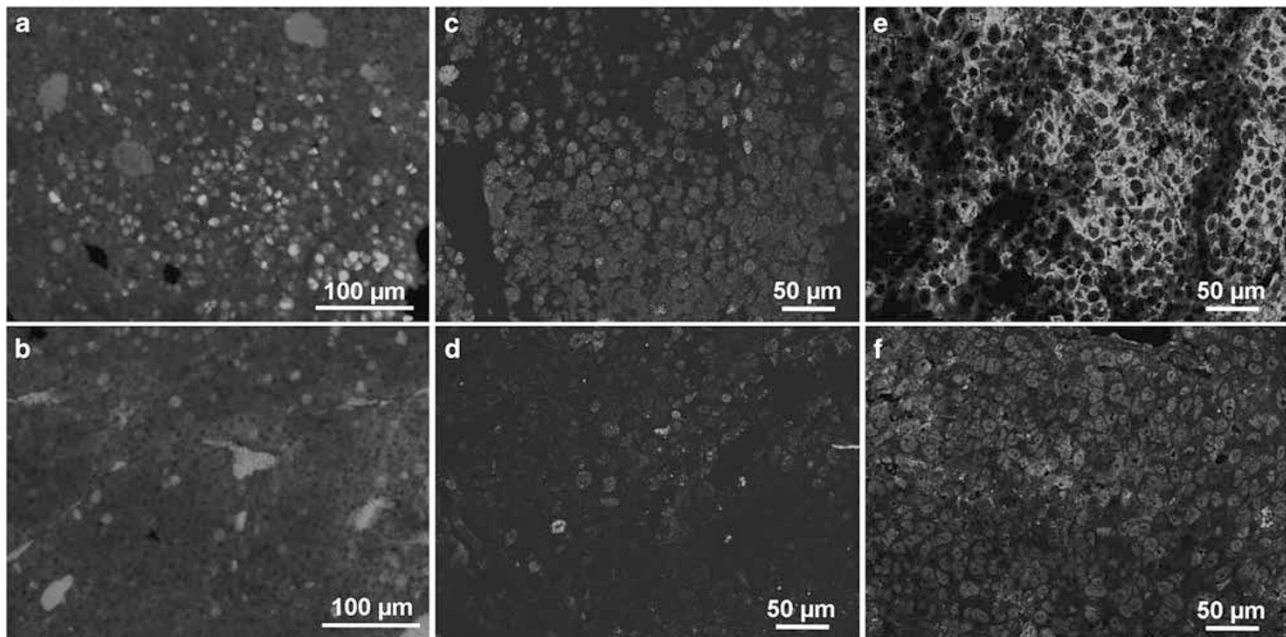


Figure 6 IBN-9 reduced cell proliferation in HCC tissue. Immunohistochemistry with the Ki67 antibody revealed an active cell proliferation in the tumor tissue (a). Treatment with IBN-9 (1.5 g/l for 21 days) resulted in a 66% ($P < 0.001$) reduction in the number of proliferating cells in the tumor (b). Similarly, the elevated expression of survivin (c) and Cdk4 (e) as seen in the untreated tumor tissues were significantly reduced in tumor tissues treated with IBN-9 (1.5 g/l for 21 days), with (d) being survivin and (f) being Cdk4.

DISCUSSION

A recent structural analysis on > 8000 FDA-approved drugs and drug candidates in the clinical trials showed that IMS is

not a major building block for the existing drugs or candidates for any indications, including cancer.²³ But in recent years, NHC-metal complex compounds with anti-tumor

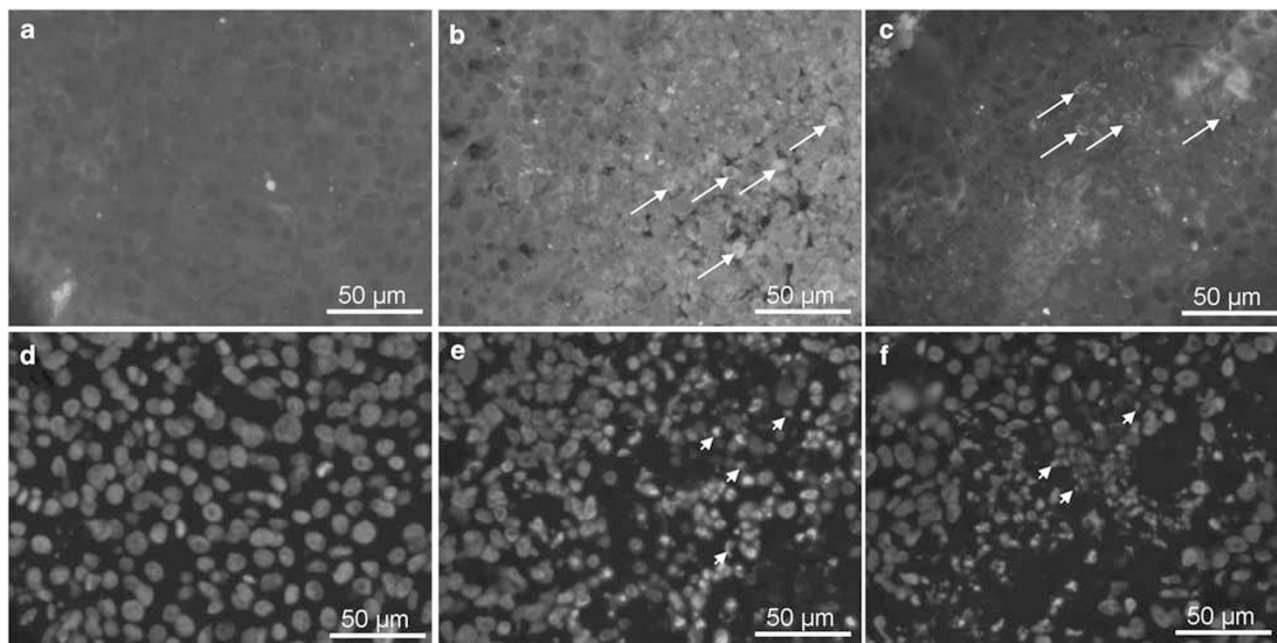


Figure 7 IBN-9 induced apoptosis in HCC tissues. No TUNEL stain was seen in the untreated tumor tissue (**a**) and the DAPI stain of the same tissue section revealed non-apoptotic nuclei (**d**). Positive TUNEL staining (green arrows) was visible in tumor tissues from two different tumor-bearing mice treated with IBN-9 (1.5 g/l for 21 days) (**b, c**). DAPI staining (blue) revealed many condensed and fragmented nuclei (going through apoptosis) (arrow heads in **e, f**) from the two identical tissue sections corresponding to (**b, c**). For color see html version.

activity have been reported.^{24–26} Most recently, a class of imidazolium-based ionic liquids with a long alkyl chain (≥ 11) at the N-3 position was shown to kill cancer cells effectively in the NCI60 panel,²⁷ which does not include any HCC cells. Here, we were independently able to show that the structurally distinctive metal-free IMS compounds, for example, IBN-1 and IBN-9, are effective anti-HCC agents *in vitro* and *in vivo*. In a preliminary structure–activity relationship (SAR) analysis, we found that the simple DPIM (Figure 1c) lacks anti-tumor activity in HCC lines, whereas IMS compounds with two benzene groups, such as IBN-1, IBN-9, and additional IBN series (IBN-19, IBN-24, IBN-25, and IBN-32; structures not shown), have significant anti-tumor activity.

Anti-cancer compounds based on the inhibition of Survivin^{5,28} and Cdk1^{12,28} have been reported and evaluated in the clinics. Among them, M₄N (tetra-*O*-methyl nordihydroguaiaretic acid) was a compound shown to arrest the transformed C3 cells in G₂-phase by simultaneously inhibiting Cdc2 (Cdk1) and Survivin expression.²⁸ On the other hand, it is not certain whether the Survivin suppressant (YM155)⁵ would also inhibit any Cdk1/Cyclins. It is clear from our results that IBN-1 and IBN-9, which are structurally distinctive from M₄N and YM155, specifically inhibit Survivin and Cdk4 *in vitro* (Figure 3) and *in vivo* (Figure 6), could be the major mechanisms for the observed cell arrest (Figure 2) and the decreased cell proliferation (Figure 6a and b). In addition, interference with the nuclear translocation (Figure 4a and b) and the progression through mitosis (Figure 4c–f)

may be another venue for the compounds to slow down the growth of cancer cells, as it was reported that Survivin in the HCC cells is mostly nuclear,^{9,10} and the nuclear location is thought to be associated with the S-phase progression.²⁹ The observation that IMS compounds (especially IBN-9) are capable of simultaneously suppressing Survivin (and its nuclear translocation) and Cdk4, thereby leading to cell arrest and apoptosis in HCC cells *in vitro* and *in vivo* represent a novel example for suppressing HCC. Although IBN-1 and IBN-9 did not significantly alter the expression of proteins relevant in p53 signaling (Supplementary Figure S2), we cannot be certain that Survivin and Cdk4 are the only two major pathways affected by the two IMS compounds.

An intriguing observation made from the current study was that IBN-1 and IBN-9 with an IC₅₀ value of around 100 μ M *in vitro* turned out to have a reasonable *in vivo* efficacy (eg, 60% tumor reduction by IBN-9). In fact, similar findings had been documented in multiple tumor types. For instance, the STAT3 inhibitor NSC74859 with an *in vitro* IC₅₀ value in monoculture close to 100 μ M effectively inhibited the growth of breast cancer³⁰ and HCC³¹ in xenograft mice. Another example was the Cdk7 inhibitor BS-181, which had an IC₅₀ of 15–30 μ M in multiple cell lines, reduced the breast tumor in mouse model by >50%.¹² The enhanced anti-tumor efficacy *in vivo* could be indirectly mediated by the compound's effect on reducing the stromal contribution to carcinoma formation and growth, with TGF- β 1 being one of the key stromal tumor-promoting components.¹⁶ It was found that HCC cells with a disrupted TGF- β 1 signaling were

more susceptible to NSC74859.³¹ A compound (Tranilast) with anti-TGF- β 1 property was recently shown to be effective in inhibiting the growth of primary mammary carcinoma and the metastasis to lung.¹⁷ Similarly, IBN-1 (also called DBZIM) with an anti-TGF- β 1 property in HSCs¹⁴ may have indirectly contributed to the anti-HCC effect *in vivo* observed in the current study. These observations cautioned us that screening and judging compounds by IC₅₀ values generated in a monoculture system *in vitro* alone may be inadequate or sometimes be misleading to obtain compound candidates with satisfactory efficacy, and PK/PD attributes *in vivo*. We also realized that the xenograft mouse model has a relatively low predictive value in developing human therapeutics, especially for compounds that may work through the stromal cells. Hence, the use of orthotopic model, ideally using primary cells derived from human HCC, would be a more accurate method for predicting compound efficacy.

In conclusion, we have synthesized and identified two metal-free IMS compounds with anti-tumor properties in HCC xenograft mouse model. Based on this study, these compounds may inhibit the HCC growth at least through the suppression of Survivin and Cdk4, as well as through the modulation of the stromal microenvironment. However, these need to be confirmed by more thorough causative experiments in future. Nevertheless, the current study reveals interesting findings on the novel anti-tumor properties of a class of unique compounds. Further, SAR and mechanistic studies on the IMS compounds would be necessary and beneficial to prove IMSs as a unique chemical pool for generating anti-tumor compound candidates with favorable mechanisms of action.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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