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# The tyrosine kinase inhibitor sorafenib sensitizes hepatocellular carcinoma cells to taxol by suppressing the HURP protein

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ABSTRACT

The hepatoma upregulated protein (HURP) represents a putative oncogene that is overexpressed in many human cancers, especially hepatocellular carcinoma (HCC). HURP plays an important role during mitotic spindle formation, a process that is targeted by various anti-cancer drugs like taxol. However, the role of HURP during the establishment of taxol chemoresistance in HCC remains unclear. In this study, we observed that high HURP protein level correlates with taxol resistance in HCC cells. Following HURP knockdown, HCC cells show a more sensitive response to taxol treatment. Notably, sorafenib, a tyrosine kinase inhibitor approved for the treatment of HCC, inhibits HURP expression primarily at the transcriptional level and sensitizes HCC cells to sub-lethal doses of taxol. By using real-time PCR and chromatin immunoprecipitation assays, we observed that the NF-κB family member c-Rel represents a putative transcription factor that activates HURP gene expression. In addition, the inhibitory effect of sorafenib on HURP expression was attributed to a reduced translation and nuclear translocation of c-Rel. Accordingly, downregulation of c-Rel using short-hairpin RNA was shown to reduce HURP protein level and enhance taxol-induced cell death. Taken together, our results indicate that HURP acts as a novel survival protein that protects HCC cells against taxol-induced cell death. In addition, the regulation of HURP gene expression by NF-κB signaling appears to be critical for the response of HCC cells to taxol.
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## 1. Introduction

The hepatoma upregulated protein (HURP) is a putative oncoprotein that is overexpressed in various cancer cells, including liver [1,2], bladder [3,4] and colon cancer cells [5]. Overexpression of HURP increases the proliferation and transformation activity of non-tumorigenic HEK293 cells [1,6] and enhances the invasiveness of hepatocellular carcinoma (HCC) cells [7]. While HURP has been used as a marker for stem cells and is abundantly expressed in poorly-differentiated cells, overexpression of HURP in embryonic stem cells inhibits their differentiation to embryoid body cells and protects the cells against apoptosis induced by serum starvation [5]. These observations suggest that HURP may function as a survival protein that protects cells against stress-induced cell death. Indeed, we recently found that HURP is induced by hepatitis B viral oncoprotein HBx, and it decreases cisplatin-induced apoptosis in hepatoma cells [8]. However, the precise cellular function and mechanisms of action of HURP are still unclear. In addition, the possibility that HURP has a protective role against other chemotherapeutic drugs like taxol has not been investigated.

HURP can form a complex with RanGTP and localize predominantly to kinetochore microtubules in vivo [9]. The localization of HURP to the mitotic spindle and its association with cellular RanGTP may promote microtubule polymerization and bipolar spindle formation when cells enter mitosis. Depletion of HURP may destabilize K-fibers, thereby delaying chromosome congression and leading to chromosome missegregation [9–11]. Notably, the Aurora kinase A phosphorylates HURP and increases its stability [2]. Furthermore, enhanced expression of Aurora kinase A promotes cell proliferation and cisplatin resistance of esophageal squamous cell carcinoma [12]. This resistant phenotype is analogous to our previous findings that a high level of HURP protein correlates with cisplatin resistance in Hep3B hepatoma

*Abbreviations:* ChIP, chromatin immunoprecipitation; DAPI, 4′,6-diamidino-2-phenylindole; DFF/ICAD, DNA fragmentation factor/inhibitor of caspase activated DFF; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; HCC, hepatocellular carcinoma; HURP, hepatoma upregulated protein; eIF4E, eukaryotic translation initiation factor 4E; Luc, luciferase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PVDF, polyvinylidene fluoride; qRT-PCR, quantitative real-time reverse transcription-PCR; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; shRNA, short-hairpin RNA.

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cells [8]. Notably, overexpression of Aurora kinase A in either HeLa cervical carcinoma cells or U2OS cells can bypass both taxol- and nocodazole-induced mitotic spindle checkpoints, as well as protect the cells against apoptosis induced by drugs that produce mitotic damage [13,14]. However, it is unknown whether HURP participates in mitotic blockage and cell death induced by drugs that disrupt the mitotic spindle.

Sorafenib – a compound recently approved by the FDA to treat HCC – represents a potent anti-cancer drug due to its anti-angiogenic properties, and is currently used in combination with other traditional chemotherapeutic drugs [15–17]. Sorafenib also functions as a multiple tyrosine kinase inhibitor, thereby decreasing cell proliferation and increasing apoptosis of cancer cells by suppressing the Raf/MEK/ERK [18–20] and PI3K/Akt pathways [21]. p38/MAPK is also a target of sorafenib in biochemical kinase assays performed in vitro [15]. In addition, knockdown of B-Raf using siRNA leads to mitotic spindle abnormality and early mitotic exit in human somatic cells [22]. Depletion of p38/MAPK also arrests HeLa cells at G2/M phase by day 2 and increases apoptosis by day 3. These findings may be explained by the notion that p38/MAPK regulates mitosis progression by activating MAP kinase-activated protein kinase 2 (MK2), which phosphorylates Pin1 at ser326 and is involved in bipolar spindle formation [23]. Moreover, these results suggest that sorafenib may perturb mitotic spindle formation as well as chromosome alignment and congression by inhibiting Raf and p38/MAPK, and possibly also by depleting HURP. In a Phase I study, a combination of sorafenib, carboplatin, and paclitaxel has been shown to represent a promising anti-tumor treatment in patients with advanced non-small cell lung cancer (NSCLC) [24]. However, it remains unclear whether sorafenib may enhance the effects of drugs like taxol that induce mitotic spindle damage in HCC cells. This possibility prompted us to study the role of HURP and sorafenib on taxol-treated HCC cells.

Here, we found that HCC cells that harbor a high level of HURP protein are spontaneously resistant to taxol. Similarly, overexpression of HURP in non-tumorigenic HEK293 increased resistance to taxol. On the other hand, knockdown of HURP using shRNA sensitized hepatoma cells to taxol. We also demonstrated that sorafenib inhibits HURP expression through downregulation of c-Rel, a putative HURP gene transactivator identified in this study. Notably, sorafenib was shown to sensitize hepatoma cells to sub-lethal doses of taxol. These results have important implications to understand the role of HURP and the effects of sorafenib on taxol-treated HCC cells.

## 2. Materials and methods

### 2.1. Cell cultures and reagents

Human embryonic kidney cells (HEK293) and hepatocellular carcinoma cells Huh7, Hep3B, and Mahlavu were maintained in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/ml; Gibco), and streptomycin (100 μg/ml; Gibco). All cells were incubated at 37 °C in a humidified atmosphere of 5% (v/v) $CO_2$ in air. The reagents used included antibodies against cleaved caspase-3 (Cell Signaling, Danvers, MA, USA), GAPDH, DFF/ICAD, ubiquitin, c-Rel, RelA/p65, eIF4E, p-eIF4E (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and HURP (kindly provided by Dr. C.-K. Chou, Chang Gung University), as well as the proteasome inhibitor MG132 (Calbiochem, San Diego, CA, USA). Sorafenib (Bayer HealthCare AG, Berlin, Germany) was kindly provided by Dr. T.-C. Chang (Chang Gung Memorial Hospital, Taoyuan, Taiwan). Unless indicated otherwise, the other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

### 2.2. Plasmids and transfection

The DNA sequence containing HURP open-reading frame (ORF) was released from pCMV6-Flag-HURP plasmid [1] (provided by Dr. C.-K. Chou) and inserted in the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) using the restriction enzymes HindIII and XbaI. The resulting expression plasmid was designated pcDNA3-HURP. Green fluorescent protein (GFP) ORF sequence was isolated by using specific primers (forward primer, 5′-GGATATCC-TACCGGTCGCCACCATG-3′; reverse primer, 5′-CCTCGAGCTTGTA-CAGCTCGTCCATGCC-3′), and ligated with pGEM-T Easy vector (Promega, Madison, WI, USA) using T4 DNA ligase. The GFP insert was released from T vector using the restriction enzyme NotI, and was inserted in the pcDNA3.1 expression vector, resulting in pcDNA3-GFP. Cells were transfected with plasmids using lipofetamine (Invitrogen), and were incubated for 48 h as described [25].

### 2.3. Quantitative real-time reverse transcription-PCR (qRT-PCR) and promoter analysis

qRT-PCR, or in short qPCR, was performed as before [25]. Reverse transcription was performed with oligo$(dT)_{16}$ on total DNase I-treated RNA (10 μg) with the Ominiscript reverse transcriptase (Qiagen, Valencia, CA, USA). Real-time PCR was performed on an ABI PRISM 7000 Sequence Detector System (Applied Biosystems, Foster City, CA, USA) using the SYBR green I master mix (Applied Biosystems) and the software SDS 1.0.1 (Applied Biosystems). PCR primers were designed with the software Primer Express 2.0.0 (Applied Biosystems). The primers were used at 100 nM and validated against glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers used were the following: HURP, forward, 5′-CCCATCTTCCCTTGAGAAAG-3′; reverse, 5′-AGGAGACATCAAGAACATGC-3′; c-Rel, forward, 5′-GGAAAAGACTGCAGAGACGG-3′; reverse, 5′-ATTGGGTTCGAGA-CAACAGG -3′; and GAPDH, forward, 5′-TCCTGCACCAC-CAACTGCTT-3′; reverse, 5′-GAGGGGGCCATCCACGTCTT-3′. PCR cycles were as follows: 2 min at 50 °C; 15 min at 95 °C; followed by 40 cycles of denaturation (15 s at 95 °C), annealing (60 s at 60 °C), and extension (60 s at 60 °C). All unknown samples and controls were done in triplicate. Relative quantification was calculated by the ΔCt method and normalized against GAPDH. Namely, the ΔCt for each candidate was calculated as ΔCt (candidate) = [Ct (candidate)–Ct (GAPDH)]. The relative abundance of the candidate gene X was expressed as $2^{\Delta Ct(X)-\Delta Ct(GAPDH)}$. The promoter region of HURP was analyzed using the TFSEARCH software (version 1.3; http://www.cbrc.jp/research/db/TFSEARCH.html; Accessed February 28, 2011).

### 2.4. Western blot analysis

To prepare whole protein extracts, cells were washed twice with phosphate-buffered saline (PBS) and lysed with a modified radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 μg/ml of aprotinin, leupeptin, and pepstatin, 1 mM $Na_3VO_4$ and 1 mM NaF) on ice for 30 min. Insoluble material was removed by centrifugation at 16,000 × *g* for 10 min at 4 °C. Protein concentration was determined using the Bradford assay [26] and the BioRad dye reagent according to instructions from the supplier (BioRad, Hercules, CA, USA). Proteins (50 μg) from each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto PVDF membrane, and incubated with the antibodies mentioned above according to the instructions of the manufacturer. The signal on membrane was revealed using enhanced chemiluminescence according to the specifications of the supplier (Pierce, Rockford, IL, USA).

### 2.5. Gene knockdown by shRNA lentivirus system

pLKO.1 plasmids expressing shRNA were purchased from the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). Luciferase shRNA (TRCN0000072244) was used as a negative control. Five plasmid clones for each gene were tested for gene knockdown efficiency in HEK293 cells. Transient transfection was done by adding 4 μg/dish (unless indicated otherwise) of shRNA plasmids along with 10 μl/well of lipofectamine (Invitrogen) into cells suspensions kept in 6-cm dish ($1 \times 10^6$ cells/dish). The shRNA plasmids used included HURP (TRCN0000062232), c-Rel (TRCN0000010421), and eIF4E (TRCN0000062576). HEK293 cells were co-transfected with the packing plasmid (pCMV-ΔR8.91), TRC library plasmid, and envelope plasmid (pMD.G) to produce shRNA lentivirus according to instructions from the supplier. The gene knockdown Mahlavu cells were selected by puromycin following transduction of the cells with the recombinant shRNA lentivirus.

### 2.6. Cell viability assay

HEK293 cells and hepatoma cells (Hep3B and Mahlavu) were seeded in quadruplicate at a density of $1 \times 10^4$ in 96-well plates. After 72 h of exposure to single drug or combination treatment, the MTT assay was used to quantify cell viability. Briefly, 20 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma–Aldrich) was added and incubated with the cells for 4 h at 37 °C. The culture medium was then removed and 100 μl of DMSO was added for 15 min at room temperature to extract the purple compound produced by living cells. The purple compound was then quantified with a spectrophotometer (OD570 nm). The OD values were normalized against the value of the control untreated group.

### 2.7. Apoptotic cells and sub-G1 phase analysis

Cells were treated with either sorafenib or taxol for the indicated incubation time. After treatment, cells were stained by DAPI and apoptosis was determined based on nuclear phenotype [27]. Sub-G1 cell population was assessed by flow cytometry as described [28].

### 2.8. Ubiquitination assay

Mahlavu cells were treated with 10 μM sorafenib for 24 h with or without 10 μM MG132 (Calbiochem) for 6 h. Equal amount of

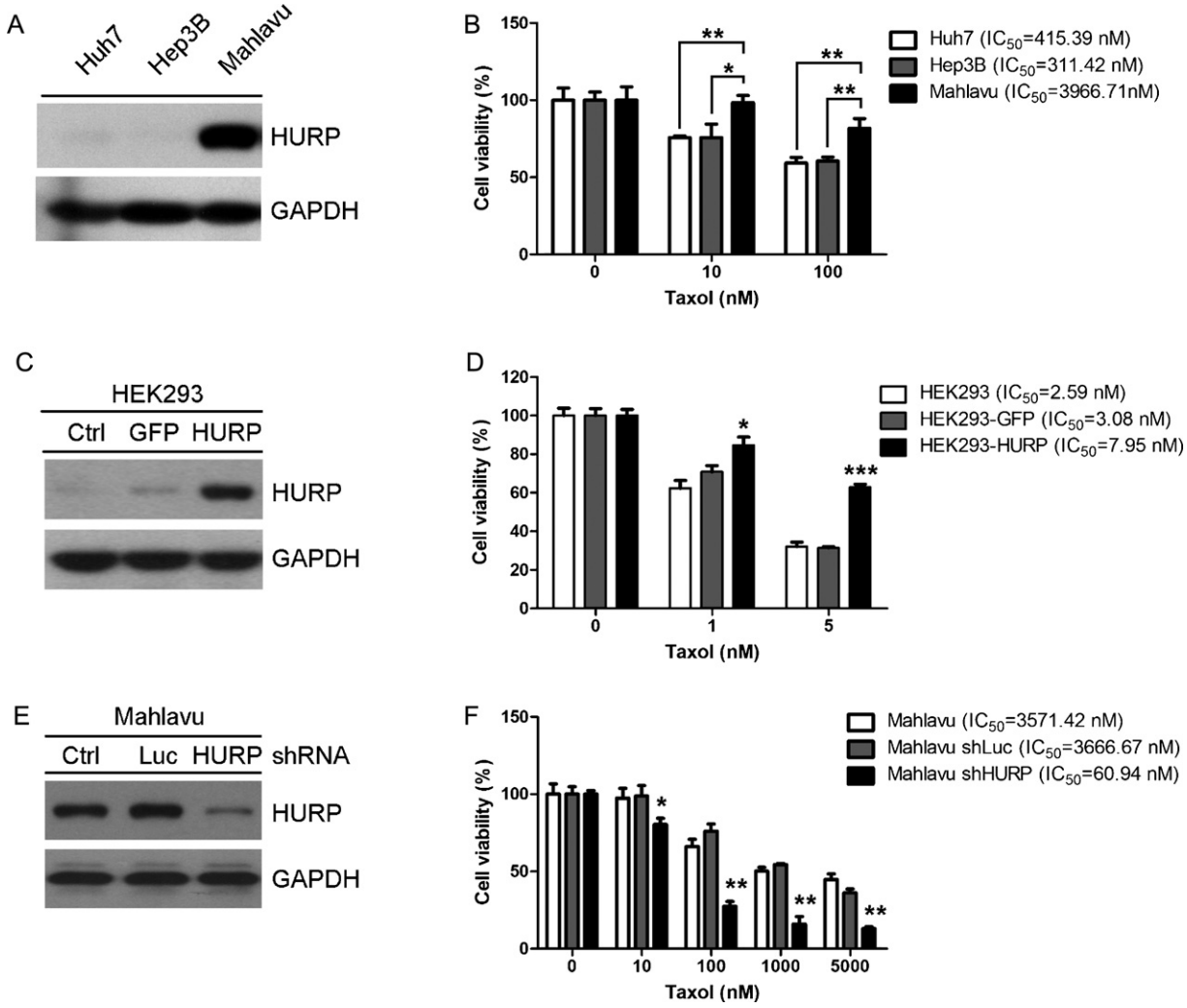


**Fig. 1.** High HURP protein levels correlate with taxol resistance in Mahlavu hepatoma cells and non-tumorigenic HEK293 cells. (A and B) HURP expression levels correlate with taxol resistance in Huh7, Hep3B, and Mahlavu cells. The level of HURP protein in these hepatoma cells was evaluated by Western blot. Cell viability following taxol treatment for 72 h was determined by using the MTT assay. (C and D) Taxol resistance of HEK293 cells following ectopic expression of HURP. HEK293 cells were transfected with the indicated expression plasmids for indicated period of time prior to Western blotting and cell viability assay. (E and F) Sensitization of Mahlavu cells to taxol by HURP knockdown with lentivirus carrying HURP shRNA sequence. Luciferase shRNA was used as a control. The results shown in (B, D, and F) represent mean values ± standard deviation of experiments performed in triplicate. Statistical significance was expressed as $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ against control cells.

total protein lysates was subjected to immunoprecipitation and Western blot with the indicated antibodies.

### 2.9. Protein degradation assay

The turnover rate of proteins was determined using cycloheximide (CHX; Sigma–Aldrich) to inhibit protein synthesis. Mahlavu cells were treated with 100 μg/ml of CHX with or without 10 μM of sorafenib. After incubation for the indicated time, the cells were harvested and lysed with the modified RIPA buffer described above. Equal amount of cell lysate (50 μg) was subjected to SDS-PAGE and analyzed by Western blotting using anti-HURP antibody.

### 2.10. Statistical analysis

The data were reported as mean values ± standard deviation (SD). Unless indicated otherwise, three independent experiments were performed. Statistical significance ($P$ value) was calculated with a two-tailed Student's $t$ test for single comparison.

## 3. Results

### 3.1. HURP promotes taxol resistance in non-tumorigenic and hepatoma cells

To investigate whether HURP is involved in taxol resistance in HCC, we analyzed three HCC cell lines, including two well-differentiated (Huh7 and Hep3B) and one poorly-differentiated (Mahlavu) cell lines. HURP expression was higher in Mahlavu cells compared to Huh7 and Hep3B cells (Fig. 1A). Notably, Mahlavu cells were more resistant to taxol than the other HCC cells tested (Fig. 1B). Next, we overexpressed HURP in non-tumorigenic HEK293 cells, and analyzed cell viability following taxol treatment. HEK293 cells that overexpressed HURP displayed significant resistance to taxol compared with either the control cells that overexpressed GFP or the parental cells (Fig. 1C and D). To further validate the function of HURP in HCC cells, we performed knockdown of HURP in Mahlavu cells by using short-hairpin RNA (shRNA) (Fig. 1E). HURP knockdown sensitized the cells to taxol compared to either the control cells overexpressing luciferase shRNA (shLuc) or the parental cells (Fig. 1F). The $IC_{50}$ values of taxol treatments were included for reference (Fig. 1B, D, and F). These data suggest that HURP may act as a survival protein and play an important role in protecting HCC cells against taxol-induced cell death.

### 3.2. Sorafenib inhibits HURP expression and enhances taxol-induced apoptosis in hepatoma cells

Sorafenib inhibits the kinase activity of B-Raf and p38/MAPK which are required for normal mitotic spindle formation during mitosis [15,22]. These observations prompted us to examine whether sorafenib affects HURP in HCC cells. Indeed, we observed that sorafenib treatment led to a dose-dependent decrease of HURP protein level (Fig. 2A). To explore the apoptotic signaling activity of sorafenib combined with taxol, we monitored the cleavage of caspase-3 and its substrate DFF/ICAD. Notably, sorafenib in combination with a sub-lethal dose of taxol (10 nM) induced a dramatic activation of caspase-3 and DFF/ICAD compared to taxol alone (Fig. 2B). Furthermore, we stained the cells with DAPI and determined the percentage of apoptotic cells based on nuclear phenotype. The level of apoptotic cells was increased following combined treatment with sorafenib and taxol when compared to either taxol or sorafenib alone (Fig. 2C, $P < 0.01$). We also investigated whether sorafenib enhances taxol-induced mitotic blockage and apoptosis by using flow cytometry. Sorafenib combined with taxol led to a major increase of apoptotic sub-G1 cells after 48 and 72 h of treatment (Fig. 2D). Next, we treated Mahlavu cells with a sub-lethal dose of taxol (10 nM) in combination with sorafenib, and analyzed cell viability using the MTT assay. While 10 μM of sorafenib decreased cell viability by about 50%, sorafenib combined with taxol induced massive cell death, and only 14% of the cells remained viable (Fig. 2E). We also observed that M-phase arrest and HURP inhibition were not found in HCC Mahlavu cells treated with 10 nM of taxol, which normally causes M-phase arrest in other cell lines that express a low level of HURP. However, HURP expression was dramatically suppressed by 10 μM of sorafenib in these HCC cells. Notably, sorafenib in combination with taxol promptly caused G2-phase arrest by 24 h, followed by accumulation of sub-G1 and apoptotic cells in HCC Mahlavu cells (Fig. 2F and G). The sensitization effect of sorafenib to taxol was not detected in either Huh7 or Hep3B cells (Figs. 2 and S1). These results are consistent with the possibility that sorafenib sensitizes HCC cells to taxol by suppressing HURP.

### 3.3. Lack of enhanced HURP degradation following sorafenib treatment in hepatoma cells

We observed that HURP mRNA level was downregulated following treatment with either 5 or 10 μM of sorafenib in Mahlavu cells (Fig. 3A). For unclear reasons, 1 μM of sorafenib slightly increased HURP mRNA in these cells. To further assess whether the reduction of HURP protein level by sorafenib involves the degradation of HURP protein, we used a cycloheximide (CHX) assay. Our results indicated that the degradation rate of HURP protein was not enhanced by the sorafenib treatment (Fig. 3B and C). In fact, HURP degradation seemed slightly slower in Mahlavu cells in the presence of sorafenib. These data suggest that sorafenib may inhibit HURP primarily at the transcriptional level.

### 3.4. Sorafenib downregulates HURP by inhibiting the c-Rel/NF-κB pathway

To assess the transcriptional regulation of HURP, we searched for putative transactivator-binding sites in the HURP promoter and found two putative c-Rel/NF-κB-binding sites (Fig. 4A). c-Rel/NF-κB is a member of the NF-κB family, which includes Rel A (p65), Rel B, c-Re, p50/p105 (NF-κB1), and p52/p100 (NF-κB2) [29]. Recent studies suggest that c-Rel promotes cell proliferation and survival against apoptosis and interferon resistance through activation of target genes like c-myc [30,31], Bcl-$X_L$ [32], and IRF-4 [33]. In addition, recent studies indicate that constitutive activation of NF-κB in HCC correlates with chemoresistance and is required for tumorigenesis and maintenance of the malignant properties of HCC [34–39]. However, it is unknown whether HURP is a target gene of c-Rel/NF-κB and whether these proteins play a role in the cellular response to taxol. To address this question, we examined the protein level of c-Rel in Mahlavu cells following treatment with sorafenib. After 24 h of treatment with sorafenib, we observed that the protein level of c-Rel was reduced by sorafenib treatment in a dose-dependent manner, whereas Rel A (p65) was not altered (Fig. 4B). As seen for c-Rel, sorafenib also induced a dose-dependent decrease of HURP protein level in Mahlavu cells (Fig. 2A), suggesting the involvement of c-Rel in sorafenib-induced HURP degradation. Furthermore, both c-Rel and HURP protein levels were reduced by the NF-κB inhibitor BAY11-7082 (Fig. 4C), which blocks activation of NF-κB by inhibiting phosphorylation of IκBα. To explore whether sorafenib regulates the translocation of c-Rel/NF-κB to the nucleus, we isolated cytoplasmic and nuclear fractions of Mahlavu cells treated with either sorafenib or DMSO. Notably, the level of nuclear c-Rel was reduced in sorafenib-treated cells (Fig. 4D). Cell fractionation was confirmed by the presence of

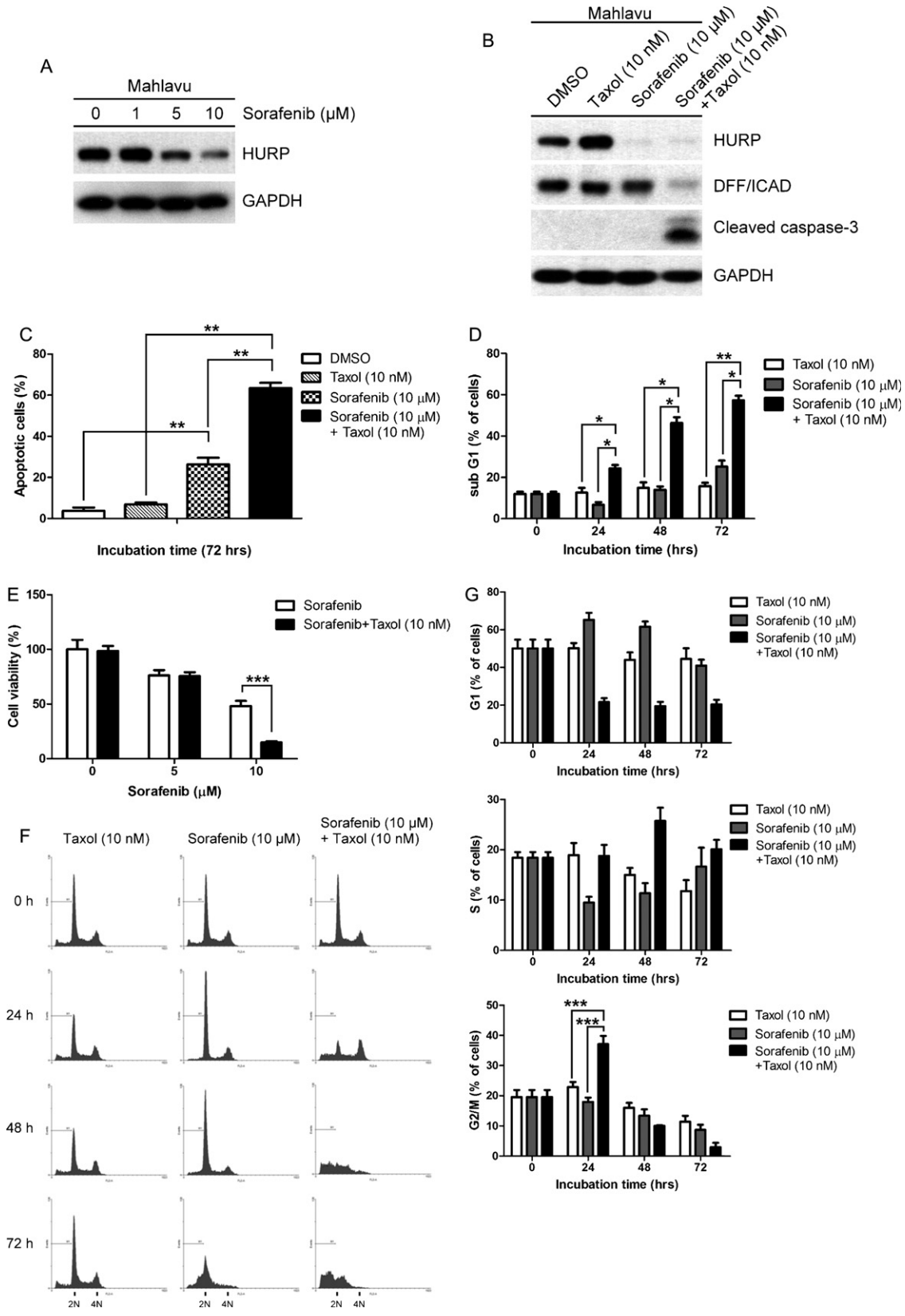


**Fig. 2.** Sorafenib suppresses HURP protein and sensitizes Mahlavu hepatoma cells to taxol. (A) Suppression of HURP protein expression by sorafenib. The HURP protein level was examined by Western blot. (B and C) Effects of sorafenib on taxol-induced caspase-3 activation and apoptosis in Mahlavu cells. Activation of caspase-3 was evaluated by cleavage of caspase-3 and DFF/ICAD. Cells were stained with DAPI, and apoptotic cells were determined by nuclear phenotype. (D) Kinetic accumulation of sub-G1 cell population of Mahlavu cells by sorafenib alone or in combination with taxol. Selected doses of sorafenib and taxol are indicated. (E) Synergistic effect of sorafenib on taxol-induced inhibition of cell growth. Mahlavu cells were co-treated with a sub-lethal dose of taxol (10 nM) and various doses of sorafenib. (F) Enhanced G2/M cell accumulation in sorafenib-treated Mahlavu cells. Representative flow cytometry profiles of Mahlavu cells treated with sorafenib alone or combined with taxol are shown. (G) Statistical analysis of cells at different phases of the cell cycle. The treatment conditions are indicated. The results shown in (C, D, E and G) represent mean values ± standard deviation of experiments performed in triplicate. Statistical significance was expressed as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ against single drug treatment.

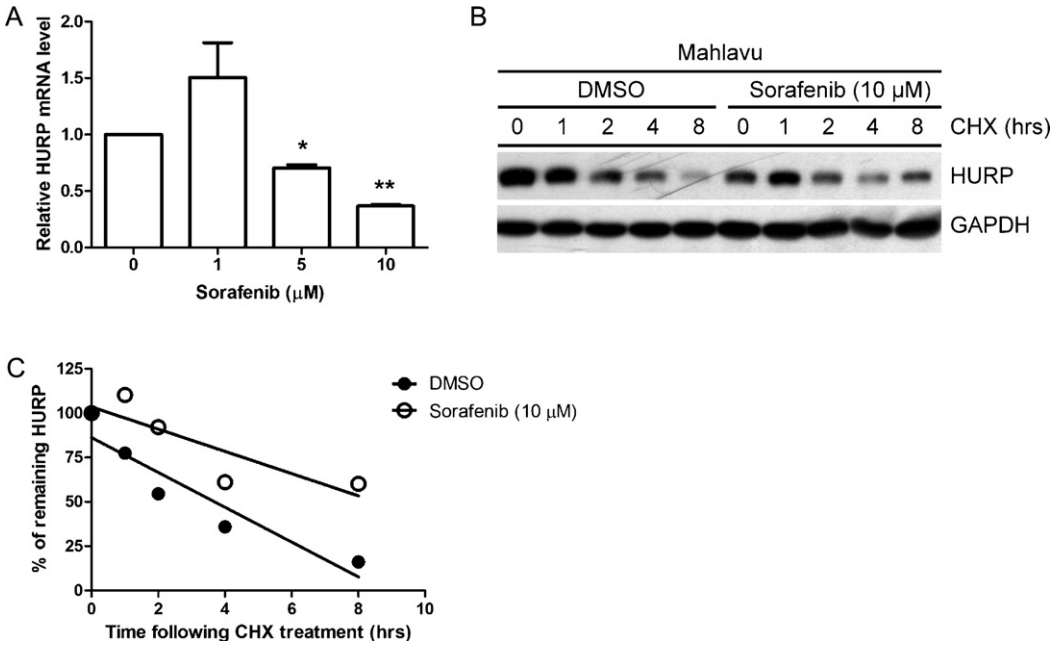


**Fig. 3.** Effect of sorafenib on HURP mRNA expression and protein stability. (A) Suppression of HURP mRNA expression by sorafenib. The HURP mRNA level was evaluated by real-time PCR. (B) No reduced degradation of the HURP protein in sorafenib-treated Mahlavu hepatoma cells. Cells were pre-incubated with cycloheximide (CHX), an inhibitor of protein synthesis, followed by the indicated incubation. (C) Linear regression of the kinetic pattern of HURP protein level shown in (B). Statistical significance was expressed as $^{*}P < 0.05$ and $^{**}P < 0.01$.

lamins in nuclear fractions and the absence of these proteins in cytoplasmic fractions. Statistical analysis indicated that nuclear translocation of c-Rel protein was significantly reduced by sorafenib compared to control (Fig. 4E; $P < 0.001$). To verify whether this finding could be duplicated in vivo, we monitored the binding activity of the c-Rel protein to c-Rel DNA-binding sites in the HURP promoter of Mahlavu cells by using chromatin immunoprecipitation (ChIP). Indeed, the PCR products of both P1 and P2 DNA regions that bind to the c-Rel protein were greatly reduced by sorafenib treatment (Fig. 4F, compare lanes 5 and 6). PCR products from control IgG were not detected (Fig. 4F, lanes 3 and 4), further confirming the validity of our ChIP assay.

To validate the direct role of c-Rel in transactivating HURP, we used a specific shRNA to knockdown c-Rel in Mahlavu cells. The protein level of HURP was reduced following partial knockdown of c-Rel (Fig. 4G). Next, we hypothesized that, if c-Rel acts as a key transactivator of HURP, c-Rel knockdown should sensitize HCC cells to taxol. Indeed, c-Rel knockdown sensitized Mahlavu cells to taxol (100 and 1000 nM) compared to Luc shRNA (Fig. 4H; $P < 0.005$). These results indicate that sorafenib may suppress HURP and sensitize HCC cells to taxol by blocking the c-Rel/NF-κB pathway.

### 3.5. Inhibition of c-Rel by sorafenib: translational inhibition

We observed earlier that sorafenib reduced the protein level of c-Rel in Mahlavu cells (Fig. 4B). To explore the mechanism underlying this phenomenon, we examined the mRNA level of c-Rel in Mahlavu cells by using real-time PCR. We found no significant changes in the mRNA level of c-Rel in Mahlavu cells with or without sorafenib treatment (Fig. 5A). The up-regulation of c-Rel gene transcription by lipopolysaccharide (LPS) and the down-regulation of c-Rel gene transcription by c-Rel shRNA were used as positive and negative controls, respectively. Another group reported earlier that the c-Rel protein is ubiquitinated and degraded by the proteasome [40]. To investigate whether the reduced protein level of c-Rel following sorafenib treatment is due to ubiquitination and proteasome degradation, we treated Mahlavu cells with the proteasome inhibitor MG132 (10 μM), and monitored the level of c-Rel by Western blotting. The protein level of c-Rel was increased in the presence of MG132 (Fig. 5B, compare lanes 1 and 3). However, the level of c-Rel was dramatically reduced in sorafenib-treated Mahlavu cells in the presence or absence of MG132 (Fig. 5B, lanes 2 and 4). In addition, we observed enhanced ubiquitination of c-Rel in cells treated with MG132 (Fig. 5C, compare lanes 1 and 3). Still, there was no further increase of c-Rel ubiquitination in MG132-treated cells after sorafenib treatment (Fig. 5C, compare lanes 3 and 4). It should be noted that MG132 treatment resulted in a clear and detectable ubiquitination of c-Rel protein (Fig. 5C, compare lanes 1 and 2 with lanes 3 and 4). Previous studies have shown that both the p50 and p65 subunits are ubiquitinated and degraded in the nucleus, thus allowing termination of NF-κB-responsive gene expression [41–43]. Since the proteasome inhibitor MG132 is also an inhibitor of the NF-κB pathway, we also investigated the effect of sorafenib on the levels of nuclear and cytoplasmic c-Rel and Rel A following MG132 treatment. Unlike c-Rel, however, the nuclear translocation of Rel A (p65) was not affected by sorafenib (Fig. 5D, compare the ratio of lanes 1 and 3 with the ratio of lanes 2 and 4). Statistical analysis indicated that nuclear translocation of c-Rel protein, but not Rel A (p65), was significantly reduced by sorafenib treatment compared to the DMSO control (Fig. 5E; $P < 0.001$). Not surprisingly, similar reduction of nuclear translocation of c-Rel and Rel A (p65) was detected in sorafenib-treated cells in the presence of MG132. These results suggest that the sorafenib-induced reduction of nuclear c-Rel may not be due to ubiquitin–proteasome degradation. Recent studies suggest that sorafenib may down-regulate several anti-apoptotic proteins such as Mcl-1 and c-FLIP$_L$ via translation inhibition mechanisms involving inhibition of phosphorylation of eIF4E, a mRNA cap-binding protein which promotes translation initiation [19,44–46]. These observations prompted us to explore the possibility that downregulation of c-Rel protein by sorafenib may be controlled by translation inhibition. The phosphorylation of eIF4E was rapidly inhibited

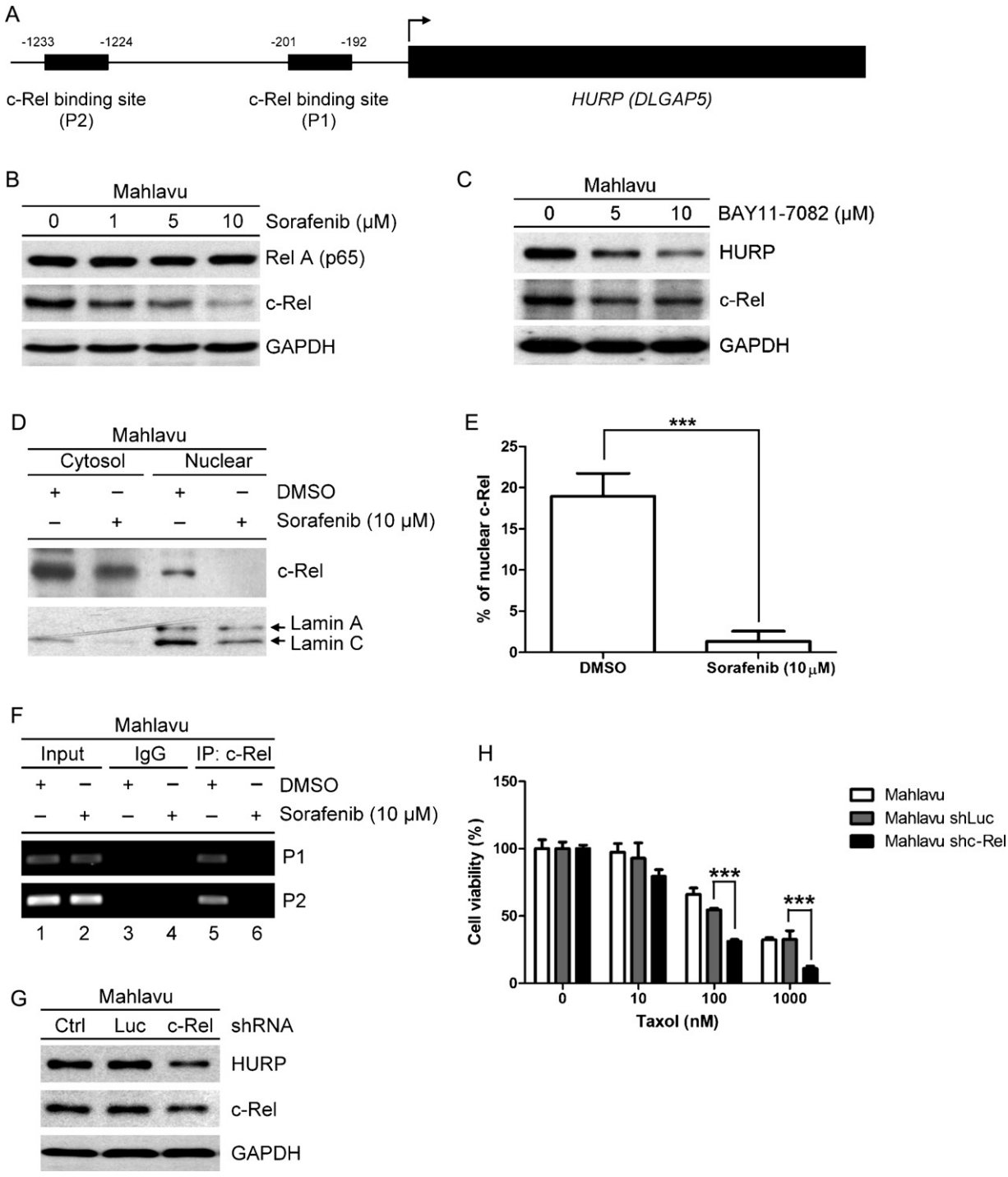


**Fig. 4.** Reduced c-Rel protein level and nuclear translocation following sorafenib treatment. (A) Predicted c-Rel binding sites in the promoter region of the HURP gene. (B) Dose-dependent inhibition of c-Rel following sorafenib treatment in Mahlavu hepatoma cells. Note that RelA (p65) was not affected by the treatment. (C) Suppression of both HURP and c-Rel expression by BAY11-7082, an inhibitor of the NF-κB pathway, in Mahlavu cells. (D) Reduction of c-Rel nuclear translocation following sorafenib treatment in Mahlavu cells. (E) Statistical analysis of nuclear c-Rel protein level in sorafenib-treated Mahlavu cells. The difference between sorafenib-induced low c-Rel and DMSO control was significant (***, $P < 0.001$). (F) Inhibition of c-Rel binding to predicted sites of HURP promoter in vivo evaluated by ChIP assay. P1 and P2 indicate the PCR products corresponding to the putative binding sites of c-Rel on the HURP promoter. (G) Reduction of HURP by c-Rel knockdown in Mahlavu cells. (H) Enhancement of taxol-induced apoptosis by sorafenib in Mahlavu cells. The results represent mean values ± standard deviation of experiments performed in triplicate. Statistical significance was expressed as $^{***}P < 0.001$ against shLuc control cells.

after 1.5 h of sorafenib treatment, and this response was accompanied by a gradual decrease of c-Rel protein level starting after 3 h of sorafenib treatment (Fig. 5F). These results support the notion that inhibition of c-Rel protein level by sorafenib may be due to a lack of available phosphorylated e-IF4E. To assess whether c-Rel translation is regulated by eIF4E in Mahlavu cells, we evaluated the protein level of c-Rel in eIF4E-knockdown cells. c-Rel protein, like HURP expression, was reduced in eIF4E-knockdown cells when compared with the parental or shLuc-control cells (Fig. 5G), suggesting that c-Rel translation is regulated by eIF4E. Nevertheless, it appears less likely that eIF4E specifically regulates c-Rel translation in Mahlavu cells, as eIF4E is an established regulator of protein translation.

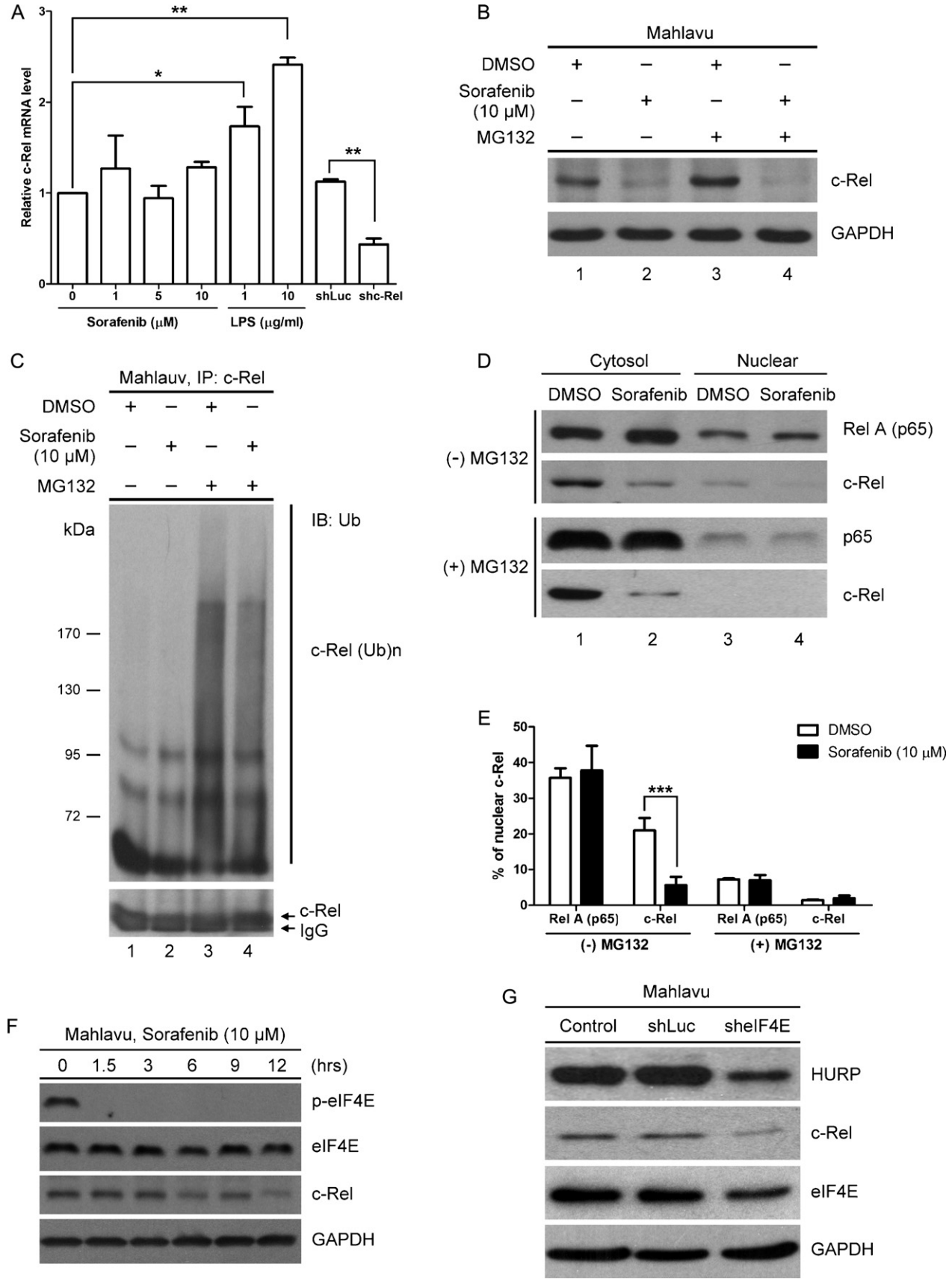


**Fig. 5.** Involvement of translation inhibition of the c-Rel protein after sorafenib treatment. (A) c-Rel mRNA level in sorafenib-treated cells was evaluated by real-time PCR. There was no significant change in the c-Rel mRNA level after sorafenib treatment in Mahlavu hepatoma cells. Up-regulation and down-regulation of c-Rel mRNA by LPS and shc-Rel, respectively, were also included as control. The results represent mean values ± standard deviation of experiments performed in triplicate. Statistical significance was expressed as $^{*}P < 0.05$, and $^{**}P < 0.01$ against no treatment. (B) Lack of reversal of sorafenib-induced c-Rel reduction by MG132, an inhibitor of proteasome, in Mahlavu cells. (C) Lack of increase in the ubiquitination of c-Rel protein by sorafenib in vivo. The c-Rel protein was immunoprecipitated followed by immunoblotting with anti-ubiquitin antibody. The

## 4. Discussion

In this study, we observed that a high level of HURP protein correlates with taxol resistance in HCC cells. Accordingly, high expression of HURP was found in taxol-resistant, poorly-differentiated Mahlavu cells, whereas low levels were observed in the relatively taxol-sensitive, well-differentiated Huh7 and Hep3B cells. Furthermore, ectopic expression of HURP in non-tumorgenic HEK293 cells rendered these cells resistant to taxol. Conversely, HURP knockdown consistently sensitized Mahlavu cells to taxol. HURP represents a novel prognostic marker in adrenocortical carcinoma and displays higher levels in patients with urinary bladder transitional cell carcinoma (TCC) undergoing recurrence [4,47]. In addition, the HURP gene is associated with invasive and metastatic activity in human HCC [7,48]. By using a bioinformatics analysis of a database originally designed to identity best-fit mouse models for human cancer genes [49], we found a positive correlation between overexpression of HURP and poor prognosis of HCC patients who received chemotherapy (Fig. S2; $P < 0.0001$). Collectively, these results strongly indicate that HURP may provide cell growth and anti-apoptosis/metastasis advantages during cancer progression.

The expression of HURP is tightly regulated with the cell cycle. HURP level is elevated when cells enter G2/M phase which is important for microtubule spindle formation and chromosome congression [1,9]. HURP then colocalizes with Cdk1 to the mitotic spindle and nine sites on the HURP protein are phosphorylated by Cdk1/cyclin B during metaphase. The phosphorylated HURP protein is then recognized by Fbx7, a subunit of $SCF^{Fbx7}$ E3 ligase complex, followed by its degradation by the proteasome [50]. HURP is also ubiquitinated by the APC/C E3 ligase complex and degraded by the proteosome when the cell cycle transitions from metaphase to anaphase [51]. Indeed, overexpression of HURP promotes proliferation of non-tumorigenic cells, such as HEK293 cells and mouse NIH3T3 fibroblasts [1,6]. Here, we showed that M-phase arrest and HURP inhibition were not observed in HCC Mahlavu cells treated with 10 nM of taxol, which normally causes M-arrest in other cell lines that express a low level of HURP. However, HURP expression was dramatically suppressed by 10 μM of sorafenib in these HCC cells. Notably, sorafenib in combination with taxol promptly caused G2-phase arrest by 24 h, followed by accumulation of sub-G1 and apoptotic cells in HCC Mahlavu cells (Fig. S3). These cellular responses were not detected in other HCC cells (Huh7 and Hep3B) which express a low level of HURP (Figs. 2 and S1). These results support the notion that HURP plays an important role in regulating M-phase cell cycle progression. Our study demonstrates for the first time that HURP also plays a protective role against mitotic stress such as taxol. Accordingly, knockdown of HURP is a promising option for the development of novel therapies for HCC cases that express abundant HURP.

Furthermore, we showed a possible molecular mechanism regulating the suppression of HURP by sorafenib. Suppression of HURP mRNA synthesis by sorafenib is likely to be due to a decrease in c-Rel-dependent NF-κB activity, including c-Rel protein level and its nuclear translocation. Many genes are regulated by RelA (p65) and other NF-κB subunits through the formation of heterodimers (p65/p50, p65/c-Rel and c-Rel/p50) or homodimers (p65/p65 and c-Rel/c-Rel). Although p65 is the most frequently detected functional NF-κB subunit, we observed that c-Rel, but not p65, was dramatically inhibited by sorafenib in Mahlavu cells (Fig. 4B). Furthermore, nuclear translocation of p65 was unaffected by sorafenib in these cells (Fig. 5D and E). For these reasons, we believe that c-Rel acts as a major NF-κB transcription factor in regulating HURP gene expression. Activation of eIF4E by phosphorylation of the protein at specific sites is important for cap-dependent translation of highly regulated cellular proteins that participate in cell cycle control and cell growth [52]. Our results indicate that phosphorylation of eIF4E was promptly inhibited by sorafenib in Mahlavu cells, followed by reduction of c-Rel protein level. These results suggest that a cap-dependent translation inhibition may be involved in the suppression of c-Rel, and subsequent reduction of transcription of HURP gene. Accordingly, depletion of c-Rel may sensitize HCC cells to taxol.

A recent study report that HURP knockout ($HURP^{-/-}$) male mice develop normally and are indistinguishable from their wild-type littermates [53]. However, $HURP^{-/-}$ female mice are infertile due to an inability to undergo decidual reaction even though ovulation, fertilization, and pre-implantation embryo development are normal [53]. It seems that inhibition of HURP may be safe for most patients undergoing chemotherapy since pregnancy is not encouraged at this stage. Since HURP plays a dispensable role for normal development, it appears that HURP may become a potential target for target therapy of cancer cells. However, targets other than HURP cannot be excluded at this time. Interactions between multi-drug resistance (MDR)-related ABC transporter and TKI (including sorafenib) are relevant to various properties of TKI drugs [54]. Indeed, MDR/P-glycoprotein level was reduced by sorafenib in HCC Mahlavu cells (Fig. S3). In addition, a recent report has shown that addition of sorafenib to conventional chemotherapy caused down-regulation of MDR genes and restored the chemosensitivity of MDR Huh7 cells [55]. However, we also observed a slight reduction of MDR protein in Huh7 cells treated with sorafenib, while the cells remained resistant to taxol treatment (Fig. S1). These results support the notion that novel mechanisms are required to account for chemoresistant HCC cells which do not express or express low levels of MDR/P-glycoprotein [56].

Conventional cytotoxic chemotherapy is not effective in most HCC cases [57]. Therefore, the development of efficient therapies that overcome drug resistance and minimize side effects becomes an urgent priority for HCC treatment. It may be useful to develop a novel approach of target therapy for treatment of HCC, which is constantly associated with taxol resistance [58]. The high mutation frequency in cancer cells, which results in altered cell cycle regulation and growth signal transduction and confer cell growth advantages, suggests that many of the aberrant molecules and pathways may be strategic targets for cancer therapy. In this sense, HURP represents a promising example for the target therapy of HCC since HURP has been found to be overexpressed in over 60% of HCC patients (C.C.-K. Chao's laboratory, unpublished data). Inhibition of HURP expression has shown impressive activity against HCC cells which express abundant HURP. This study shows that sorafenib alone resulted in better cell-killing activity than taxol alone in HCC cells that overexpress HURP. This notion may also be applicable to the treatment of a broad range of human cancers growing in tissue culture and in human tumor xenograft models. Important challenges during clinical development include the ability to predict which tumors will be sensitive to the combined therapy.

region corresponding to poly-ubiquitinated c-Rel was indicated. The input c-Rel protein (10%) was shown below. The nearly co-migrated IgG protein was also indicated. (D) Reduction of c-Rel nuclear translocation following sorafenib treatment in Mahlavu cells in the presence of MG132. (E) Statistical analysis of nuclear c-Rel protein level in sorafenib-treated Mahlavu cells in the presence of MG132. The difference between sorafenib-induced low c-Rel and DMSO control was significant (***, $P < 0.001$). (F) Immediate inhibition of the phosphorylation of eIF4E by sorafenib along with a gradual reduction of c-Rel protein level in Mahlavu cells. (G) Reduction of HURP and c-Rel by eIF4E knockdown in Mahlavu cells.

## Conflict of interest

The authors state no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.04.008.

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