Bis-benzylidene Piperidone RA190 Treatment of Hepatocellular Carcinoma via Binding RPN13 and Inhibiting NF-κB Signaling

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Abstract

Background Hepatocellular carcinoma (HCC) is the fifth most common malignancy and the third leading cause of cancer mortality worldwide. The development of new anticancer agents targeting different pathways is imperative to improve the advanced HCC. The aberrant metabolism and aggressive growth of cancer cells can render them particularly susceptible to proteasome inhibition, as first demonstrated by the success of bortezomib treatment for multiple myeloma. However, resistance does emerge and this 20S proteasome inhibitor has not proven active against HCC. The bis-benzylidene piperidone RA190 represents a novel class of proteasome inhibitor that covalently binds to cysteine 88 of RPN13, a ubiquitin receptor subunit of the proteasome’s 19S regulatory particle. RA190 treatment inhibits proteasome function, causing rapid accumulation of polyubiquitinated proteins. Methods Human HCC cell lines were treated by RA190 in vitro in different concentration and time frame. We checked the killing effect and the possible mechanisms that lead the tumor apoptosis. We also performed the orthotopic HCC animal model to show the RA190 had significant killing effect in vivo. Results We showed RA190 is also toxic to HCC cells by triggering the rapid build-up of polyubiquitinated proteins, resulting in endoplasmic reticulum stress and the induction of cell death via apoptosis. Considerable evidence suggests that nuclear factor κB (NF-κB) signal is essential for promoting inflammation-associated cancer. Here, we showed that RA190 inhibited the NF-κB pathway in HCC by preventing the degradation of IκBα via the proteasome. Treatment of mice bearing an orthotopic HCC model with RA190 significantly reduced tumor growth. We therefore explored combining RA190 with a tyrosine kinase inhibitor currently used to the treat HCC, Sorafenib. Conclusions
RA190 and Sorafenib exhibited synergetic killing of HCC cells in vitro, suggesting further exploration of such a combination treatment of HCC is warranted.

Background

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and the third leading cause of cancer mortality worldwide[1]. The Barcelona Clinic Liver Cancer Staging (BCLC) treatment guidelines recommend surgical resection, liver transplantation, or radio frequency ablation for the management of early stage of HCC, whereas transarterial chemoembolization or systemic chemotherapy are used for patients with intermediate or advanced stage HCC[2]. Systemic chemotherapy for HCC is limited to only a few conventional drugs (Sorafenib[3, 4], regorafenib[5], lenvatinib, cabozantinib and ramucirumab) with unsatisfactory objective response rates[6, 7]. These drugs target multiple tyrosine kinase pathways with the exception of ramucirumab that targets VEGF-R2. Even immunotherapy (nivolumab) targeting programmed cell death protein-1 (PD-1) exhibits only a 15-20% objective response rate in advanced HCC[8]. Thus, new anticancer agents with a distinct mechanism of action are urgently needed to improve outcomes for advanced HCC patients.

The ubiquitin-proteasome system (UPS) is a complex and tightly controlled system that mediates protein homeostasis by degrading 80-90% of cellular proteins and it is central to regulating key cellular functions through targeted protein degradation. The high metabolic and protein synthesis rates that sustain the aggressive growth of cancer cells place increased burden on the UPS, thereby creating a therapeutic window. Furthermore, several driver oncogenic pathways are dependent on the UPS to mediate signaling, providing further rationale to UPS inhibition as cancer therapy[9]. Indeed, the therapeutic potential of targeting UPS in cancer has been
borne out by the licensure of three PSMB5 20S proteasome subunit-targeted inhibitors, beginning in 2003 with bortezomib for the treatment of multiple myeloma. Recently, accumulating evidence suggests that small molecules targeting alternative components of the UPS, including the receptors for its ubiquitinated substrates, also have potential for the treatment of HCC[10].

The bis-benzylidene piperidone RA190 covalently binds to cysteine 88 of RPN13 (ADRM1), a key ubiquitin receptor in the 19S regulatory particle of the proteasome, and inhibits its function causing rapid accumulation of polyubiquitinated proteins. RA190 showed a therapeutic effect in multiple myeloma, cholangiocarconoma, ovarian and cervical cancer models in previous studies [11]. In this study, we examine the impact of RA190 on HCC, including the levels of polyubiquitinated proteins, Endoplasmic Reticulum Stress, apoptosis and its therapeutic potential.

Nuclear factor κB (NF-κB) is important for promoting inflammation-associated cancer[12, 13]. Its activation triggers the transcription of numerous genes with κB binding sites, most of them are involved in the regulation of inflammation, immune responses and cell survival [14]. IkBα is the most prominent IkB family member bound to NF-κB, inhibiting its activity in the cytoplasm. Phosphorylation of IkBα by the IKK complex at two different serine residues (Ser32 and Ser36) marks IkBα for subsequent degradation through an ubiquitin-dependent pathway in the proteasome, thereby liberating NF-κB for nuclear entry, DNA-binding and transcriptional activation[15]. Inhibition of IkBα degradation by proteasome inhibitors prevents the release and nuclear entry of NF-κB. RA190 was previously reported to block NF-κB signaling via stabilization of p-IkBα. Here, we show that RA190 inhibits the NF-κB pathway by preventing IkBα degradation by the proteasome and causing the cytoplasmic accumulation of NF-κB. Finally, we also
show that combining two drugs, RA190 and Sorafenib, with distinct mechanisms of action (proteasome and tyrosine kinase inhibition respectively) had a synergetic effect in treating an orthotopic xenograft model of HCC.

Methods

Cell lines

HepG2 and Hep3B human hepatocellular carcinoma cell lines were purchased from Bioresource Collection and Resource Center (BCRC numbers RM60025 and 60434), Taiwan and were grown in DMEM medium supplemented with 10% FBS (Invitrogen), 100 μg/mL penicillin, and 100 U/mL streptomycin.

Animal

Mouse experiments were conducted with the ethics approval from Animal Care and Use Committee in Chang Gung Memorial Hospital, Keelung. Male nude mice CAnN.Cg-Foxn1nu/CrlNarl (4–6 weeks old, 20 g) were purchased from the National Laboratory Animal Center (Taipei, Taiwan). All mice were sacrificed by using CO₂ for 20 min. All animal procedures were conducted in Abnova Co., and were performed according to approved protocols and by recommendations for the proper use and care of laboratory animals.

Chemicals

RA190 and RA190B were synthesized in-house and purified to 99% as previously described [11]. Sorafenib was purchased from L.C. laboratories.
Total RNA was isolated from cells using the RNeasy mini kit (Qiagen). Extracted RNA was normalized for concentration and reverse transcribed using iScript cDNA synthesis kit (Bio-Rad). Taqman gene expression assays measured Bip-1, ATF-4, CHOP10, RPN13, iNOS, GAPDH expression levels utilizing Taqman gene expression master mix (Applied Biosystems) per the manufacturer’s instructions. Spliced XBP1 mRNA was assayed with SsoFast EvaGreen Supermix (Bio-rad) following the recommendations for the iCycler System. Forward and reverse primer was: 5'-TGCTGAGTCCGCAGCAGGTG and 5'-TGGGTCCAAGTTGTCCAG AATGCC. Calculations were done according to the Livak method and normalized to reference gene GAPDH. Each condition was replicated three times; each sample was run in triplicate. 2 μL cDNA sample was used for PCR amplification with iQ™ SYBR® Green Supermix (Bio-Rad Laboratories) according to the manufacturer's protocol.

Biotin labeling assay

HepG2 cells (5X10^6) were lysed using MPER (Pierce) lysis buffer (1 mL) according to the manufacturer protocol. Cell lysates were centrifuged at 10,000 rpm briefly (2 min) at 4 °C to remove cell debris. Lysate supernatant (100 μL) was pre-cleared with streptavidin dyna beads (20 μL) for one hour at 4 °C to remove non-specific biotin binding and incubated with compounds (indicated concentrations) at 4 °C for 1 hr. An equal amount of each sample (20 μL of lysate) was mixed with the same volume of Laemmli sample buffer (20 μL) (BioRad) and was boiled for 5 min. The proteins were separated using 4-15% Bio-Rad Mini-PROTEAN SDS-PAGE gel (1 hr at 100 V) and transferred to the membrane overnight at 4 °C (24 V). The membrane was blocked with 5% BSA in PBST (phosphate buffered saline containing 0.1% Tween 20)
for 1 hr at room temperature and washed for 20 min (3X in PBST). Then the membrane was probed with HRP-streptavidin (1:10,000 in PBST) for 1 hr at room temperature and soaked for 30 min (3X in PBST) and developed using HyGLO chemiluminescent detection reagent (Denville) for biotin recognition.

**Western blot analysis**

50 μg/well of protein from the HepG2 cell lysate was separate using SDS-PAGE and transferred to a nitrocellulose membrane (G.E. Bioscience). After blocking with 5% skim milk in PBST for 1 hr at room temperature, membranes were incubated overnight with primary antibody at 4°C. Layers were then washed with PBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody before visualization with ECL plus (G.E. Bioscience). All antibodies, including NF-κB (Cell signaling D14E12), IkBα (Cell signaling L35A5), poly-Ub (Enzo FK2), P21 (Cell Signaling 12D1), Actin (Abcam EPR16769), lamin A (Abcam EPR4068), and tubulin (Abcam EPR13796) were diluted in 5% BSA buffer. The dilution ratio of the antibody followed the manufacturer's recommendation.

**Clonogenicity Assay**

The clonogenicity assay was performed as previously described[16]. HepG2 cells (500 cells/well) were plated in a 6-well plate at day 1. Cells were treated on day 2 with RA190 at the indicated concentrations. After 14-days incubation, colonies were stained with crystal violet (0.5% w/v) and imaged.

**Immunofluorescence stain**

1.5 x10^4 cells of HEpG2 per well were seeded in the 8-chamber slide in 500 μL of
culture medium, incubated at 37°C under humidified 95% air/5% CO₂ for 18 hours. After treatment for 30 minutes, the slides were fixed with 4% paraformaldehyde (in PBS pH 7.4) for 10 min, and permeabilized with 400 μL of 0.1% Triton-X-100 in 1X PBS at room temperature for 5 min. After blocking with 3 mL 10 % donkey serum in PBS pH 7.4 at R.T. 30 min, the slides were incubated overnight with the primary antibody, IκBα (Cell Signaling 4812S), NF-κB (B.D. 558393) and proteasome (19S, NOVUS NB100-1483) at 4°C. After washing slides with PBST pH 7.4, 2° Ab was added (anti-Rabbit—DyLight 550 (Bethyl), Proteasome-Goat—DyLight650 (Bethyl)) in 1% donkey serum in PBS pH7.4 for 2 hr. Finally, NF-κB—FITC (Biorbyt) in 1% donkey serum in PBS pH7.4 was added for 2 hr. The nucleus was stained with DAPI (Thermo) in 1% donkey serum in PBS pH7.4 for 10 min, and the slides mounted using antifade. The immunofluorescence signal was visualized by confocal microscopy using a Leica 2000 and edited by Leica 2000 software.

**Flow cytometry analysis**

Cell were stained with Annexin V (P.E., B.D. 560930), propidium iodide (B.D. 556463), or Active Caspase 3 (P.E., B.D. 561011) and flow cytometric analyses performed on a Becton-Dickinson FACScan with CELLQuest software (Becton-Dickinson Immunocytometry System, Mountain View, CA) and Flowjo 10 software.

**Orthotopic tumor implantation model**

Male nude mice, 4–6 weeks old, were anesthetized via i.p. administration of 80 mg/kg ketamine and 10 mg/kg xylazine. While under demonstrable anesthesia, an upper midline incision of the abdomen was made and 5x10⁵ HepG2-Luc cells mixed
with Matrigel (1:1) in 20 µL was injected into the left lobe of the liver in male nude mice through a 23-gauge syringe by laparotomy, six in each group. To avoid the leakage of the tumor and seeding into the peritoneum, the injection site was compressed by cotton swab for 2 minutes until no bleeding was evident from the liver surface [17]. After tumor inject, the abdominal wound was closed by interrupted stitches. To monitor the tumor growth in mice, the tumor was imaged by the IVIS system as previously described[18].

**Statistical analysis**

All data are expressed as mean ± S.E. where indicated and are representative of at least two separate experiments. In the tumor treatment experiments, the outcome of interest was the duration of survival until euthanasia based on the animal protocol (in stress, weight change greater than 20%). The event-time distributions for different mice were compared using the Kaplan–Meier method and the log-rank statistic by Prism 6 software. All p-values < 0.05 were considered significant. The statistics were calculated by Prism 6 software.

**Results**

RA190 had a superior effect against HepG2 cell growth compared to Sorafenib

Cells seeded at the concentration of 2,500 cells/well in 100 mL DMEM medium supplemented with 10% FBS in 96-well plate. Twenty-four hours post seeding cells were treated with RA190 and Sorafenib at specified concentrations. Seventy-two hours after treatment, cells were incubated according to the manufacturer’s protocol with the MTT reagent for 1hr and absorbance at 570 nm measured to assess inhibition of cell growth. The IC\textsubscript{50} for RA190 (0.15 µM) was significantly lower than for Sorafenib (9.7 µM) using HepG2 cells (Figure 1A). In a clonogenicity assay,
HepG2 cells treated with RA190 exhibited a reduced number of tumor colonies with an IC$_{50}$ of 0.1 µM (Figure 1B).

**RA190 binds to RPN13 in HepG2 cells**

To identify RA190’s cellular target in HepG2 cells, biotin was covalently linked to RA190 via its free amine functionality (RA190B), as previously described. HepG2 cell lysate was treated with RA190B (at 0, 5, 10, or 25 µM), subjected to SDS-PAGE, and probed with streptavidin-peroxidase following protein transfer to a polyvinylidene difluoride (PVDF) membrane. The streptavidin-peroxidase bound to biotinylated cellular proteins, and a new band at 42 kDa was found in treated samples (Figure 2A) that is consistent with our previous data in other cancer cell lines [11] and indicates that RA190 binds to the 42kDa RPN13 protein in HepG2 cells with specificity.

**RA190 triggers accumulation of polyubiquitinated proteins**

Since compounds related to RA190 are proteasome inhibitors [11], we examined its impact on the levels of polyubiquitinated proteins in HepG2 cells by ubiquitin immunoblot analysis. RA190 treatment (1 µM, 2 µM) of HepG2 cells (12hr) dramatically increased the levels of polyubiquitinated proteins and in a dose-dependent manner (Figure 1C and 1D). Because RPN13 also acts to promote UCH37’s deubiquitinase function, the molecular weight of the accumulated polyubiquitinated proteins observed following exposure to RA190 was higher than seen in bortezomib-treated cells (not shown).

**Rapid accumulation of polyubiquitinated proteins leads to ER stress and apoptosis**
In addition, with the rapid accumulation of polyubiquitinated unfolded proteins, RA190 treatment also triggered the rapid elevation of BIP-1, ATF-4, CHOP10 and Spliced XBP-1 transcript expression levels (Figures 3A-D), consistent with an ER stress response. HepG2 cells after RA190 treatment thereafter also significantly increased the proportion of Annexin V/PI double positive cells (Figures 4A-C), suggesting activation of apoptosis by an unresolved ubiquitin proteasome stress response. Indeed, caspase 3 (Figure 4C) and PARP (Figure 1C) cleavage and p21 expression (Figure 1D) were also considerably increased in HepG2 cells after RA190 treatment providing further biochemical evidence of activation of apoptosis.

Autophagy is a potentially compensatory pathway to mitigate the impact of proteasome inhibition. Formation of the lipidated LC3-II, a biomarker of autophagy, was not elevated within 8hr after RA190 2µM treatment (Figure S1), although this was seen upon addition of 10 µM chloroquine, a positive control. Taking together, the rapid accumulation of polyubiquitinated proteins after RA190 treatment caused ER stress that could not be counteracted by the induction of autophagy, leading to apoptosis of the HepG2 cells.

Impact of RA190 on NF-κB pathway

To examine whether RA190 blocked IκBα degradation and thereby the entry of NF-κB to the nucleus, we used immunofluorescence to visualize IκBα and NF-κB at 30min after treating with RA190 or the 20S proteasome inhibitor MG132 and compared to DMSO (vehicle)-treated cells. IκBα was readily detectable in the cytoplasm of RA190 or MG132 treated cells (Figure 5). In the DMSO treated, IκBα was almost undetected, consistent with its rapid degradation by the proteasome. Most of the IκBα is co-located with the proteasome (Figure 5B) in RA190 or MG132-
treated cells. In the DMSO group, the majority of NF-κB protein was nuclear. While much was still in the nucleus, the NF-κB protein was significantly increased in the cytoplasm in the RA190 treated group (Figure 5). This result was also examined 60min post-treatment by immunoblot of the cytoplasmic vs. nuclear cellular factions, and a similar pattern was observed (Figure 6). The NF-κB was significantly accumulated in the cytoplasm at 60min after RA190 treatment (Figure 6A-B), and a similar finding was evident in MG132 treated HepG2 cells (Figure 6C-D).

**RA190 possess superior *in vivo* tumor growth inhibition**

HepG2-Luc cells (1x10^5 cells in 20 µL) were injected into the left lobe of the liver at day 0. Once the tumor signal was detected at day 7 by i.p. injection of luciferin and IVIS imaging, the mice were randomized into two groups (6 mice in each group). Upon randomization, treatment (RA190 20mg/kg in the active arm, and DMSO in the control arm, intraperitoneal injection) was initiated once daily for 21 days. The tumor was visualized and bioluminescence quantified after injection of luciferin by the IVIS imaging system again at day 11, 14, 21, 28, 35 and 42. Two mice in the DMSO group were sacrificed early due to tumor burden at day 35. Surviving mice in both groups were sacrificed at day 42 and the tumor volume was smaller in the liver specimen of the RA190-treated mice (Figure 7A). Figure 7B shows the signal change in individual mice. The bioluminescence intensity in RA190 groups was significantly lower than the DMSO group (*P*=0.02) at day 35 time point.

**RA190 and Sorafenib combination is synergistic**

To test whether combination treatment may have a synergetic killing effect we reduced the RA190 concentration to 1 µM and Sorafenib to 10 µM. After treating
18hr, cell viability was still around 80% with individual drugs. However, combining RA190 and Sorafenib, significantly improved the killing effect and cell viability dropped lower than 40% (Figure 8A). Under a checkerboard analysis and the ZIP synergy score prediction model using a synergy finder application we further sought the optimal combination ratios. In this experiment HepG2 cells were treated with Sorafenib (first 48hr) then RA190 was added (last 24hr) for a total assay time of 72hr. It showed the ZIP synergy score of 2.31 which indicates a synergetic effect (Figure 8B,C) [19].

Discussion

The ubiquitin-proteasome pathway is a central component in maintaining protein homeostasis. The increased metabolism of cancer cells is associated with aberrant protein expression, including the accumulation of misfolded or deleterious proteins. This proteotoxic stress renders cancer cells more reliant upon proteasome function to degrade the over-produced/mis-folded protein accumulation, thereby creating a therapeutic window and providing a rationale for proteasome inhibition as a means to shift this delicate equilibrium towards cell death[20]. Proteasome inhibitors also applied in HCC therapy in some preclinical studies and showed promising results[21].

NF-κB acts as a central link between hepatic injury, fibrosis, and HCC, and it likely represents an important target for the treatment of liver fibrosis and/or HCC[12]. The activation of NF-κB drives transcription of hundreds of genes with κB binding sites that are involved in the regulation of inflammation, immune responses and cell survival[14]. NF-κB is held in an inactive cytoplasmic complex with an inhibitory IκB protein. To activate NF-κB, the phosphorylated IκBα is detached from the NF-κB
complex and transferred to the proteasome for degradation. The malfunction of IκBα is an important driver of aberrant constitutive NF-κB in solid tumors[22], including HCC[13]. NF-κB activity is dependent on the RPN13-UCH37 axis in the proteasome because it contributes to the recognition of polyUb-tagged IκBα as a substrate for the proteasome. Removal of the polyUb tag (deubiquitination) occurs ahead of substrate transfer to the catalytic subunit for degradation. Upon substrate recognition, RPN13 activates the deubiquitinase function of its binding partner UCH37. Therefore inhibiting RPN13 function blocks both substrate recognition and deubiquitination activity, and this has been shown critical for NF-κB activation via RPN13 knockdown and RPN13 inhibitors such as RA183 and RA190.

Overexpression of ADRM1 mRNA and/or RPN13 was reported in many types of malignancy, including multiple myeloma, diffuse large B-cell lymphoma, gastric cancer, ovarian cancer, and intrahepatic cholangiocarcinoma and high expression of RPN13 indicated poor prognosis[23]. The overexpression of ADRM1 mRNA and RPN13 was detected in HCC, and NF-κB activity was reduced therein by knockdown of ADRM1/RPN13 [24]. We further observed that RA190 inhibited IκBα protein degradation and led the IκBα accumulation in the proteasome (Figure 5A, B), consistent with a role for RPN13. It prevents the NF-κB nuclei entry (Figure 4C, Figure 5) and likely contributes to HCC cell death. Indeed, we found that RA190 also had a significant therapeutic effect in HCC, as seen in other cancer types[11]. Consistent with an on-target effect, biotinylated RA190 also bound with specificity to RPN13 in HepG2 cells. RA190 treatment also led to the rapid accumulation of poly-ubiquitinated proteins in HCC cells, and initiated apoptosis (Figure 4) that is consistent with inhibition of proteasome function and unresolved proteotoxic stress. While RPN13 has previously been seen to regulate cisplatin-induced autophagy,
RA190 did not activate the autophagy pathway (Figure S1) in HCC. While RA190 had a significant tumor control effect in the orthotopic tumor implant model in vivo (Figure 6), it was not curative. This suggests the need for combination therapy. Sorafenib is used clinically to treat HCC, and we observed synergy with RA190 in vitro.

Conclusions

In summary, RA190 binds to RPN13 in HCC cells and inhibits proteasome function therein. This triggers apoptosis in the HCC cells because of a rapid accumulation of poly-ubiquinated protein accumulation and resultant unresolved endoplasmic reticulum stress, as well as the inhibition of NF-κB, a critical oncogenic signal in HCC, by preventing the degradation of IκBα. While RA190 treatment controlled the growth of an orthotopic HCC xenograft model, synergy seen in studies in vitro suggest that an RPN13 inhibitor might fruitfully be combined with Sorafenib as a salvage therapy for HCC patients.

Declarations

Ethics approval and consent to participate

All animal procedures were performed according to approved protocols and by recommendations for the proper use and care of laboratory animals.

Consent for publication

Not applicable.

Availability of data and materials
Not applicable.

**Competing interests**

The authors declare the following competing financial interest(s): Under a licensing agreement between Pontifax/PI Therapeutics and Johns Hopkins University, Drs. Anchoori and Roden are entitled to royalties on an invention (US patent application 20190175572) described in this article. This arrangement has been reviewed and approved by Johns Hopkins University in accordance with its conflict of interest policies. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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**Authors’ Contributions**

Drs. SRS and RA designed the research. Drs. CYC, Mr. TSC and Ms. HYL performed the experiments and analyzed the data. Drs. SRS, SYC and RR wrote this article. Dr. SYC and all performed the research. All authors of the manuscript have read and agreed to its content and are accountable for all aspects of the accuracy and integrity of the manuscript.
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Abbreviations

Hepatocellular carcinoma (HCC), nuclear factor κB (NF-κB), Barcelona Clinic Liver Cancer Staging (BCLC), programmed cell death protein-1 (PD-1), ubiquitin-proteasome system (UPS).

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Figure 1

Impact of RA190 upon HepG2 cell viability, colony formation, polyubiquitinated proteins, PARP, and P21.
RA190B binds to RPN13 in HepG2 cells (A) Immunoblot with HRP-streptavidin on HepG2 cell lysates were incubated with 0, 5, 10 or 25 μM RA190B, separated by SDA-PAGE, transferred to a membrane and probed with HRP-streptavidin. After development, RPN13 (42kb) expression was quantified. (B) mRNA expression as determined by quantitative RT-PCR was significantly increased after 4 hr RA190 treatment.
Figure 3

Elevation of mRNA levels of UPR genes after RA190 treatment (A,B,C,D) The mRN.
RA190 triggers apoptosis in HepG2 cells (A,B) The percentage of PI/Annexin V+ H
RA190 causes accumulation of IκBα and NF-κB in the cytoplasm, co-localized with
NF-κB is significantly accumulated in the cytoplasm after treating RA190 in a immunoblot assay. HepG2 cells were treated with RA190 for 60 min, and nuclear and cytoplasmic fractions were separated and each fraction was subjected to Western blot analysis with antibodies to NF-κB, lamin A (a nuclear marker), and tubulin (a cytoplasmic marker). (A) NF-κB showed significant accumulation in the cytoplasmic fraction of cells treated with RA190 for 60 min, as compared with DMSO. The quantified data is presented in a bar graph (B).
Figure 7

RA190 showed superior tumor control effect in vivo. (A) Bioluminescence imaging
RA190 combination with Sorafenib showed synergy against HepG2 cells. (A) Bar graph depicting the cell viability of RA190 and Sorafenib.

(B, C) A surface plot of the Synergy score showed the optimal cell killing at 0.3 μM RA190 and 0.5 mM Sorafenib.
Supplementary Files

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S1 Figure.pptx