Synergistic Interaction between the HDAC Inhibitor, MPT0E028, and Sorafenib in Liver Cancer Cells In Vitro and In Vivo

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Abstract

**Purpose:** To investigate the antitumor activities of a histone deacetylase (HDAC) inhibitor, MPT0E028, plus sorafenib in liver cancer cells in vitro and in vivo.

**Experimental Design:** Different liver cancer cell lines were exposed to sorafenib in the presence or absence of MPT0E028, and cell viability was determined by MTT assay. Effects of combined treatment on cell cycle and intracellular signaling pathways were assessed by flow cytometry and Western blot analysis. The Hep3B xenograft model was used to examine the antitumor activity in vivo.

**Results:** Our data indicate that sorafenib and MPT0E028 synergistically reduced cell viability in liver cancer cells, and also markedly induced apoptotic cell death in these cells, as evidenced by the cleavage of caspase-3, PARP, and DNA fragmentation. MPT0E028 altered the global modifications of histone and nonhistone proteins regardless of the presence of sorafenib. However, sorafenib blocked MPT0E028-induced Erk activation and its downstream signaling cascades, such as Stat3 phosphorylation (Ser727) and Mcl-1 upregulation. Ectopic expression of constitutively active Mek successively reversed the apoptosis triggered by the combined treatment. Pharmacologic inhibition of Mek by PD98059 potentiated MPT0E028-induced apoptosis, suggesting that the synergistic interaction between MPT0E028 and sorafenib occurs at least partly through inhibition of Erk signaling. The data demonstrated that transcriptional activation of fibroblast growth factor receptor 3 (FGFR3) contributes to MPT0E028-mediated Erk phosphorylation. Finally, MPT0E028 plus sorafenib significantly improved the tumor growth delay (TGD) in a Hep3B xenograft model.

**Conclusions:** These findings suggest that MPT0E028 in combination with sorafenib has significant anti-hepatocellular carcinoma activity in preclinical models, potentially suggesting a novel therapeutic strategy for patients with advanced hepatocellular carcinoma. Clin Cancer Res; 20(5); 1274–87. ©2014 AACR.
Translational Relevance

Hepatocellular carcinoma remains a major health problem that is ranked as the sixth most common cancer and the third most common cause of cancer-related deaths worldwide. Sorafenib is currently the only drug for patients with advanced hepatocellular carcinoma, but some of them who do not have response or become insensitive, resulting in tumor progression. Therefore, an approach that improves therapeutic efficacy is urgently needed. The present study provides the evidence that a histone deacetylase (HDAC) inhibitor, MPT0E028, and sorafenib exhibited a synergistic interaction in killing hepatocellular carcinoma cells, inducing marked apoptosis via a caspase-dependent pathway. Mechanistically, sorafenib-mediated inhibition of the MPT0E028-activated fibroblast growth factor receptor 3 (FGFR3)/Erk signaling pathway may be a major component of the observed synergism. Moreover, the combined treatment significantly improves the tumor growth delay (TGD) and decreases tumor volume in the Hep3B xenograft model. These findings indicate that the MPT0E028/sorafenib combination warrants future development for potential therapeutic applications in patients with hepatocellular carcinoma.

Despite of the success of sorafenib in the treatment of some patients with hepatocellular carcinoma, a majority of these patients do not respond to sorafenib, and some patients who initially respond to sorafenib subsequently become insensitive, resulting in tumor progression (14). Given that hepatocellular carcinoma is a complex and heterogeneous tumor with aberrant activation of several signaling pathways, researchers have sought to target hepatocellular carcinoma with a combination of sorafenib plus chemotherapy or another targeted therapeutic agent (15). In the latter context, the strategy of combining sorafenib with an HDAC inhibitor is particularly interesting. High-level expression of HDAC1 is reportedly correlated with a higher incidence of cancer cell invasion, a more advanced tumor–node–metastasis (TNM) stage, and a lower survival rate in patients with hepatocellular carcinoma (16). Preclinical studies have shown a potential for synergistic or additive effects when sorafenib is combined with HDAC inhibitors, and some ongoing clinical studies are evaluating the benefits of this treatment for patients with advanced hepatocellular carcinoma (17–19).

This study was undertaken to evaluate the preclinical efficacy of the HDAC inhibitor, MPT0E028, in combination with sorafenib in human hepatocellular carcinoma cells. We herein report, for the first time, that MPT0E028 and sorafenib exhibited a synergistic interaction in killing hepatocellular carcinoma cells, inducing marked apoptosis via a caspase-dependent pathway. Our data suggest that the sorafenib-mediated inhibition of the MPT0E028-activated fibroblast growth factor receptor 3 (FGFR3)/Erk signaling pathway may be a major component of the observed synergism. Moreover, we show that the combined treatment significantly improves the tumor growth delay (TGD) and decreases tumor volume in the Hep3B xenograft model, compared with treatment by either drug alone. Taken together, these findings indicate that our combined treatment warrants further development for potential therapeutic applications in patients with hepatocellular carcinoma.

Materials and Methods

Materials

Sorafenib (purity ≥ 99%) was purchased from Biovision. MPT0E028 and vorinostat (purity ≥ 98%) were synthesized by Dr. Jing-Ping Liou’s Laboratory (Taipei Medical University, Taipei, Taiwan; ref. 13). EGTA, EDTA (disodium salt), leupeptin, dithiothreitol, propidium iodide, MTT, phenylmethylsulfonylfluoride (PMSF), ribonuclease A, z-VAD–FMK, Ac-DEVD–CHO, PD98059, PD173074, and all of the other chemical reagents were obtained from Sigma. RPMI-1640 medium, Dulbecco’s Modified Eagle Medium (DMEM), FBS, penicillin, streptomycin, and all other tissue culture reagents were obtained from GIBCO/BRL Life Technologies. The following antibodies were used: caspase-8, caspase-9, p21, histone H3, acetyl-a-tubulin, phospho-Erk, and Erk (Cell Signaling Technology); McI-1, PARP, and FGF3 (Santa Cruz Biotechnology); acetyl-histone H3 and pan-actin (Millipore); caspase-3 (Imgenex); phospho-stat3-
Tyr\textsuperscript{705} and phospho-stat3-Ser\textsuperscript{727} (Epitomics); and Stat3 (BD Biosciences).

**Cell culture**

Hep3B, HepG2, and PLC/PRF/5 cells were purchased from the American Type Culture Collection. These hepatocellular carcinoma cell lines were cultured in RPMI-1640 medium (Hep3B and PLC/PRF/5) or DMEM (HepG2) supplemented with 10% FBS (v/v) and penicillin (100 U/mL)/streptomycin (100 μg/mL)/amphotericin B (0.25 μg/mL). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO\textsubscript{2}/95% air.

**MTT assay**

Cells were seeded in 96-well plates (5,000 cells/well) and incubated overnight for attachment, and were then treated with indicated agents in 10% FBS-supplemented medium for 72 hours. The medium was replaced with MTT (0.5 mg/mL) at 37°C for 2 hours. After removal of medium, the cells were lysed with 200 μL per well dimethyl sulfoxide (DMSO), and absorbance at 550 nm was measured and the values of 50% inhibition concentration (IC\textsubscript{50}) for each drug were determined. The combination index value was determined from the fraction-affected value of each combination according to the Chou–Talalay method by using CompuSyn software (ComboSyn, Inc.), and a combination index value below 1 represents synergism (20).

**Cell proliferation assay**

Cell proliferation was assessed by bromodeoxyuridine (BrdU) incorporation assay using the Proliferation Assay Kit (Millipore). Cells were seeded in 96-well plates (5,000 cells/well) with 10% FBS in culture medium, and treated with indicated agents for 72 hours. BrdU was added during last 2 hours of incubation period. The assay was performed according to the manufacturer’s instructions.

**Flow cytometric analysis**

After drug treatment, the cells were harvested by trypsinization, washed with PBS, then pellets were resuspended and fixed in ethanol (70%, v/v) at -20°C overnight, and washed once with PBS. After centrifugation, the cells were incubated for 15 minutes at room temperature in 0.1 mL of phosphate-citric acid buffer (0.2 mol/L NaHPO\textsubscript{4}, 0.1 mol/L citric acid, and pH 7.8). Cells were stained with propidium iodide staining buffer containing Triton X-100 (0.1%, v/v), RNase A (100 μg/mL), and propidium iodide (80 μg/mL) for 30 minutes in the dark. Cell-cycle distribution was analyzed by flow cytometry with CellQuest software (Becton Dickinson).

**Apoptosis assay**

Drug-induced apoptosis was assessed using the Cell Death Detection ELISA Kit (Roche Applied Science), which quantitates cytoplasmic histone–associated DNA fragments in the form of mono-/oligonucleosomes. Cells were treated with indicated agents for 72 hours in 10% FBS-supplemented medium and then analyzed according to the manufacturer’s instructions.

**Transient transfection and Western blot analysis**

The cells were transfected with lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Silencer select siRNA against ERK was purchased from Ambion. Plasmid expressing constitutively active Mek was prepared as described previously (21). Western blot analysis was performed as previously described (13). Briefly, cells were harvested by scraping with lysis buffer (1 mmol/L EDTA, 150 mmol/L NaCl, 1% Triton X-100, 0.1 mmol/L sodium pyrophosphate, 1 mmol/L PMSF, 1 mmol/L Na\textsubscript{3}VO\textsubscript{4}, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 5 mmol/L NaF in 20 mmol/L Tris-HCl buffer, pH 7.5). Cell lysates were centrifuged at 13,000 × g for 30 minutes. Total protein was determined and equal amounts of protein were separated by SDS-PAGE and immunoblotted with specific antibodies. Proteins were visualized by enhanced chemiluminescence (Amersham).

**RNA isolation and real-time PCR analysis**

Total RNA was isolated with TRIzol reagent according to the manufacturer’s protocol (Invitrogen). Aliquots of 5 μg of total RNA from each sample were reverse-transcribed to cDNA by with M-MLV RT reagent (Promega). Real-time PCR (RT-PCR) was carried out by FastStart Universal SYBR Green Master (Roche) and cDNA amplification was detected by the StepOne RT-PCR System (Applied Biosystems). Relative gene expression was normalized to 18S and calculated by using the 2\textsuperscript{[-ΔΔCt]} method (22). The sequences of all primers used are listed in Supplementary Table S1.

**LDH assay**

Cells were treated with drugs at indicated concentrations for 72 hours followed by the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) at 490 nm to measure the levels of LDH (lactate dehydrogenase) release according to the manufacturer’s protocol.

**In vivo studies and immunohistochemistry**

Eight-week-old female nude athymic mice were fed ad libitum water (reverse osmosis, 1 ppm Chlorine) and PicoLab Rodent Diet (20.0% crude protein, 9.9% crude fat, and 4.7% crude fiber). The mice were group-housed under conditions of constant photoperiod (12 hours light/12 hours dark) at 21°C to 23°C and 60% to 85% humidity. All animal experiments were carried out in accordance with protocols approved by the Animal Use and Management Committee of National Taiwan University (Taipei, Taiwan; IACUC approval no: 20100225).

Hep3B cells used for implantation were harvested during log-phase growth and resuspended in PBS at 5 × 10\textsuperscript{6} cells/mL. Each mouse was inoculated subcutaneously with 1.0 × 10\textsuperscript{7} cells (0.2 mL cell suspension). As tumors became established, mice were randomized to four groups that received the following agents by gavage: (i) vehicle, (ii)
sorafenib, (iii) MPT0E028, and (iv) MPT0E028 plus sorafenib. Tumors were monitored twice weekly and then daily as their volumes approached 1,200 mm³. Tumor/volume (mm³) = \( (w^2 \times l)/2 \), where \( w \) is the width and \( l \) is the length (mm) of the tumor. The time to endpoint (TTE) for each mouse was determined by the following equation: TTE = \( \log_{10} \text{endpoint volume} - b/m \), where TTE is expressed in days, endpoint volume is in mm³, \( b \) is the intercept, and \( m \) is the slope of the line obtained by linear regression of a log-transformed tumor growth dataset. The dataset consists of the first observation that exceeded the study endpoint volume and the three consecutive observations that immediately preceded the attainment of the endpoint volume. Treatment efficacy was determined from TGD, which is defined as the increase in the median TTE for a treatment group compared with the control group and multiplied by 100. The mice were then daily as their volumes approached 1,200 mm³.

Effects of sorafenib in combination with MPT0E028 on cell viability and proliferation in liver cancer cells

To examine the antitumor activity of the combined treatment in a preclinical setting, we first conducted a concentration–response study. Three liver cancer cell lines were treated with different concentrations of sorafenib in the presence or absence of MPT0E028 for 72 hours, and cell viability was determined by MIT assay. Our results revealed that MPT0E028 significantly and concentration-dependently enhanced sorafenib-mediated cytotoxicity in Hep3B cells (Fig. 2A). To determine whether the combined treatment had a synergistic impact on cell viability, the combination index values of each dose were calculated by the CompuSyn software (Supplementary Table S3). At concentrations below the IC₅₀ value (0.3125 and 0.625 μmol/L), MPT0E028 exhibited a synergistic effect in combination with sorafenib (Fig. 2A, right). A synergistic effect was also observed when sorafenib was combined with vorinostat, although a higher concentration of vorinostat was required to achieve the same effect (Supplementary Fig. S1). The synergistic interaction between MPT0E028 and sorafenib was also observed in PLC/PRF/5 and HepG2 cells, indicating that this was not a cell line–specific effect (Fig. 2B and C). Moreover, the combined treatment significantly enhanced the antiproliferative effects in all three tested cell lines as evidenced by BrdU incorporation assay (Supplementary Fig. S2A). Notably, the effects of combination on cell proliferation and viability were also observed in a time-dependent manner (Supplementary Fig. S2B and S2C). Taken together, our results indicate that cotreatment with MPT0E028 and sorafenib synergistically increased cytotoxicity and improved the antiproliferative effects in liver cancer cell lines.

Cotreatment with MPT0E028 and sorafenib enhances apoptotic cell death via a caspase-dependent pathway

As sorafenib and HDAC inhibitors reportedly induce apoptosis in hepatocellular carcinoma cells (7, 24), we...
examined the impact of our drug combination on programmed cell death. First, the effect of coadministration of MPT0E028 and sorafenib on cell-cycle distribution was analyzed by fluorescence-activated cell sorting analysis. No appreciable cell-cycle arrest was observed after sorafenib treatment (Fig. 3A, top). However, the combined treatment significantly enhanced the percentage of sub-G₁ phase cells over that seen in cells treated with either drug alone, suggesting that apoptosis is the main cause of cell death in the cotreated Hep3B cells (Fig. 3B). Meanwhile, the possibility of necrotic effect was excluded by examining LDH leakage in the supernatants of drug-treated Hep3B cells (Supplementary Fig. S1C). To confirm this effect, nucleosome formation (representing DNA fragmentation) was determined in drug-treated cells. The results showed that sorafenib dramatically enhanced MPT0E028- or vorinostat-induced nucleosome formation in Hep3B cells (Fig. 3C).
We further investigated the mechanism of cotreatment-induced apoptosis by Western blot analysis. When cells were cotreated with sorafenib plus MPT0E028 or vorinostat at concentrations lower than the IC50 value, we observed cleavage of PARP and caspase-3, indicating the occurrence of apoptosis (Fig. 4A). Moreover, cotreatment with the pan-caspase inhibitor, z-VAD–FMK, attenuated PARP cleavage in a concentration-dependent manner, suggesting that the observed apoptosis was triggered via a caspase-dependent pathway (Fig. 4B). Moreover, caspase-8 and caspase-9 were also activated by the combined treatment (Fig. 4C), indicating that both intrinsic and extrinsic apoptotic pathways are involved in this phenomenon. Overall, these data indicate that the synergism between sorafenib and MPT0E028 in killing hepatocellular carcinoma cells might be achieved through caspase-dependent induction of apoptosis.
Figure 3. Effects of cotreatment on cell-cycle progression and nucleosome formation in Hep3B cells. A, Hep3B cells were treated with the indicated drugs for 72 hours, and cell-cycle distribution was analyzed by flow cytometry. B, statistical analysis of sub-G1 phase in Hep3B cells exposed to DMSO (a), sorafenib alone (d and g), MPT0E028 alone (b and c), or in sorafenib/MPT0E028 combination (e, f, h, and i). Data, mean ± SD (n = 4). C, Hep3B cells were treated with sorafenib (5 μmol/L) alone or in combination with MPT0E028 (0.3125 μmol/L) or vorinostat (2.5 μmol/L) for 72 hours. Nucleosome formation was measured using the Cell Death ELISA Kit (Roche Applied Science). Data, mean ± SD (n = 3; *P < 0.01; **P < 0.001 compared with the control group).
Effects of MPT0E028 plus sorafenib on multiple signaling pathways in Hep3B cells

To gain insights into the mechanisms underlying the synergistic interaction between MPT0E028 and sorafenib in Hep3B cells, we examined whether the combined treatment enhanced the signaling pathways affected by each agent alone. There was no profound enhancement of the acetylation of histone H3 or α-tubulin following the combined treatment, but the induction p21 by MPT0E028 or vorinostat was markedly attenuated in the presence of sorafenib (Fig. 5A). Therefore, the observed synergistic effects may not result from augmentation of the epigenetic effects triggered by MPT0E028. Sorafenib has been shown to inhibit the Raf/Mek/Erk pathway, and patients with hepatocellular carcinoma with higher levels of phospho-Erk have a better survival rate (7, 25). Furthermore, loss of Mcl-1 (through inhibition of translation) has been associated with sorafenib-induced apoptosis (8), and Stat3 was recently reported to be a major kinase-independent target of sorafenib (11). Therefore, we postulated that the combination of MPT0E028 and sorafenib might impact these signaling pathways. To our surprise, MPT0E028 alone dramatically elevated the level of phosphorylated-Erk (p-Erk) and increased its downstream signaling, as reflected by increases in phosphorylated-Stat3-Ser727 (p-Stat3-Ser727) and upregulation of Mcl-1 (Fig. 5A). The same pattern was observed when sorafenib was combined with vorinostat in Hep3B cells (Fig. 5B). Forcing expression of constitutively active Mek attenuated the cotreatment-induced downregulation of p-Erk, Mcl-1, and PARP cleavage, suggesting that the sorafenib-mediated inactivation of Erk may play a pivotal role in MPT0E028-mediated apoptosis (Fig. 5B). Pharmacologic inhibition of Mek by PD98059-potentiated MPT0E028- or vorinostat-induced apoptosis, as evidenced by PARP cleavage (Fig. 5C), confirming the importance of Erk activation in the synergistic interaction between MPT0E028 and sorafenib. We further investigated the mechanism behind Erk activation by cDNA microarray to examine differential expressed genes affected by MPT0E028 (0.625 μmol/L) in Hep3B cells. The result showed that FGFR3 induction correlated with the activation of the mitogen-activated protein kinase pathway (unpublished data). We confirmed the data by RT-PCR, and the mRNA level of FGFR3 was upregulated by MPT0E028, but this phenomenon was abrogated in the presence of sorafenib (Fig. 5D). The protein levels of FGFR3 and p-Erk were also upregulated by MPT0E028 in a concentration-dependent fashion (Fig. 5E). Coadministration of FGFR3 inhibitor (PD173074) attenuated MPT0E028-induced Erk phosphorylation, suggesting that transcriptional activation of FGFR3 may contribute to Erk activation in Hep3B cells (Fig. 5F). These data reinforce the results reported by Lachenmayer and colleagues in the mechanistic aspect, which showed additive preclinical efficacy of panobinostat with sorafenib in hepatocellular carcinoma (17). Collectively, these data suggest that the synergistic interaction between MPT0E028 and sorafenib is achieved at least partially via inhibition of the FGFR3/Erk signaling pathway.

In vivo antitumor activity of MPT0E028 plus sorafenib in a Hep3B xenograft model

To evaluate whether the synergistic effect of MPT0E028 plus sorafenib could be clinically relevant, we examined the
antitumor activity of this cotreatment in athymic nude mice bearing established Hep3B tumor xenografts. Once a tumor was palpable (approximately 55 mm³), mice were randomized into vehicle control and treatment groups (n = 7/group). All mouse tumors were allowed to reach an end-point volume of 1,200 mm³, and in vivo antitumor efficacy was expressed as TGD (Fig. 6A and Table 1). The median TTE in the control group was 34.8 days. There was no improvement of TTE in mice orally treated with sorafenib (60 mg/kg daily). Oral treatment with MPT0E28 alone (100 mg/kg daily) increased the median TTE to 40.8 days, corresponding to a TGD of 17%. In the sorafenib plus MPT0E28 group, the median TTE was further prolonged to 53.1 days, for a TGD of 53%. Log-rank analysis showed that the cotreatment exhibited significant antitumor activity in the Hep3B xenograft model (P = 0.0001). MPT0E28 alone or in combination with sorafenib also suppressed tumor growth, with TGI of 33.3% and 47.6%, respectively (Fig. 6B). This effect was also observed in the PLC/PRF/5 xenograft model, with a TGI of 65.3% (Supplementary Fig. S3A). Notably, the mice tolerated all of the treatments without overt signs of toxicity; no significant body weight difference or other adverse side effect was observed (Fig. 6C and Supplementary Fig. S3B). To correlate the in vivo antitumor activity of MPT0E28 plus sorafenib alters multiple signaling pathways in Hep3B cells. A, cells were treated with various concentrations of sorafenib alone or in combination with MPT0E28 (left) or vorinostat (right). B, Hep3B cells were transfected with control vector or HA-Mek for 24 hours and then exposed to sorafenib in combination with MPT0E28 or vorinostat for 72 hours. C, cells were exposed to MPT0E28 or vorinostat in the presence or absence of PD98059 for 72 hours. Whole-cell lysates were collected and subjected to Western blot analysis (A–C). D–F, cells were treated with indicated agents for 72 hours. Relative mRNA levels of FGFR3 were determined by RT-PCR (D). Data, mean ± SD (n = 3; **, P < 0.01; *** P < 0.001 compared with the control group). Whole-cell lysates were subjected to Western blot analysis (E and F).
Synergistic Interaction between MPT0E028 and Sorafenib

Table 1. Summary of treatment response for the antitumor activity of drug combination in the human Hep3B hepatocellular carcinoma xenograft model

<table>
<thead>
<tr>
<th>n</th>
<th>Agent</th>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Schedule</th>
<th>Median TTE (days)</th>
<th>T – C</th>
<th>% of TGD</th>
<th>Log-rank significance</th>
<th>P (vs. control)</th>
</tr>
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<tbody>
<tr>
<td>7</td>
<td>Control</td>
<td>—</td>
<td>po</td>
<td>qd to endpoint</td>
<td>34.8</td>
<td>—</td>
<td>—</td>
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<td>—</td>
</tr>
<tr>
<td>7</td>
<td>Sorafenib</td>
<td>60</td>
<td>po</td>
<td>qd to endpoint</td>
<td>34.8</td>
<td>0</td>
<td>0%</td>
<td>ns</td>
<td>0.1767</td>
</tr>
<tr>
<td>7</td>
<td>MPT0E028</td>
<td>100</td>
<td>po</td>
<td>qd to endpoint</td>
<td>40.8</td>
<td>6</td>
<td>17%</td>
<td>a</td>
<td>0.0012</td>
</tr>
<tr>
<td>7</td>
<td>Sorafenib + MPT0E028</td>
<td>60 + 100</td>
<td>po</td>
<td>qd to endpoint</td>
<td>53.1</td>
<td>18.3</td>
<td>53%</td>
<td>b</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

NOTE: Study endpoint = 1,200 mm³; days in progress = 54.

Abbreviations: n, number of animals in a group not dead from accidental or unknown causes, or euthanized for sampling; ns, not significant; po, oral administration; % of TGD, [(T – C)/C] × 100; qd, once daily; statistical significance, log-rank test; T – C, difference between median TTE (days) of treated versus the control group.

*P < 0.01, compared with the control group.

**P < 0.001, compared with the control group.

effects with the mechanisms identified in vitro, intratumoral biomarkers were assessed by Western blot analysis. As shown in Fig. 6D, the combined treatment markedly induced caspase-3 activation and PARP cleavage in tumors, indicating elevated apoptosis. Consistent with our in vitro data, MPT0E028 alone increased the level of p-Erk, and this phenomenon was attenuated in the presence of sorafenib. In addition, immunostaining of Ki-67 and CD31 revealed diminished proliferation and angiogenesis within tumors from mice treated with sorafenib in combination with MPT0E028 (Fig. 6E). Taken together, these data indicate that cotreatment with MPT0E028 and sorafenib significantly enhanced the antitumor activity in vivo.

Discussion

Hepatocellular carcinoma is a complex and heterogeneous tumor that has been associated with genomic aberrations. The key signal transduction pathways that have been implicated in the pathogenesis of hepatocellular carcinoma are the EGFR, Ras/Raf/Mek/Erk, phosphoinositide 3-kinase/Akt, mTor, HGF/c-Met, Wnt, and Hedgehog signaling cascades (15). Drugs that selectively target these molecules might, therefore, have therapeutic potential. Sorafenib, a multikinase inhibitor found to increase the survival time of patients with hepatocellular carcinoma, represented a breakthrough in the treatment of this disease and is now the standard treatment for patients with advanced TNM stage, and a lower survival rate in patients with hepatocellular carcinoma (16). Thus, HDACs are promising drug targets for the treatment of hepatocellular carcinoma. Several HDAC inhibitors are currently being tested against a variety of solid and hematologic malignancies (29). In addition to being tested as a single therapeutic agent, HDAC inhibitors are also being assessed in combination with chemotherapy, radiotherapy, and molecular targeted agents (12, 30).

MPT0E028, an orally available N-hydroxycrylamide–derived HDAC inhibitor, was found to exhibit antiproliferative effects in a panel of NCI-60 cancer cell lines. Furthermore, in a HCT116 tumor xenograft model, it proved to be more potent than vorinostat in delaying tumor growth, without triggering body weight loss or adverse effects (13). It has been shown that Hep3B and PLC/PRF/5 are most resistant to sorafenib among different liver cancer cell lines (31). In this study, we confirmed the fact by showing that Hep3B and PLC/PRF/5 cells have higher IC50 than HepG2 cells (Fig. 1A) In addition, Hep3B has hepatitis B virus gene integration that has been reported to be a good model to investigate hepatocellular carcinoma research (32). Furthermore, Hep3B cells produce much higher α-fetoprotein than PLC/PRF/5 cells, suggesting Hep3B may represent patients with highly malignant tumors (33). On the basis of these findings, we used Hep3B cells in most of the mechanistic studies to elucidate the

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Figure 6. Antitumor activity of MPT0E028 plus sorafenib in a Hep3B xenograft model. Athymic nude mice bearing subcutaneously established Hep3B xenograft tumors were randomized to four groups \(n = 7\), and received the indicated treatments by gavage. All tumors grew to the 1,200 mm\(^3\) endpoint volume. A, tumor volumes were measured regularly and the percentage of TGD was calculated as described in the Materials and Methods section. (Continued on the following page).
Synergistic Interaction between MPT0E028 and Sorafenib

The synergistic effects of sorafenib and MPT0E028 in hepatocellular carcinoma.

In this study, we observed a synergistic interaction between MPT0E028 and sorafenib in human hepatocellular carcinoma cells. Therefore, MPT0E028 not only has a broad spectrum of action against different types of cancer, it also offers a new tool for treating hepatocellular carcinoma when combined with sorafenib.

Sorafenib executes its antitumor activities, which include triggering cell apoptosis and blocking tumor angiogenesis, by targeting the Raf/Mek/Erk pathway. A previous study showed that patients with high levels of p-Erk have a greater survival rate (7). To our surprise, we observed that MPT0E028, at the concentrations below IC₅₀ value (0.3125 and 0.625 μmol/L), dramatically activated Erk and aspects of its downstream signaling, such as p-Stat3-Ser²³³ and upregulation of Mcl-1. MPT0E028-mediated Erk activation was concentration-dependently abrogated by sorafenib. Ectopic expression of constitutively active Mek reversed the apoptotic cell death triggered by the cotreatment in Hep3B cells. Furthermore, blockade of Erk activation by treatment with the Mek inhibitor, PD98059, potentiated MPT0E028-induced apoptosis. Therefore, we postulated that treatment with low concentrations of MPT0E028 may render hepatocellular carcinoma cells more dependent on Erk signaling and increase their sensitivity to sorafenib. Our observations are in accordance with previous studies showing that interruption of Erk signaling by Mek inhibitors sensitized tumor cells to HDAC inhibitor–induced apoptosis (34–37). In this study, we observed the transcriptional activation of FGFR3 by MPT0E028, and that was abrogated in the presence of sorafenib. MPT0E028-induced Erk activation was abrogated by FGFR3 inhibitor (PD173074), suggesting that FGFR3 induction may be the underlying mechanism of Erk phosphorylation. Lachenmayer and colleagues have reported the additive preclinical efficacy of sorafenib and HDAC inhibitor panobinostat through decreased cell proliferation, induction of apoptosis-autophagy, as well as decreased vessel density and tumor volume in hepatocellular carcinoma xenografts (17). In the present study, we provide compelling evidence that combined treatment with the HDAC inhibitor, MPT0E028, plus sorafenib synergistically inhibits the growth of human liver cancer cells in vitro and in vivo. Moreover, we further reinforce the data by elucidating the underlying mechanisms by which sorafenib enhances MPT0E028-mediated apoptosis by inhibiting the FGFR3/Erk signaling pathway.

Mcl-1 is a Bcl-2-like antiapoptotic protein that is crucial for cancer development and chemoresistance in a number of human malignancies (38). Mcl-1 is overexpressed in about half of patients with hepatocellular carcinoma, suggesting that it is a potential target for hepatocellular carcinoma therapy (39). Translational inhibition of Mcl-1 plays a central role in the proapoptotic effects of sorafenib through dephosphorylation of eIF4E (8, 9), and down-regulation of Mcl-1 in leukemic cells potentiates the apoptosis triggered by HDAC inhibitors (40). In this study, we observed that both MPT0E028 and vorinostat induced accumulation of Mcl-1 at concentrations below the IC₅₀ value, but that this was overcome when combined with sorafenib. From our preliminary data, overexpression of Mcl-1 rescued the apoptosis induced by the cotreatment in Hep3B cells (data not shown). It has been reported that Erk increases Mcl-1 protein stability through phosphorylating at two consensus residues (Thr⁹² and Thr¹⁸⁵), and blockade of Erk signaling by sorafenib overriders Mcl-1–mediated chemoresistance (41). Previous articles have shown that upregulation of Mcl-1 is associated with resistance to the Bcl-xl inhibitor, ABT-737, but this phenomenon was abrogated by cotreatment with sorafenib or Mek inhibitors (42, 43). Therefore, Mcl-1 upregulation may also play a crucial role in limiting the efficacy of HDAC inhibitor–mediated apoptosis in human hepatocellular carcinoma.

In this study, we found that sorafenib did not potentiate the epigenetic effects of MPT0E028 (e.g., the acetylation of histone H3 and α-tubulin), but did downregulate MPT0E028-induced p21 expression. The ability of HDAC inhibitors to induce p21 expression has been described in numerous cell types, and has been shown to occur through promoter hyperacetylation (44). Disruption of p21 expression reportedly potentiates the lethality of HDAC inhibitors and DNA-damaging agents in different cancer cell types, such as leukemia and renal cell carcinoma (45–47). It has been proposed that p21 is cleaved by caspase-3 during DNA damage–induced apoptosis (48). However, sorafenib-induced p21 attenuation was not rescued by caspase-3 inhibitor (Ac-DEVD-CHO), suggesting that the mechanism may not result from protein cleavage (Supplementary Fig. S4A). Moreover, RT-PCR showed that the mRNA level of p21 was significantly upregulated by MPT0E028 and this phenomenon was abrogated in the presence of sorafenib (Supplementary Fig. S4B). Therefore, sorafenib-mediated transcriptional repression may contribute to the downregulation of p21. Beyond its role as a cyclin-dependent kinase inhibitor, recent findings suggest that p21 may...
participate in DNA repair by regulating the interaction between PARP-1 and base excision repair factors, thereby participating in resistance to chemotherapeutic agents [49, 50]. It will therefore be important to clarify whether the sorafenib-mediated downregulation of p21 contributes to the synergistic interaction between MPT0E028 and sorafenib.

In conclusion, we herein show that MPT0E028 and sorafenib interact in a synergistic manner to induce cell death in human hepatocellular carcinoma cells, triggering pronounced apoptosis via a caspase-dependent pathway. Moreover, we also show that sorafenib-mediated inhibition of MPT0E028-induced Erk activation might play a pivotal role in this synergistic effect. These results suggest that the combination of MPT0E028 and sorafenib could be an attractive strategy for treating patients with advanced hepatocellular carcinoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
Synergistic Interaction between MPT0E028 and Sorafenib

49. Cazzalini O, Scovassi AI, Savio M, Stivala LA, Prosperi E. Multiple roles of the cell cycle inhibitor p21(CDKN1A) in the DNA damage response. Mutat Res 2010;704:12–20.
Synergistic Interaction between the HDAC Inhibitor, MPT0E028, and Sorafenib in Liver Cancer Cells \textit{In Vitro} and \textit{In Vivo}

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