Ibrutinib Potentiates Antihepatocarcinogenic Efficacy of Sorafenib by Targeting EGFR in Tumor Cells and BTK in Immune Cells in the Stroma


Hepatocellular carcinoma (HCC), the most prevalent primary liver cancer, is a leading cause of cancer-related death worldwide because of rising incidence and limited therapy. Although treatment with sorafenib or lenvatinib is the standard of care in patients with advanced-stage HCC, the survival benefit from sorafenib is limited due to low response rate and drug resistance. Ibrutinib, an irreversible tyrosine kinase inhibitor (TKI) of the TEC (e.g., BTK) and ErbB (e.g., EGFR) families, is an approved treatment for B-cell malignancies. Here, we demonstrate that ibrutinib inhibits proliferation, spheroid formation, and clonogenic survival of HCC cells, including sorafenib-resistant cells. Mechanistically, ibrutinib inactivated EGFR and its downstream Akt and ERK signaling in HCC cells, and downregulated a set of critical genes involved in cell proliferation, migration, survival, and stemness, and upregulated genes promoting differentiation. Moreover, ibrutinib showed synergy with sorafenib or regorafenib, a sorafenib congener, by inducing apoptosis of HCC cells. In vivo, this TKI combination significantly inhibited HCC growth and prolonged survival of immune-deficient mice bearing human HCCLM3 xenograft tumors and immune-competent mice bearing orthotopic mouse Hepa tumors at a dose that did not exhibit systemic toxicity. In immune-competent mice, the ibrutinib–sorafenib combination reduced the numbers of BTK+ immune cells in the tumor microenvironment. Importantly, we found that the BTK+ immune cells were also enriched in the tumor microenvironment in a subset of primary human HCCs. Collectively, our findings implicate BTK signaling in hepatocarcinogenesis and support clinical trials of the sorafenib–ibrutinib combination for this deadly disease.

Introduction
Liver cancer is an aggressive disease; there has been a dramatic increase in incidence and mortality in the last several decades worldwide (1, 2). Between 1990 and 2015, there was a ~75% increase in liver cancer incidence globally (3). In 2015, incidence of liver cancer was 854,000 cases and 810,000 patients died of this disease. Hepatocellular carcinoma (HCC), which accounts for approximately 90% of all primary liver cancers, is the fourth major cause of cancer-related death worldwide and the fastest growing cause of cancer-related mortality in the United States (2). Hepatitis B or hepatitis C virus infections, alcoholic liver disease, and nonalcoholic fatty liver disease, a complication of obesity, are the major risk factors for HCC (2). High mortality rate is, in part, due to late diagnosis of this aggressive tumor in patients with chronic liver disease.

Ibrutinib (PCI-32765) is the first-in-class small-molecule inhibitor targeting Bruton’s tyrosine kinase (BTK; ref. 11), a key non-receptor tyrosine kinase in the B-cell receptor signaling pathway, and critical for the survival of malignant B cells. The FDA designated ibrutinib as a breakthrough therapy, and it was approved for the treatment of relapsed and refractory mantle cell lymphoma, relapsed and refractory chronic lymphocytic leukemia and Waldenström’s Macroglobulinemia (12). Ibrutinib irreversibly inhibits Tyr-223 kinase activity by covalently binding to Cys-481 adjacent to the ATP-binding pocket of BTK (13). ibrutinib exhibits both

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Mol Cancer Ther 2020;19:384–96
doi: 10.1158/1535-7163.MCT-19-0135
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antitumorigenic and immunomodulatory and antitumorigenic functions because of its ability to inhibit tyrosine kinases, for example, BTK, ITK, BLK, TEC, BMX, and JAK3 expressed in innate and adaptive immune cells (13) and ErbB family members, for example, EGFR/ErbB1, Her2/ErbB2, and ErbB4 (14), predominantly expressed in epithelial cells. Several reports demonstrated ibrutinib’s efficacy in preclinical models of a variety of solid tumors (15). Ibrutinib exhibits lasting tumor suppression by activating CD8+ T cells (16–18) and suppressing myeloid-derived suppressor cells (MDSC; ref. 19). Currently, ibrutinib in combination with chemother or immunotherapy is being assessed in clinical trials in several solid tumors.

In this study, we assessed the antitumorigenic efficacy of ibrutinib alone and in combination with sorafenib in vitro and in vivo and determined the underlying molecular mechanisms. We found that ibrutinib cooperates with sorafenib by inactivating its substrate EGFR in tumor cells and BTK in immune cells in the tumor microenvironment. Our study also demonstrated that the BTK+ immune cells are enriched in the tumor stroma in a subset of primary human HCCs.

Materials and Methods

Cell culture and drug treatment

HCC cell lines HepG2, Hep3B, PLC/PRF/5, SNU-182, SNU-449, and BNL 1ME A.7R.1 (BNL) were obtained from the ATCC. Huh-7, Hepa1-6 (Hepa), and HCCLM3 cells were provided by Drs. James Taylor (Fox Chase Center), Gretchen Darlington (previously at Baylor Hepa1-6 (Hepa), and HCCLM3 cells were provided by Drs. James Taylor (Fox Chase Center), Gretchen Darlington (previously at Baylor Heps, and BNL 1ME A.7R.1 (BNL) were obtained from the ATCC. SNU-182, SNU-449, and BNL 1ME A.7R.1 (BNL) were obtained from the ATCC. Hep3B, PLC/PRF/5, SNU-182, SNU-449, and BNL 1ME A.7R.1 (BNL) were obtained from the ATCC. Huh-7, Hepa1-6 (Hepa), and HCCLM3 cells were provided by Drs. James Taylor (Fox Chase Center), Gretchen Darlington (previously at Baylor Heps, and BNL 1ME A.7R.1 (BNL) were obtained from the ATCC.

Primary human HCCs.

Cell culture and drug treatment

Primary human HCCs. HCC cells were seeded into 12-well plates (1 to 2 × 104 cells/well). After 24 hours, cells were treated with sorafenib, ibrutinib, combination of both or vehicle for 5 to 7 days. The culture medium and drugs were replaced every other day. Cells were fixed in 4% paraformaldehyde and colony formation was visualized with 0.05% crystal violet dye.

Plasmid transfection

HCC cells were plated in a 6-well plate at 3 × 104 cells/well. After 24 hours, cells were transfected with 2 μg of Myr-Akt-HA or wild-type Akt plasmid DNA using the Lipofectamine 3000 reagent (Thermo Fisher Scientific).

RNA interference

HCC cells plated overnight in a six-well plate at 3 × 105 cells/well were transfected with siEGFR (catalog no. M-003114-03; Dharmacon) or control siRNA (catalog no. D-001206-13; Dharmacon; final concentration, 50 nmol/L) using RNAiMAX (Thermo Fisher Scientific). After 48 hours, cells were treated with the drugs or vehicle for another 48 hours, and cell viability was measured with CellTiter-Glo Luminescence Cell Viability Assay.

RNA-sequence analysis

RNA-sequencing (RNA-seq) was performed in PLC/PRF/5 cells treated with sorafenib (2 μmol/L), ibrutinib (4 μmol/L), combination of both or vehicle (DMSO) for 12 hours. Total RNA was isolated with TRIzol reagent (Thermo Fisher Scientific) followed by selection of positive clones with puromycin (5 μg/mL) treatment for 4 weeks. For treatment of cells in culture, sorafenib and ibrutinib were dissolved in DMSO.

Cell survival assay

HCC cells seeded into 96-well plates (3,000 cells/well) were allowed to grow overnight followed by treatment with sorafenib (LC Laboratories), ibrutinib (Cayman Chemicals and Acorn PharmaTech), or combination of both. Cell viability was assessed after 72 hours of drug exposure using CellTiter-Glo Luminescence Cell Viability Assay (Promega). Each treatment was done in quadruplicate.

Statistical analysis of drug interaction

The two drugs (A and B) are considered to act synergistically if the biological response (cell survival in this study) to A (sorafenib) and B (ibrutinib) cotreatment is greater than the sum of the response to A and B alone. A two-way ANOVA was used to test this hypothesis (μboth – μneither) > (μA – μneither) + (μB – μneither), where μ is the mean response to each treatment and the vehicle control. P values <0.05 are considered as significant synergistic interactions between the two drugs (21).
transcription PCR (qRT-PCR) was performed using 0.01 to 0.1 μg cDNA with SYBR Green in a thermocycler. The fold difference in target gene mRNA levels was calculated using the ΔΔCt method and normalized to 18S rRNA or β-actin mRNA. The primer sequences are provided in the Supplementary Table S1.

Flow cytometry

Cells at distinct phases of cell cycle were distinguished by staining DNA with propidium iodide (PI) and quantified by flow cytometry. HCC cells treated with the drugs alone, in combination, or vehicle for 24 hours were collected, washed with PBS, and fixed in ice-cold 70% ethanol and stored at −20°C overnight. The cells were centrifuged, washed with PBS and resuspended in 0.5 mL PBS. To the single-cell suspension, 50 μL of RNase A (1 mg/mL in PBS) was added and incubated at 37°C for 30 minutes, followed by the addition of 50 μL of PI (500 μg/mL in PBS) with gentle mixing and incubation in the dark at room temperature for 15 minutes and subjected to flow cytometry using a LSRII flow cytometer (BD Biosciences). Cell apoptosis was determined with PI and Annexin V double staining using Annexin V/Dead Cell Apoptosis Kit (Invitrogen) following manufacturer’s instruction.

Mouse strains, animal husbandry, and treatment

NSG (NOD scid Il2rg−/−) mice were purchased from Target Validation Shared Resource core facility at the Ohio State University, and C57BL/6N mice were purchased from Charles River Laboratories. All animals were housed in a temperature-controlled room under a 12-hour light/12-hour dark cycle and under helicobacter-free conditions and fed normal chow diet. All animal studies were reviewed and approved by the Ohio State University Institutional Animal Care and Use Committee. Both male and female mice were used for experiments. For drug treatment, ibrutinib stock (40 mg/mL in DMSO) was diluted with 10% hydroxypropyl beta-cyclodextrin (HPBCD; CTD Holdings) to the final concentration of 4 mg/mL immediately before injection. Sorafenib was diluted in Cremophor EL/ethanol/water (1:1:6) to 3 mg/mL before delivery through oral gavage. For subcutaneous xenografts, 10 to 12 weeks old NSG mice were injected subcutaneously with HCCLM3 (5 × 10^5) cells into the right flank. When tumor volume reaching 150 mm³, mice were randomized into four groups and injected intraperitoneally with ibrutinib (20 mg/kg/day), sorafenib (15 mg/kg/alternate day) administrated through oral gavage, combination of both, and respective vehicles (DMSO in HPBCD for ibrutinib and Cremophor EL/ethanol/water for sorafenib), respectively. Tumor volumes based on digital caliper measurements were calculated by the ellipsoidal formula \[1/2(length \times width^2)\]. After 4 weeks of treatment, mice were euthanized and tumor tissues were collected, weighed, and photographed.

For orthotopic model, 10 to 12 weeks old NSG or C57BL/6N mice were injected with 1 million HCCLM3-Luc or 0.05 million Hepa-Luc cells into the left liver lobe, and the mice were monitored by IVIS Lumina (Caliper Life Sciences) every week. Once the luciferase signal was detected, mice were randomized into four groups and treated with the vehicle, sorafenib (15 mg/kg/alternate day), ibrutinib (20 mg/kg/day), or their combination as described above. Survival was determined using Kaplan–Meier curve.

Bioluminescent imaging

Mice were anesthetized with 2% isoflurane and 100% O₂ with a flow rate of 2 L/minute, injected with luciferin (150 mg/kg, i.p.) and imaged with 1 minute exposure from the dorsal perspective using an IVIS Lumina (Caliper Life Sciences). Acquisition and analysis of images were performed by using the Living Image Software.

Analysis of systemic and tissue toxicity in mice

C57BL/6N mice were treated with the vehicle, sorafenib (15 mg/kg/alternate day), ibrutinib (20 mg/kg/day), or their combination for 4 weeks. Serum collected from mice by cardiac puncture and analyzed with the Vet Accel Chemistry Analyzer (Alfa Wasserman) for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatine kinase (CK), blood urea nitrogen (BUN), albumin, and globulin.

Western blot analysis

A total of 7 × 10^4 HCC cells were plated overnight in 60-mm dish and treated with sorafenib and ibrutinib alone or in combination for 1 hour or 24 hours. Whole-cell extracts were prepared in cell lysis buffer (CLR; catalog no. 9803; Cell Signaling Technology) containing protease inhibitor cocktails (Sigma). The cell lysates were incubated at 4°C for 10 minutes and centrifuged at 4°C for 10 minutes to collect clear supernatants. The tumor lysates from snap-frozen tissues were prepared by suspension in CLB followed by sonication before centrifugation. Protein concentrations in the extracts were measured using a Bio-Rad Protein Assay Kit (catalog no. 500-0006) with BSA as the standard. Equal amounts of protein (10–50 μg) from whole cell or tissue lysates were separated by SDS-polyacrylamide (10% or 4% to 20% gradient) gel electrophoresis (Bio-Rad), transferred to nitrocel lulose membranes (GE Healthcare), incubated using blocking buffer (LI-COR) followed by immunoblotting with phospho-Akt (Cell Signaling Technology, #4060), total Akt (Cell Signaling Technology, #9272), phospho-BRCA1 (Cell Signaling Technology, #4370), total ERK (Cell Signaling Technology, #9102), phospho-EGFR (Cell Signaling Technology, #3777), total EGRF (Santa Cruz Biotechnology, #sc-03) PARP (Cell Signaling Technology, #9532), BTK (Cell Signaling Technology, #8547), β-actin (Santa Cruz Biotechnology, #sc-73778), tubulin (ProteinTech, #66031), or GAPDH (Cell Signaling Technology, #5174). Following incubation with appropriate secondary antibody (IRD-680 or IRD-800), the specific immune-reactive bands were visualized using Odyssey CLX Imaging System (LI-COR) and quantified using Image Studio software.

IHC and tissue microarray

After fixation in 4% paraformaldehyde, tumor tissues were processed, embedded in paraffin, and sectioned at 4-μm thickness. Hepa tumor and human HCC tissue microarray (TMA; catalog nos. LV242 and HLHv090BO01; USBiomax) sections were baked at 60°C for 30 minutes, de waxed in xylene, and rehydrated in graded ethanol. Antigen retrieval was processed in citrate buffer (0.01 mol/L, pH 6.0) at 95°C for 30 minutes followed by incubation with the Ki-67 (Cell Signaling Technology, #9027) or BTK antibodies (Cell Signaling Technology, #8547), and color was developed by the DAB method. Images were taken by a phase contrast microscope (BX41TT; OLYMPUS) and camera (DP71; OLYMPUS) and the Ki-67–positive tumor cells were counted using ImageJ software.

Results

Ibrutinib inhibits HCC cell proliferation and clonogenic survival by inhibiting EGFR signaling

To test the antihepatocarcinogenic functions of ibrutinib, a tyrosine kinase inhibitor (TKI), we determined its IC50 in seven human [HepG2, HCCLM3, PLC/PRF/5, Huh7, sorafenib-resistant Huh7}
Ibrutinib cooperates with sorafenib to reduce HCC cell survival

There is a lot of impetus to develop novel combinations to improve therapeutic efficacy of sorafenib, a first-line treatment for advanced HCCs. We, therefore, examined if ibrutinib could sensitize HCC cells to sorafenib. To this end, we first treated multiple HCC cell lines with both drugs at concentrations ranging from 0.25- to 2-fold of respective IC50. The combination treatment decreased cell viability more significantly compared with individual drugs (Fig. 2A and B; Supplementary Figs. S2A–S2F). We determined the degree of synergy between sorafenib and ibrutinib using the widely accepted Chou-Talalay combination index (CI) method (31). A CI value <1 indicates that the drugs are acting synergistically; a lower CI value indicates a greater degree of synergy. CI values for sorafenib–ibrutinib combinations were <1 in all human and mouse HCC cell lines tested (Fig. 2C; Table 1), suggesting that these drugs cooperatively suppressed HCC cell survival. Among these cell lines, PLC/PRF/5, SNU-449, and HepG2 cells were more sensitive to the cotreatment (CI < 0.5; Table 1). Importantly, the two TKIs also inhibited proliferation of sorafenib-resistant Huh7-SR and HCCLM3 cells and ibrutinib-resistant HepG2 cells more effectively than individual drugs. Statistical analysis confirmed significant synergy between the two TKIs in inhibiting HCC cell survival (data for three cell lines shown in the Supplementary Table S2).

Next, we tested whether ibrutinib can synergize with regorafenib, recently FDA-approved as a second-in-line therapy for patients with HCC who progress on sorafenib. Notably, ibrutinib exhibited synergy in combination with regorafenib in all three cell lines tested (Huh7, PLC/PRF/5, and HCCLM3), as demonstrated by much lower IC50 of the combination compared with individual TKIs (Supplementary Figs. S2G–S2I). This is not surprising considering the fact that regorafenib is structurally identical to sorafenib except for one fluorine atom in the central phenyl ring (32).

FACS analysis of PI-stained cells revealed a substantial increase in sub-G1 cell population in PLC/PRF/5, HCCLM3, and HepG2 cells with concomitant decrease in G1 population upon ibrutinib-sorafenib cotreatment at their respective IC50 (Fig. 2D; Supplementary Figs. S3A and S3C). In PLC/PRF/5 and HCCLM3 cells, each drug increased sub-G1 population, however, the effect was more pronounced with the drug combination. In contrast, sub-G1 population increased only after TKI cotreatment in HepG2 cells (Supplementary Fig. S3C). Intriguingly, the population of HepG2 cells at G1 increased without significant changes in sub-G1 when treated at half of the IC50 dose (Supplementary Fig. S3D), suggesting that the cells were arrested at G1 at lower drug concentrations. Elevated cleaved PARP levels in PLC/PRF/5 cells and Annexin/PI-positive HCCLM3 cells (Fig. 2E; Supplementary Fig. S3B) confirmed enhanced apoptosis induced by this drug combination.
Investigation of the molecular mechanism underlying synergy revealed that the ibrutinib–sorafenib combination reduced the phosphorylation of Akt in HCC cells when compared with ibrutinib only (Fig. 2F; Supplementary Fig. S4A). Akt signaling, critical for chemoresistance in HCC (33), is highly activated in sorafenib resistant cells (27, 34). To confirm the role of Akt pathway in mediating the cooperation of these two TKIs, Akt signaling was activated in Huh7 cells by transfecting the wild-type (WT-Akt) or constitutively active, myristoylated Akt (Myr-Akt) expression vector, followed by drug treatment. As expected, Myr-Akt was stabilized and the phospho-/total-Akt ratio increased 3-fold compared with WT-Akt in Huh7 cells (Supplementary Fig. S4B). Assessment of cell viability after 72 hours of drug exposure showed that Myr-Akt expressing cells became resistant individual drugs (IC50 of each TKI could not be reached at the drug concentration tested).

Figure 1.
Effect of ibrutinib (IBT) on HCC cell survival and EGFR, Akt, and ERK phosphorylation. A, HCC cell lines were incubated with ibrutinib at concentrations ranging from 0.5 to 60 μmol/L for 72 hours, and cell viability was determined using CellTiter-Glo Kit, which measures cellular ATP level (n = 4/group, data are mean ± SD). B, Huh7 and Huh7-SR cells were treated with ibrutinib for 1 hour, and the lysates were subjected to Western blotting with specific antibodies. C, HCCLM3 cells were treated with EGF (20 ng/mL) along with ibrutinib in DMSO or DMSO alone for the indicated times, and phospho-/total-EGFR and Akt levels were determined by immunoblotting. The signals were quantified using Image Studio software. D, HCCLM3 cells were transfected with EGFR-siRNA (siEGFR) or control siRNA (siCon) for 48 hours followed by ibrutinib (24 μmol/L) or vehicle treatment for 1 hour, and the lysates were subjected to immunoblotting, and cell viability of siRNA-transfected cells treated with ibrutinib at indicated concentrations for 72 hours was measured (t-test, data are mean ± SD). E, F, The heatmap depicting expression of several critical genes whose expression was significantly altered in IBT-treated PLC/PRF/5 cells, as identified by RNA-seq analysis. G, qRT-PCR validation of selected genes in PLC/PRF/5 cells treated with ibrutinib (2 μmol/L) for 24 hours (t-test, data are mean ± SD).
Table 1. IC50 and CI when fraction affected (FA) is 50%.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Ibrutinib (IC50) (μmol/L)</th>
<th>Sorafenib (IC50) (μmol/L)</th>
<th>CI at 0.5 FA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLC/PRF/5</td>
<td>1</td>
<td>14</td>
<td>0.36</td>
</tr>
<tr>
<td>Hepa</td>
<td>1.7</td>
<td>14</td>
<td>0.53</td>
</tr>
<tr>
<td>SNU-449</td>
<td>3</td>
<td>4</td>
<td>0.39</td>
</tr>
<tr>
<td>Huh7</td>
<td>6</td>
<td>3</td>
<td>0.64</td>
</tr>
<tr>
<td>Huh7-SR</td>
<td>7</td>
<td>12</td>
<td>0.73</td>
</tr>
<tr>
<td>Huh7-5R</td>
<td>7</td>
<td>12</td>
<td>0.73</td>
</tr>
<tr>
<td>HCCLM3</td>
<td>24</td>
<td>12</td>
<td>0.69</td>
</tr>
<tr>
<td>BNL</td>
<td>52</td>
<td>9</td>
<td>0.64</td>
</tr>
<tr>
<td>HepG2</td>
<td>40</td>
<td>4</td>
<td>0.43</td>
</tr>
</tbody>
</table>

concentrations tested; Fig. 2G and H). Notably, CI values at different concentrations of the two drugs in combination increased in Myr-Akt compared with WT-Akt expressing cells (Fig. 2I), suggesting that the constitutively active Akt abrogated drug synergy.

Ibrutinib and sorafenib combination inhibits survival of liver cancer stem cells in vitro

Suppression of SOX2 and SOX9 expression (Fig. 1F and G) led us to hypothesize that the ibrutinib–sorafenib combination could inhibit cancer stem cell (CSC) properties of HCC cells. The tumor spheroid model has been widely used to elucidate the mechanism of chemoresistance (35, 36), and to uncover CSC characteristics that are not manifested in monolayer cultures. To determine the effect of the ibrutinib–sorafenib combination on CSC properties of HCC cells, we first established HCC spheroid cultures in ultra-low attachment plates (Fig. 3A) and measured expressions of CSC marker genes, for example, CD13, CD44, CD133, SOX2, SOX9, EPCAM, and KLF4 in self-renewing spheroids. As expected, compared with monolayers, expressions of these genes were highly upregulated in PLC/PRF/5 spheroids (Fig. 3B). Survival analysis revealed increased resistance of spheroids to both drugs compared with monolayers (Fig. 3C and D). Nevertheless, both drugs cooperatively (CI < 1) inhibited the growth of HCC spheroids (Fig. 3E), which correlated with marked decrease in expressions of CD13, CD44, KLF4, SOX2, and SOX9 by this TKI combination compared with the vehicle controls (Fig. 3F). This drug combination also inhibited survival of Hepa and HCCLM3 spheroids (Supplementary Figs. S4C and S4D). These results suggest that this combination therapy could potentially reduce HCC tumor recurrence by suppressing CSC survival.

Ibrutinib and sorafenib combination inhibits the growth of human HCC xenografts and increases median survival of NSG mice

To validate our in vitro findings, we tested the efficacy of the ibrutinib–sorafenib combination therapy in NSG (immunodeficient) mice bearing subcutaneous HCCLM3 xenograft tumors. We chose HCCLM3 cells because these cells form tumors within a short time with ~100% penetrance. When the tumor volume reached ~150 mm3, we started treating mice with the drugs or vehicle and measured body weights and tumor volumes weekly. To determine drug synergy, we treated mice with sorafenib at 15 mg/kg/alternate day and ibrutinib at 20 mg/kg/day (Fig. 4A), which are much less than usual doses of sorafenib (37, 38) and ibrutinib (39, 40) used to treat tumor-bearing mice. The loss of body weight in mice due to tumor burden was <5% and it was not significantly affected by the drug treatment (Supplementary Fig. S4E). After 4 weeks of treatment, tumor volume was significantly reduced by the TKI combination compared with the vehicle controls or single drugs (Fig. 4B and C). Although decrease in tumor weight was significant in mice treated with sorafenib (35%) or ibrutinib (36%), cotreatment showed more pronounced decrease (60%) in tumor weight (Fig. 4D). Scoring Ki-67 positive cells showed significant decrease in proliferating tumor cells in combination therapy compared with the monotherapy (Fig. 4E and F). Immunoblot analysis showed that the treatment with each drug reduced phospho-EGFR/Akt/ERK levels in tumors, which was more prominent in tumors developed in mice treated with both drugs (Supplementary Figs. S4F and S4G).

Monitoring NSG mice bearing orthotopic HCCLM3-Luc xenografts showed that tumor growth was significantly (P = 0.011) inhibited by the ibrutinib–sorafenib combination compared with the vehicle (Fig. 5A and B). Notably, the median survival of mice increased from 27 to 40 days (P = 0.0007) upon treatment with both drugs compared with vehicle controls (Fig. 5C).

Ibrutinib and sorafenib act in concert to increase median survival of immune-competent mice bearing orthotopic Hepa tumors

Because ibrutinib exhibits immunomodulatory functions, we next tested therapeutic efficacy of these drugs in immunocompetent C57BL/6N mice bearing orthotopic tumors developed by transplanting Hepa-Luc cells. These cells form tumors within 1 to 2 weeks when inoculated in the livers (Fig. 6A; ref. 41). When the tumor volume reached ~150 mm3, we started treating mice with the drugs as described in Fig. 5A and monitored their survival. Notably, tumor-bearing mice treated with the TKI combination significantly (P = 0.0004) prolonged survival compared with the vehicle control (Fig. 6B); the median survival increased from 8 to 38 days in mice treated with both drugs.

Next, we determined systemic toxicity of this treatment regimen (Fig. 4A) by monitoring body weights and assessing organ functions in C57BL/6N mice. Body weights of these mice were not significantly affected by these drugs alone or in combination (Supplementary Fig. S5A). The serum levels of liver enzymes, for example, ALP, ALT, AST, and cardiac enzyme, for example, CK were not significantly affected by the TKIs alone or in combination (Supplementary Figs. S5B–S5E). Only BUN level was slightly decreased with single drugs; however, it was not further affected by cotreatment (Supplementary Fig. S5F). Unaltered serum albumin and globulin levels indicate that the protein synthesis capacity of the liver was not compromised by these TKIs in mice (Supplementary Figs. S5G and S5H). Microscopic examination of hematoxylin and eosin (H&E)-stained tissue sections did not detect any significant difference in the structure of liver, lung, spleen, and kidney among the drug-treated and control mice (Supplementary Figs. S5I–S5L).

Given that the main function of BTK is in the immune cells, we assessed the influence of ibrutinib on the immune cells in the tumor microenvironment. Indeed, IHC analysis of Hepa orthotopic tumor sections showed enrichment of BTK+ cells in the tumor stroma (Fig. 6C and D). Importantly, the BTK+ cell population was profoundly decreased in mice subjected to combination therapy compared with monotherapy or vehicle. Immunoblot results of the representative tumor lysates showed that ibrutinib treatment reduced BTK protein level, which was more significant in mice treated with both drugs (Fig. 6E and F). These results indicate that ibrutinib and sorafenib combination therapy is effective in suppressing HCC growth in the liver microenvironment of immune-competent mice.
To determine the relevance of these findings to human patients with HCC, we probed two human primary HCC TMAs with a BTK-specific antibody. Results showed that BTK-expressing (BTK+)) cells were enriched in the stroma of a subset of HCCs, whereas few such cells were detected only in the sinusoids of normal livers (Fig. 6G; Supplementary Figs. S6A and S6B). Although, some BTK+ cells were recruited in the benign inflamed liver tissues adjacent to the HCCs (Supplementary Fig. S6C), enrichment of these cells was significantly higher (P = 0.029) in the tumor microenvironment (Fig. 6H). Scoring two TMAs revealed that 27% of HCCs (23 of 86) and only 4% (1 of 24) benign livers exhibited score 3. These results clearly demonstrate that BTK+ immune cells are recruited in the tumor stroma of a subset of HCCs.
patients with HCC. Collectively, these results implicate BTK expressing immune cells in HCC pathogenesis and provide rationale for ibrutinib–sorafenib combination therapy.

Discussion

Although systemic treatment with sorafenib is a standard of care option in patients with advanced-stage HCC, its overall survival benefit is limited, with <3 months improvement in the SHARP study (42) and median overall survival of 12.3 months in the REFLECT study (5). Targeted therapy directed towards crucial survival pathways in both cancer cells and tumor microenvironment is an emerging treatment strategy in cancer. In this study, we have demonstrated that ibrutinib exhibits antitumorogenic effects on both sorafenib-sensitive and resistant HCC cell lines by irreversibly inactivating EGFR signaling. Ibrutinib also inactivates BTK in immune cells, and thereby modulating the tumor microenvironment. EGFR, a receptor tyrosine kinase (RTK), is stimulated by multiple ligands resulting in the activation of various signaling pathways that primarily control cell proliferation, differentiation and survival. EGFR signaling plays a key role in tumor cell growth, angiogenesis, and metastasis.
role in liver generation after acute and chronic liver damage. Also, it plays a key role in cirrhosis and HCC. Furthermore, HCC metastasis and production of inflammatory cytokines have been shown to be regulated by EGFR-regulated pathways (43). Unlike other clinically approved EGFR inhibitors directed towards its kinase domain, ibrutinib can irreversibly inactivate both the wild-type and kinase-mutant EGFR (44, 45). Inhibition of EGFR, Akt, and ERK signaling in HCC cells by ibrutinib treatment holds great therapeutic potential because activation of these signaling pathways is associated with the tumorigenic potential of various cancers including HCC (26).

Activation of Akt signaling is involved in sorafenib resistance (27). Ability of ibrutinib to suppress EGFR, Akt, and ERK pathways suggests a great potential for ibrutinib to overcome sorafenib resistance. More importantly, ibrutinib and sorafenib combination therapy inhibited...
HCC cell survival of highly aggressive and sorafenib-resistant HCCLM3 cells in NSG mice. This was accomplished at a dose much lower than the dose of each drug alone without causing notable systemic toxicity to the mice. These data obtained in preclinical models suggest that ibrutinib–sorafenib combination therapy will be effective as a first line of treatment for patients with sorafenib resistance often associated with activated EGFR/Akt/ERK signaling. The improved survival (27 to 40 days) of the immune cell-deficient NSG mice bearing orthotopic HCC-LM3 xenografts with this combination therapy indicates that the inhibition of EGFR signaling in the tumor cells is a key to the mechanism of action of ibrutinib.

Currently, ibrutinib is undergoing clinical trials in several solid tumors, because of its immunomodulatory effects targeting the tumor microenvironment. Several reports underscore the relevance of understanding the role of tumor-infiltrating immune cells including B cells in hepatocarcinogenesis (46–48). Ibrutinib inhibits the function of all BTK expressing hematopoietic cells, for example, B cells, basophils, monocytes, MDSCs, and NK cells. In fact, it inhibits T helper cells as well due to its action on ITK (13, 16). Thus, ibrutinib has broad immune modulatory effects (19, 49). Immune microenvironment plays a critical role in the pathogenesis of HCC due to its prototypical inflammation-associated nature where prolonged hepatitis accounts for approximately 90% of the HCC burden. Therefore, targeting immune cells in the tumor microenvironment is expected to be a rewarding strategy in the treatment of HCC. Indeed, although not significant \( (P = 0.059) \), ibrutinib alone increased median survival of immune-competent C57BL/6N mice bearing orthotopic Hepa tumors from 8 to 21 days, which was increased to 38 days in combination with sorafenib \( (P = 0.004) \). IHC analysis showed that infiltration of BTK\(^{+}\) immune cells in human primary HCCs and mouse Hepa orthotopic tumors. Precipitous reduction in BTK\(^{+}\) cells in the Hepa tumor stroma upon combination therapy implicates their role in modulating HCC growth. However, the role of BTK\(^{+}\) immune cells in hepatocarcinogenesis has not been elucidated. Nonetheless, the efficacy of ibrutinib in potentiating anti-HCC action of sorafenib both in NSG and C57BL/6N mouse models suggests that it functions by inhibiting distinct tyrosine kinase signaling both in the tumor cells and immune cells in the tumor microenvironment. Studies are ongoing to determine the cell type–specific role of BTK in HCC development using cell type–specific knockout mice. It has been reported that infiltration of PD-1\(^{+}\) B cells in the stroma is associated with poor prognosis of patients with HCC (50). It would be of interest to monitor PD-1 expression in BTK\(^{+}\) cells before and after the drug treatment.

There is an unmet need to develop better therapeutic options in the first-line setting when patients are most fit to receive therapy, to improve the survival and response rate in HCC. Although data with
PD-1 inhibitors show improved results for HCC treatment in the second-line setting, in recently completed clinical trials, PD1 inhibitors failed to improve HCC patient survival in the first-in-line setting. In addition, not all patients are candidates for immune therapies, either due to history of orthotopic liver transplant or autoimmune disorders. Thus, developing novel combination therapy to improve survival benefit of sorafenib is important and relevant. Our data in preclinical models clearly show that ibrutinib may potentiate anti-HCC therapeutic efficacy of sorafenib.

**Disclosure of Potential Conflicts of Interest**

Anne M. Noonan is a member of the advisory board of Exelixis, QED Therapeutics, and Helsinn. No potential conflicts of interest were disclosed by the other authors.

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**Conception and design:** C.-H. Lin, X.-F. Bai, K. Ghoshal

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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** C.-H. Lin, K.H. Elkholy
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): C.-H. Lin, K.H. Elkholy, J.M. Barajas, L. Yu, X. Zhang, W.N. Khan


Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.-H. Lin, K.H. Elkholy, N.A. Wani, D. Li, P. Hu

and bioluminescence imaging of tumors. This work was supported, in part, by grants R01CA193244 (to K. Ghoshal) and R01CA086978 (to S.T. Jacob and K. Ghoshal) from the NCI, a seed grant from Translational Therapeutics program of OSUCCC and Intramural Funding Program from Sylvester Comprehensive Cancer Center, the University of Miami (to W.N. Khan). J.M. Barajas was a Howard Hughes Medical Institute Gilliam fellow. K.H. Elkholy was supported by a PhD scholarship from the Ministry of Higher Education and National Research Center in Egypt.

Acknowledgments

We thank Dr. Amy Johnson and Dr. Hui-Lung Sun for kindly providing ibrutinib and Akt plasmids, respectively. We thank Jianmin Zhu and Pietrzak Maciej for their assistance with flow cytometry and RNA-seq analysis, respectively. We thank analytic cytometry, target validation, and small-animal imaging shared resources at the Ohio State University Comprehensive Cancer Center (supported by P30CA016058 from NCI) for providing FACS analysis, NSG mice, and the assistance of the Jackson Laboratory.

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Received February 9, 2019; revised August 12, 2019; accepted September 27, 2019; published first October 3, 2019.
Ibrutinib Potentiates Antihepatocarcinogenic Efficacy of Sorafenib by Targeting EGFR in Tumor Cells and BTK in Immune Cells in the Stroma
