Tirapazamine Sensitizes Hepatocellular Carcinoma Cells to Topoisomerase I Inhibitors via Cooperative Modulation of Hypoxia-Inducible Factor-1α

Tian-Yu Cai, Xiao-Wen Liu, Hong Zhu, Ji Cao, Jun Zhang, Ling Ding, Jian-Shu Lou, Qiao-Jun He, and Bo Yang

Abstract

Topoisomerase I inhibitors are a class of anticancer drugs with a broad spectrum of clinical activity. However, they have limited efficacy in hepatocellular cancer. Here, we present in vitro and in vivo evidence that the extremely high level of hypoxia-inducible factor-1α (HIF-1α) in hepatocellular carcinoma is intimately correlated with resistance to topoisomerase I inhibitors. In a previous study conducted by our group, we found that tirapazamine could downregulate HIF-1α expression by decreasing HIF-1α protein synthesis. Therefore, we hypothesized that combining tirapazamine with topoisomerase I inhibitors may overcome the chemoresistance. In this study, we investigated that in combination with tirapazamine, topoisomerase I inhibitors exhibited synergistic cytotoxicity and induced significant apoptosis in several hepatocellular carcinoma cell lines. The enhanced apoptosis induced by tirapazamine plus SN-38 (the active metabolite of irinotecan) was accompanied by increased mitochondrial depolarization and caspase pathway activation. The combination treatment dramatically inhibited the accumulation of HIF-1α protein, decreased the HIF-1α transcriptional activation, and impaired the phosphorylation of proteins involved in the homologous recombination repair pathway, ultimately resulting in the synergism of these two drugs. Moreover, the increased anticancer efficacy of tirapazamine combined with irinotecan was further validated in a human liver cancer Bel-7402 xenograft mouse model. Taken together, our data show for the first time that HIF-1α is strongly correlated with resistance to topoisomerase I inhibitors in hepatocellular carcinoma. These results suggest that HIF-1α is a promising target and provide a rationale for clinical trials investigating the efficacy of the combination of topoisomerase I inhibitors and tirapazamine in hepatocellular cancers. Mol Cancer Ther; 13(3); 630–42. ©2013 AACR.

Introduction

Hepatocellular carcinoma is the most frequently occurring primary liver cancer, with an incidence of half a million cases per year worldwide (1). Although surgery remains the treatment of choice for hepatocellular carcinoma, tumor size and hepatic functional reservation might restrict surgical ablation. Conventional radio/chemotherapies are generally not prescribed for advanced hepatocellular carcinoma due to their low efficacies (2). We and other groups (3, 4) have found that the expression of HIF-1α is increased in most hepatocellular carcinomas and is associated with poor outcomes. Because fibrogenesis during the development of cirrhosis ultimately destroys the normal blood supply to the liver, the blood supply is limited in hepatocellular carcinoma with cirrhosis, which ultimately leads to local hypoxia (5). The insufficient blood supply of the rapidly growing tumor tissues also induces hypoxia in the central region of the tumor. Hypoxia leads to the activation of hypoxia-inducible factor 1α (HIF-1α), which acts as the transcriptional factor for a variety of essential genes in the hypoxic microenvironment, including those encoding for VEGF and basic fibroblast growth factor (6–8).

HIF-1α is a master regulator of tumor growth, angiogenesis, metastasis, and resistance to anticancer drugs (9–11). Although the proteasome pathway rapidly degrades HIF-1α under normoxia, this protein is stable under hypoxia, where it translocates to the nucleus and binds to hypoxia response elements (HREs) within the promoter of its target genes. Significant evidence indicates that HIF-1α plays an important role in the resistance to chemotherapy of hepatocellular carcinoma (12, 13). Hence, inhibition of the HIF-1α pathway is a promising approach for the treatment of hepatocellular carcinoma.
Camptothecin, a natural product isolated from *Camptotheca acuminata* Decne, was originally discovered because of its antitumor activity, and the mechanism of action was later demonstrated to be the accumulation of topoisomerase I–DNA adducts, observed both in vitro and in vivo (14–17). Camptothecins bind the covalent 3-phosphotyrosyl intermediate and specifically block DNA religation, thus inducing DNA damage. During DNA replication, the replication fork is thought to collide with the "trapped" topoisomerase I–DNA complexes, resulting in double-strand breaks and ultimately cell death. Irinotecan is a topoisomerase I inhibitor registered for the treatment of gastrointestinal cancer in humans and has been shown to exert activity on many cancer cell lines in vitro, but it fails to provide definitive information in vivo, particularly through intravenous infusion in patients with hepatocellular carcinoma (18). Similar to other cancer types, hepatocellular carcinoma progresses and develops drug resistance via the upregulation of stress-induced molecules such as HIF-1α. We surmised that HIF-1α may play an important role in the resistance to topoisomerase I inhibitors in hepatocellular carcinoma.

Tirapazamine (3-amino-1,2,4-benzotriazine 1,4 dioxide, SR4233) is a bioreductive drug belonging to the aromatic N-oxide family that has a selective toxicity for hypoxic cells (19). In cell suspensions, tirapazamine is 50- to 500-fold more toxic to hypoxic cells compared with cells in normoxia. The hypoxic selectivity of tirapazamine is due to the rapid reoxidation of the initial radical to the parent prodrug by O2. Thus, tirapazamine functions as a hypoxia-activated agent (20, 21), exhibiting the capability of potentiating the antitumor efficacy of many anticancer drugs (22–25). The putative mechanism may be attributed to its suppression of the accumulation of HIF-1α protein. Thus, we hypothesized that the combination of tirapazamine (HIF-1α–downregulating agents) with topoisomerase I inhibitors may be a potential therapeutic regimen for hepatocellular carcinoma. In our study, we show for the first time that HIF-1α is strongly correlated with resistance to topoisomerase I inhibitors in hepatocellular carcinoma. Using both in vitro and in vivo models of hepatocellular carcinoma, we demonstrate the potential for synergistic activity of tirapazamine in combination with topoisomerase I inhibitors based on the modulation of the HIF-1α protein.

Materials and Methods

**Reagents and cell lines**

CPT-11, SN-38, TPT, HCPT, and MONCPT were kindly provided by Dr. Wei Lu (East China Normal University, Shanghai, China) and tirapazamine was purchased from Topharmen Shanghai Co. Ltd. All of the drugs were dissolved in dimethyl sulfoxide (DMSO) and stored at aliquots (20 mmol/L) at −20°C. Primary antibodies to cleaved caspase-3, H2AX, p-H2AX, Chk1, p-Chk1, Chk2, p-Chk2, ATM, p-ATM, Rad51, and β-Actin were obtained from Cell Signaling Technologies. Antibodies to Caspase-3, PARP, and HRP-labeled secondary anti-goat, anti-mouse, and anti-rabbit antibodies were from Santa Cruz Biotechnology. Antibody to HIF-1α was obtained from BD Biosciences. DMSO, propidium iodide (PI), sulfonrodamine B (SRB), DAPI (4′, 6-diamidino-2-phenylindole), JC-1, and CoCl2 were obtained from Sigma-Aldrich.

The five hepatocellular carcinoma cell lines, HepG2, Hep3B, Huh 7, Bel-7402, and SMMC-7721, were purchased from Cell Bank of China Science. A normal liver cell line, Chang liver, was obtained from Cell Bank of China Science. Following receipt, cells were grown and frozen as a seed stock as they were available. All six cell lines were passaged for a maximum of 2 months after which new seed stocks were thawed. All six cell lines were authenticated using DNA fingerprinting (variable number of tandem repeats), confirming that no cross-contamination occurred during this study. All six cell lines were tested for mycoplasma contamination at least every month. The six cell lines were cultured in RPMI-1640 medium/DMEM containing 10% FBS and 1% antibiotics in a humidified atmosphere with 5% CO2 at 37°C. Hypoxia treatment was carried out by placing the cells in a CO2-Water Jacketed Incubator (Thermo Forma) filled with a mixture of 0.6% O2, 5% CO2, and 94.4% nitrogen.

**Cell proliferation assay**

Cell proliferation was evaluated by the Sulforhodamine B (SRB) protein staining assay (26). Briefly, treated cells in 96-well plates were fixed with 10% TCA and stained with SRB. SRB in the cells was dissolved in 10 mmol/L Tris-HCl and measured at 515 nm using a multiwell spectrophotometer (Thermo Electron Co.). The rate of inhibition of cell proliferation was calculated for each well as (A515control cells –A515treated cells)/A515control cells ×100%. The average IC50 values were determined by the logit method from at least three independent tests.

**Flow cytometric analysis of apoptosis and mitochondrial membrane potential (∆Ψm)**

Cells were treated with SN-38, tirapazamine, or their combination under normoxia or hypoxia for 12 hours. After harvesting and washing twice with cold PBS buffer, the Annexin V-FITC/PI apoptosis Detection Kit was used to analyze apoptotic cells. Analysis of the sub-G1 phase after PI staining was also used to assess apoptosis. For PI staining, treated cells were harvested and fixed with 70% ethanol at −20°C, then incubated with RNaseA followed by PI in the dark for 30 minutes. For determination of mitochondrial potential, cells were resuspended in PBS containing 0.1 μmol/L JC-1 and were incubated at 37°C for 15 minutes in the dark. All samples were analyzed using a FACS-Calibur cytometer (Becton Dickinson).

**Clonogenic assays**

Cells treated with tirapazamine or SN-38/TPT/HCPT/MONCPT or their combination were plated in 60 mm dishes in triplicate on soft agar. Once set, the dishes were overlaid with 2.5 mL of medium and incubated at 37°C for...
10 days in hypoxic conditions, at which time the colonies were scored and photographed.

**Quantitative real time RT-PCR analysis**

Total RNA was prepared with Trizol (Invitrogen) and cDNA was synthesized from 2 μg of RNA with Reverta Ace (Toyobo). Quantitative real-time RT-PCR (qRT-PCR) was performed with SYBR Green PCR Kits (Qiagen Inc.). The primers used were as follows: **HIF-1α**, forward primer 5′-TCACCCACAGCACTACAGGATC-3′ and reverse primer 5′-CCACCAAGTTAAAGCATCAGGT-TCC-3′; **VEGF**, forward primer 5′-AGGAGGCGAAT- CATCACG-3′ and reverse primer 5′-CAAGGCCACA- GGGATTTCTC-3′; **GAPDH**, forward primer 5′-GTCACTCATGACACCTGAGG-3′ and reverse primer 5′-GAGC- CTTGACAAAAGTGTG-3′. In all experiments, two negative controls were also performed. Values are expressed as fold increases relative to the reference sample.

**Immunofluorescence**

Cells were plated onto glass culture slides and incubated with SN-38/tirapazamine or SN-38 or vehicle [0.1% DMSO (v/v)] for different time periods. After blocking with 5% bovine serum albumin for 30 minutes, cells were incubated with primary HIF-1α or γ-H2AX antibodies (1:100 dilution) for 1 hour, washed three times with PBS and then incubated with Alexa Fluor 488-conjugated and rhodamine secondary antibodies (Invitrogen), respectively, in the dark. Nuclei were visualized by staining with DAPI (4′,6-diamidino-2-phenylindole). Fluorescence signals were analyzed using an Olympus Fluoview 1000 confocal microscope.

**Plasmid-based overexpression of HIF-1α**

Transfection with **HIF-1α** plasmids or empty vector (EV) obtained form Origene was performed using the FuGENE HD Transfection Reagent (Roche Applied Science) according to the manufacturer’s instructions.

**shRNA knockdown of HIF-1α**

shRNA targeting **HIF-1α** was purchased from Origene with a GFP-tag and puromycin resistance. Transfection of shRNA into Bel-7402 cells was performed using Lipofectamine 2000 (Invitrogen). Individual clones stably expressing the shRNA were generated by single-cell sorting into 96 round-bottom wells and antibiotic selection with puromycin (500 ng/mL). Western blot analysis and an Olympus Fluoview 1000 confocal microscope were used to select clones with stable knockdown of **HIF-1α**.

**Immunoblot analysis**

Immunoblotting of biomarkers in cell lysates and tumor tissue homogenates was performed as previously described (27).

**Luciferase reporter assays**

Bel-7402 cells were seeded into 96-well plates and then cotransfected with HRE-luciferase-pGL3 and Renilla luciferase (internal control for transfection efficiency) plasmids using Lipofectamine 2000 according to the manufacturer’s instructions. Luciferase activity was measured using the Dual-Luciferase reporter assay system. In the assay, firefly luciferase activity was normalized by Renilla luciferase.

**Measurement of in vivo activity**

Tumors were established by injection of Bel-7402 cells (5 × 10⁶ cells per animal, subcutaneously into the arm pit) into 5- to 6-week-old BALB/c male athymic mice. Treatments were initiated when tumors reached a mean group size of about 100 mm³. Tumor volume (mm³) was measured with calipers and calculated as (W²×L)/2, where W is the width and L is the length. Athymic mice were intraperitoneally injected with CPT-11 (2.5 mg/kg) dissolved in physiologic saline once daily and tirapazamine (25 mg/kg) dissolved in a cremophor:ethanol:0.9% sterile sodium chloride solution (1:1:8, volume) every 2 days. Mouse weight and tumor volumes were recorded every 2 days until the animals were sacrificed. Animal care was in accordance with institutional guidelines.

**Statistical analyses**

The results are expressed as the mean ± SD of at least three independent experiments. Differences between means were analyzed using Student t test and were considered statistically significant when P < 0.05.

**Results**

**HIF-1α protein level is strongly correlated with resistance to topoisomerase I inhibitors in hepatocellular carcinoma cell lines**

In our study, we first investigated the strength of the association between expression of HIF-1α and resistance to topoisomerase I inhibitors in hepatocellular carcinoma cell lines. We measured Bel-7402 cell growth under hypoxic or normoxic culturing conditions in the presence of topoisomerase I inhibitors. Compared with normoxia, the IC₅₀ values for SN-38, TPT, HCPT, and MONCPT were 7.6-, 6.2-, 5.4-, and 4.3-fold higher, respectively, under 0.6% O₂. Then, we decided to investigate whether the accumulation of HIF-1α induced by hypoxia was a key factor in this resistance; thus, an HIF-1α–deficient Bel-7402 cell line was established. The transfection of a HIF-1α–targeting shRNA construct almost completely suppressed the protein expression of HIF-1α even under hypoxia, whereas HIF-1α expression in EV-transfected Bel-7402 cells remained intact (Fig. 1A). Compared with normoxia, the IC₅₀ for SN-38, TPT, HCPT, and MONCPT were similar under 0.6% O₂ for Bel-7402/HIF-1α (-), hypoxia-induced resistance disappeared in HIF-1α gene silencing cells (Fig. 1B and Supplementary Fig. 51). Meanwhile, cells treated with 200 μmol/L CoCl₂, which resulted in a robust induction of intracellular HIF-1α,
showed no statistical difference in the IC50 for SN-38 between cells treated with CoCl2 and hypoxic cells (Fig. 1C). These data demonstrate that hypoxia can induce resistance to topoisomerase I inhibitors in hepatocellular carcinoma cell lines and HIF-1α may play an important role.

Tirapazamine synergistically increases topoisomerase I inhibitor inhibition of cell proliferation in hepatocellular carcinoma cell line

The above observations indicate the therapeutic potential treatment with the combination of topoisomerase I inhibitors and HIF-1α inhibitors, which may overcome the resistance to topoisomerase I inhibitors in hepatocellular carcinoma cell lines. It has been reported that tirapazamine can inhibit the accumulation of HIF-1α protein (28), and we confirmed this effect in our system using luciferase reporter gene assays and immunoblot analysis. As shown in Fig. 2B, luciferase activity was markedly increased under hypoxia compared with normoxia, while this activity was suppressed by tirapazamine in a dose-dependent manner after 6-hour treatment, ranging from 10.2% (0.31 μmol/L) to 66.2% (10 μmol/L). Similarly, the protein level of HIF-1α was reduced upon tirapazamine treatment (Fig. 2A). Taken together, these data imply that tirapazamine is indeed inhibiting HIF-1α accumulation.

Because tirapazamine can inhibit the accumulation of HIF-1α protein, we determined the cytotoxicity of tirapazamine and each of the four topoisomerase I inhibitors alone and of tirapazamine combined with each of the other drugs at clinically achievable concentrations in the Bel-7402 cell line using the SRB cytotoxicity assay. Combination index (CI) values were calculated at the fixed-ratio drug concentrations used for cytotoxicity assays. Dose–response curves to tirapazamine, the four topoisomerase I inhibitors and tirapazamine combined with each topoisomerase I inhibitor are shown in Fig. 2C–F. The combinations of tirapazamine plus topoisomerase I inhibitor all showed strong synergy (CI < 0.3) or synergy (CI < 0.7) in the Bel-7402 cell line, achieving about 80% cell death. In
addition, we determined the cytotoxicity of tirapazamine (2.5 μmol/L) in combination with various concentrations of topoisomerase I inhibitors in Bel-7402 cells. As shown in Supplementary Fig. S2, treatment of cells with tirapazamine (2.5 μmol/L) significantly enhanced cell sensitivity to topoisomerase I inhibitors. To analyze the antitumor effect of the combination treatment of tirapazamine with the four topoisomerase I inhibitors, a clonogenic assay was performed. As shown in Supplementary Fig. S3, tirapazamine and the topoisomerase I inhibitors both weakly suppressed colony formation of Bel-7402 cancer cells (55%–85%). Significantly, tirapazamine enhanced the antitumor effect of topoisomerase I inhibitors and drastically suppressed colony formation (11%–23%). Thus, the combination effect of tirapazamine and topoisomerase I inhibitors was quite dramatic and this effect had to be confirmed in other hepatocellular carcinoma cell lines.

Tirapazamine synergistically increases SN-38–induced cell proliferation inhibition in different hepatocellular carcinoma cell lines

Because tirapazamine synergized most strongly with SN-38 (active metabolite of irinotecan), and irinotecan is a widely used drug in clinical practice, we determined the effects of tirapazamine, SN-38, and their combination on various hepatocellular carcinoma cell lines, including Bel-7402, SMMC-7721, HepG2, Hep3B, and Huh-7. Survival curves to tirapazamine, SN-38, and their combination are shown in Fig. 3A–E. As shown in Supplementary Table S1, SN-38 plus tirapazamine showed distinct synergy in all cancer cell lines tested, with all CI values below 0.8, indicating the much stronger antiproliferative abilities achieved by the combination of SN-38 and tirapazamine. However, in a normal liver cell line, the combination effect was minimal (Fig. 3F).
Tirapazamine sensitizes hepatocellular carcinoma cells to SN-38–triggered caspase-dependent apoptosis

To further understand mechanisms of enhanced cytotoxicity observed with the combination of tirapazamine and SN-38, we examined their effects on apoptosis, mitochondrial membrane depolarization, and caspase activation. We first investigated whether the observed cytotoxicity was due to apoptosis. DAPI staining was used to visualize the apoptosis induced by the cotreatment of tirapazamine and SN-38. After exposure to SN-38 or/and tirapazamine of the indicated concentrations for 12 hours, the nuclear DNA of Bel-7402 cells were permeabilized and stained with DAPI. As shown in Fig. 4A, 10 μmol/L tirapazamine plus 0.1 μmol/L SN-38 triggered more apoptosis, as indicated by the apoptotic bodies, than either tirapazamine or SN-38 induced alone. We next investigated the effects of...
treatment with the single agents or the combination on mitochondrial membrane potential. Mitochondrial membrane depolarization, as determined by the JC-1 mitochondrial probe, was induced by tirapazamine and SN-38 as single agents 12 hours after treatment, and the combination resulted in a greater than additive effect ($P < 0.05$) compared with the single agents (Fig. 4C).

Furthermore, we examined the effect of tirapazamine, SN-38, and their combination on the activation of caspase-3 and cleavage of PARP. We found that although tirapazamine and SN-38 had little effect on caspase-3 and PARP, the two together induced a more significant cleavage of PARP and caspase-3 (Fig. 4E). Together, our results demonstrate that the combination of tirapazamine and SN-38 elicited more apoptosis via mitochondrial membrane depolarization, resulting in increased cell death.

**Tirapazamine and SN-38 combination therapy suppresses HIF-1α expression**

Our results indicate that high levels of HIF-1α confer resistance to SN-38; thus, we were interested in examining the involvement of HIF-1α in the efficacy of the...
tirapazamine and SN-38 combination treatment. Western blot analysis and immunofluorescence microscopy (Fig. 5A and B) showed that tirapazamine and SN-38 alone weakly inhibited the accumulation of HIF-1α protein in vitro. Strikingly, combination therapy resulted in complete inhibition of HIF-1α accumulation. The combination did not have an effect on the HIF-1β and HIF-2α proteins. Then, we analyzed the
mRNA levels of HIF-1α and VEGF by RT-PCR. The results showed that exposure to tirapazamine or SN-38 reduced the expression of VEGF, a target gene of HIF-1α (Fig. 5C). This effect was at least additive when the two agents were administered together, similar to their effect on HIF-1α protein levels. HIF-1α binds to the HRE cis-elements within the promoters of hypoxia-responsive genes to regulate their expression. To investigate whether the tirapazamine and SN-38 combination treatment could affect the activity of HIF-1α, we examined its effect on HIF-1α binding to DNA using a reporter gene assay. After transfection, cells were exposed to the single agents or the combination for 4 hours under hypoxia. As shown in Fig. 5D, luciferase activity was markedly induced under hypoxia compared with normoxia, and this activity was both inhibited by increasing doses of tirapazamine or SN-38. This inhibition was dramatically increased when tirapazamine and SN-38 were combined, and at the maximum combination dose (10 μM plus 0.1 μmol/L) there was a 92% decrease of HIF-1α transcriptional activity.

Next, we investigated the effect of the combination on HIF-1α posttranscriptional regulation. As shown in Supplementary Fig. S4A, the degradation rates of HIF-1α were similar in treated and untreated cells, indicating that the combination did not significantly affect HIF-1α degradation. To exclude the possibility that the inhibition of HIF-1α accumulation was attributed to HIF-1α protein degradation, Bel-7402 cells were pretreated with MG132 (a specific proteasome inhibitor) or chloroquine (CQ, a lysosome inhibitor) before the drugs were added to prevent HIF-1α degradation. As shown in Supplementary Fig. S4B, MG132 and CQ treatment failed to reverse the decrease of HIF-1α protein levels in the combination treatment.

To more directly assess the effects of the combination on the rate of de novo synthesis of the HIF-1α protein, cells were pretreated with cycloheximide (CHX) for 3 hours under normoxia to inhibit new protein synthesis and then incubated in fresh medium. At this point, most HIF-1α proteins were degraded, and a limited amount of protein remained. These CHX-pretreated cells were then exposed to hypoxic conditions, followed by treatment with tirapazamine, SN-38, or the combination for different periods of time, and HIF-1α protein levels were analyzed by Western blot analysis. As shown in Fig. 5E, significantly less HIF-1α protein accumulated in cells treated with the combination than in cells treated with tirapazamine or SN-38 alone at all times tested. This result further confirmed that the combination decreases the rate of HIF-1α protein synthesis.

There is a functional link between HIF-1α variation and DNA repair. To assess the effect of tirapazamine, SN-38, and their combination on DNA, the formation of DSBs was measured by immunostaining of γ-H2AX. The cells treated with tirapazamine or SN-38 showed several γ-H2AX foci after a 4-hour treatment. Cells treated with the combination treatment for 4 hours showed a more than 3-fold increase in the number of γ-H2AX foci (Fig. 5B), which is similar to the results for γ-H2AX protein levels (Fig. 5A). To investigate whether the effect of the combination treatment on HIF-1α expression might affect DNA repair, shRNA-mediated repression of HIF-1α was performed in Bel-7402 cell line. Bel-7402/HIF-1α(-) and Bel-7402/HIF-1α(+) cells were pretreated with SN-38 for 2 hours under hypoxia to form DSBs and then incubated in fresh medium without drug for 6 hours. The formation of DSBs was measured by immunostaining. As shown in Fig. 5F, the Bel-7402/HIF-1α(-) cells contained more DSB-indicative γ-H2AX than those Bel-7402/HIF-1α(+) cells, indicating HIF-1α knockdown causes an inhibition of DNA repair, resulting in a larger population of persistently unrepaired DSBs.

Camptothecin-induced DSB are predominantly repaired by homologous recombination (HR) in mammalian cells (29–31). We attempted to detect which repair pathway was inhibited by HIF-1α in the current model to repair the DSBs elicited by camptothecin. As shown in Fig. 5A, ATM, Chk1, and Chk2 were phosphorylated after 4 hours of exposure to SN-38 or tirapazamine. The Rad51 and phosphorylation of Chk1 at Ser317 were both decreased in the combination treatment of tirapazamine and SN-38. The close correlation between loss of RAD51 and DSB accumulation suggested that the inhibition of the RAD51-mediated HR repair pathway was likely responsible for the accumulation of DSBs in Bel-7402 cells.

The combination of tirapazamine and irinotecan arrests tumor growth

Because the tirapazamine plus SN-38 exhibited the most effective synergistic effect with the lowest CI values on human hepatocellular carcinoma Bel-7402 cells (Supplementary Table S1), the in vivo efficacy of the tirapazamine and irinotecan combination therapy was chosen to test against Bel-7402 xenografts in nude mice. As shown in Fig. 6A and B and Supplementary Table S2, the intra-peritoneal administration of tirapazamine at a dose of 25 mg/kg every 2 days or irinotecan at a dose of 2.5 mg/kg every day produced no significant difference in mean RTV compared with that of the control group. However, tirapazamine plus irinotecan caused marked tumor growth inhibition (T/C value: 36.9%) that was significantly greater than that caused by tirapazamine (T/C value: 88.1%) or irinotecan treatment alone (T/C value: 86.4%). Furthermore, compared with the initial body weights, the mice treated with the combination showed no significant body weight loss on day 26 (Fig. 6C). Thus, the combination of tirapazamine and irinotecan exerted more potent tumor growth inhibitory effects compared with the monotherapy groups, but caused no extended bodyweight loss to the animals.

The combination therapy induces apoptosis and downregulates HIF-1α in tumor tissues

Hematoxylin and eosin (H&E) staining for formalin-fixed paraffin-embedded tissues was used to distinguish
tumor tissues from adjacent normal tissues. In mice treated with tirapazamine and irinotecan, most tumor cells were severely damaged or destroyed (Fig. 6E). We next aimed to explore the effect of monotreatment or combined treatment of tirapazamine and irinotecan on the protein levels of apoptosis-related proteins in tumor tissues from drug-administrated mice. Notably, the caspase cascade was activated in the tissues from the nude mice treated with combination therapy (Fig. 6D). Importantly, the expression of HIF-1α, as determined by Western blot analysis (Fig. 6D) and immunohistochemistry (Fig. 6F), was consistent with the aforementioned cell culture data (Fig. 5A and 5B), highlighting the involvement of these proteins in the tumor growth inhibitory effects exerted by tirapazamine and irinotecan in vivo.

Discussion
Topoisomerase I inhibitors are a class of anticancer agents with a mechanism of action involving the disruption of DNA replication in cancer cells, the result of which is cell death. They are important components in the current treatment of colorectal cancer; however, in clinical studies of topoisomerase I inhibitors with advanced hepatocellular carcinoma, they had modest activity in advanced hepatocellular cancer. Accumulating evidence demonstrates that overexpression of HIF-1α plays an important role in chemoresistance and that hypoxia has...
a protective effect against DNA damage induced by topoisomerase inhibitors (32, 33). As shown in Supplementary Fig. S5A, the protein level of HIF-1α in Bel-7402 cells in hypoxia is much higher than the levels in two colon cancer cell lines HT29 and SW480, which were reported to be susceptible to SN-38 (34) in hypoxia owing to their decreased levels of HIF-1α. Thus, we speculated that the hepatocellular carcinoma resistance to topoisomerase 1 inhibitors is correlated with levels of HIF-1α. As expected, under hypoxia, Bel-7402 cells were resistant to SN-38, TPT, HCPT, and MONCPT. Furthermore, our results using shRNA or cobalt chloride (CoCl2) are also consistent with our hypothesis that HIF-1α is an important element in the limited efficacy of topoisomerase 1 inhibitors on hepatocellular carcinoma. Accordingly, selective down-regulation of HIF-1α has been documented to be a mechanism underlying increased sensitization to topoisomerase 1 inhibitors.

Tirapazamine is an anticancer drug that is activated to a toxic radical specifically in hypoxic microenvironments. Cells in hypoxic areas are resistant to killing by most anticancer drugs, and tirapazamine has been shown to potentiate the antitumor efficacy of many anticancer drugs in hypoxia. It was reported that tirapazamine can induce a reduction in HIF-1α protein levels; thus the combination of tirapazamine with topoisomerase 1 inhibitors was of particular interest to us.

As expected, in our in vitro study, synergistic anticancer effects of tirapazamine plus topoisomerase 1 inhibitors were observed in human hepatocellular carcinoma cells. CI values and the significant decline of the survival curves in the combination group strongly demonstrated that tirapazamine potentiated the SN-38-induced cytotoxicity in hepatocellular carcinoma cells. In addition, the data from both the flow cytometry and Western blot analyses indicated that tirapazamine plus SN-38 synergistically increased the execution of caspase-dependent apoptosis. We also compared the cytotoxicity of tirapazamine plus SN-38 in hepatocellular carcinoma cell lines to the Chang liver cell line (normal liver cells). The data suggest that the combination of tirapazamine plus SN-38 kills hepatocellular carcinoma cells efficiently but minimally affects normal liver cells. In our in vivo experiment, this synergistic effect of the drug combination was also observed in the Bel-7402 xenograft nude mice model (Fig. 6B). The coadministration of tirapazamine and irinotecan arrested tumor growth by 60.9%. Moreover, there was no significant difference in body weight loss between combination and irinotecan treatment groups. These results suggest that the tirapazamine and irinotecan combination synergistically inhibited tumor growth and had minimal toxicity in vivo.

In this work, we have shown that the combination of tirapazamine and topoisomerase 1 inhibitors has a major antitumor effect in vivo and induces massive cell death in vitro under hypoxic conditions. These effects are correlated with a potent inhibition of HIF-1α accumulation in tumor cells. Adding tirapazamine to SN-38–based therapeutics was accompanied by inhibition of HIF-1α accumulation in vivo and in vitro as well as a dramatic reduction of tumor volume and massive tumor cell death. This effect was observed even at low doses of tirapazamine and SN-38 due to a cooperative effect of the two agents on HIF-1α expression. The combination of tirapazamine and SN-38 did not affect the mRNA levels of HIF-1α but did decrease the rate of HIF-1α protein synthesis and inhibited HIF-1α transcriptional activity and the accumulation of HIF-1α protein, resulting in a decrease in expression of target genes, such as VEGF.

The antitumor activity of topoisomerase I inhibitors is generally believed to result from stabilization of covalent topoisomerase I–DNA complexes (35), which are converted to DNA double-strand breaks upon collision with the replication fork. Tirapazamine, a hypoxia-selective cytotoxin, has demonstrated activity in cancer clinical trials. Under hypoxic conditions, tirapazamine is reduced to a topoisomerase Ilox poison that leads to DNA double-strand breaks, single-strand breaks, and base damage (20). The typical mechanism by which both tirapazamine and topoisomerase I inhibitors work is through binding to the topoisomerase molecule, which blocks the topoisomerase from binding to the DNA, causing the DNA damage and ultimately leading to cell death.

HIF-1α plays an important role in the prevention and repair of drug-induced DNA damage and can cause chemoresistance. In the work by Kang and colleagues (36), transcript levels of two DNA-PK complex members, DNA-PKcs and Ku80, were found to be downregulated in HIF-1α–deficient cells that were previously found to have a higher susceptibility to DNA DSB-inducing chemotherapeutics. L.E. Huang reported (37) that HIF-1α upregulates DNA repair genes by counteracting c-Myc activities through c-Myc displacement. In recent research (38), modulating expression of HIF-1α by small interfering RNA or cobalt chloride markedly reduced or increased transcription of XPA in lung cancer cell lines, and XPA has a dual role in sensing and recruiting other DNA repair proteins to the damaged template. Therefore, we hypothesized that modulation on HIF-1α is involved in the synergistic effect of the tirapazamine and SN-38 combination treatment. To test this hypothesis, we next asked whether the synergism could be achieved in cells with forced HIF-1α overexpression. Bel-7402 cells forced to overexpress HIF-1α were treated with tirapazamine, SN-38, or their combination. We found that the synergistic effects were weakened when cells were transfected with the HIF-1α plasmid (Supplementary Fig. S6).

In our research, the well-established DNA damage marker γ-H2AX was used to investigate genetic instability. Because of the presence of HIF-1α, tirapazamine, or SN-38 by itself in a low dose did not induce much DNA damage, but the combination treatment of tirapazamine and SN-38 greatly increased the amount DNA damage observed in the cells. The complete inhibition of HIF-1α accumulation may be responsible for γ-H2AX induction in our model because HIF-1α can induce a DNA damage
repair response. Furthermore, the first phase occurred in both Bel-7402/HIF-1α (−) and Bel-7402/HIF-1α (+) cells after 4 hours of SN-38 treatment, which represented a fast increase in foci. This rapid increase in γ-H2AX foci suggests DNA damage is occurring in the tumor cells. In the second phase, the γ-H2AX foci levels dramatically decreased in Bel-7402/HIF-1α (+) cells but remained the same in Bel-7402/HIF-1α (−) cells. These data may be explained by the following mechanistic model: the addition of SN-38 induced DNA DSBs; DNA repair mechanisms were ongoing, which eliminated the increase of foci production; after the cells had been placed in fresh medium without SN-38 for 4 hours, the γ-H2AX foci were eliminated; the knockdown of HIF-1α by short hairpin RNA (shRNA) impaired DNA repair, a larger population of persistently unrepaired DSBs remained. We also observed that ATM, Chk1, and Chk2 were phosphorylated after 4 hours exposure to SN-38 or tirapazamine. However, Rad51 and phosphorylation of Chk1 at Ser317 were decreased in the combination treatment of tirapazamine and SN-38. The strong correlation between RAD51 removal and DSB accumulation suggested that the inhibition of the RAD51-mediated HR repair pathway was most likely responsible for the accumulation of DSBs in Bel-7402 cells. Therefore, our observations suggested that through cooperative inhibition of HIF-1α accumulation, the combination of tirapazamine and SN-38 inhibited the RAD51-mediated homologous recombination repair pathway, induced the accumulation of topoisomerase inhibitor-induced DNA damage, exhibited synergistic cytotoxicity and triggered significant apoptosis in hepatocellular carcinoma cell lines.

In summary, we report for the first time that HIF-1α is strongly correlated with hepatocellular carcinoma cell line resistance to topoisomerase I inhibitors. The combination significantly improved the anticancer activities of the drugs, as indicated by the synergistic inhibitory effects on cancer cell proliferation, the sensitized execution of apoptosis, and the enhanced in vivo antitumor efficiency. We believe these results can be attributed to the down-regulation of HIF-1α. Collectively, these data favor the regimen of combining tirapazamine and topoisomerase I inhibitors as a promising therapeutic strategy, and further preclinical and clinical studies of this novel combination are warranted.

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

Authors’ Contributions
Conception and design: T.-Y. Cai, X.-W. Liu, J. Cao, Q.-J. He, B. Yang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.-Y. Cai, X.-W. Liu, J. Zhang, L. Ding
Analysis and interpretation of data (e.g., statistical analysis, biosististics, computational analysis): T.-Y. Cai, X.-W. Liu, J. Cao, J. Zhang, L. Ding
Writing, review, and/or revision of the manuscript: T.-Y. Cai, X.-W. Liu, H. Zhu, Q.-J. He
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T.-Y. Cai, J. Cao, J. Zhang, J.-S. Lou, B. Yang
Study supervision: T.-Y. Cai, H. Zhu, Q.-J. He, B. Yang

Grand Support
This work was supported by grants from National Natural Science Foundation of China No. 81302789 (to J. Lou), Zhejiang Provincial Program for The Cultivation of High-Level Innovative Health Talents (to Q. He), the Department of Education of Zhejiang Province No. Y201226213 (to L. Ding) and the Fundamental Research Funds for the Central Universities No. 2012QNA7021 (to L. Ding)

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 17, 2013; revised November 21, 2013; accepted December 12, 2013; published OnlineFirst December 20, 2013.


Molecular Cancer Therapeutics

Tirapazamine Sensitizes Hepatocellular Carcinoma Cells to Topoisomerase I Inhibitors via Cooperative Modulation of Hypoxia-Inducible Factor-1α

Tian-Yu Cai, Xiao-Wen Liu, Hong Zhu, et al.


Updated version Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-13-0490

Supplementary Material Access the most recent supplemental material at: http://mct.aacrjournals.org/content/suppl/2013/12/23/1535-7163.MCT-13-0490.DC1

Cited articles This article cites 38 articles, 11 of which you can access for free at: http://mct.aacrjournals.org/content/13/3/630.full#ref-list-1

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.