Sorafenib–mediated targeting of the AAA\(^+\) ATPase p97/VCP leads to disruption of the secretory pathway, endoplasmic reticulum stress and hepatocellular cancer cell death.

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**Running title:** Sorafenib targets VCP in hepatocellular cancer cells

**Keywords:** sorafenib, endoplasmic reticulum stress, VCP, tyrosine phosphorylation, HCC

**List of abbreviations** - \(\alpha_1\)AT – \(\alpha_1\) antitrypsin; CTL – control; CNX – calnexin; ER – endoplasmic reticulum; ERAD – ER-associated degradation; HCC – hepatocellular carcinoma; IRE1-DN – IRE1\(\alpha\) dominant negative mutant; Tun – tunicamycin; UPR – unfolded protein response

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Conflict of interest: The authors declare no conflict of interest related to the present work.
Abstract
The molecular mechanisms and cellular targets of Sorafenib, a multikinase inhibitor used for the treatment of hepatocellular carcinoma (HCC), remain to be fully characterized. Recent studies have shown that Sorafenib induces tumor cell death through the activation of endoplasmic reticulum (ER) stress signaling and/or autophagy in various cellular models. Using liver cancer-derived cell lines, we specifically demonstrate that the IRE1 and PERK arms of the Unfolded Protein Response (UPR) become activated upon Sorafenib treatment, whereas the ATF6 arm is inhibited. Our results also reveal that Sorafenib treatment causes disruption to the secretory pathway, as witnessed by the fragmentation of the Golgi apparatus and the induction of autophagy. Based on these observations we tested the relevance of the AAA+ ATPase p97/VCP as a potential functional target of Sorafenib. Our results show that p97/VCP tyrosine phosphorylation is prevented upon Sorafenib treatment, and that this can be correlated with enhanced membrane association. Moreover, we show that DBeQ, a recently discovered inhibitor of p97/VCP, enhances Sorafenib-mediated toxicity in cultured cells. Our data demonstrate a novel mechanism for Sorafenib-mediated cell death in HCC, which depends on the integrity of the secretory pathway; and we identify p97/VCP phosphorylation as a potential target for improved Sorafenib treatment efficacy in patients.
Introduction

Hepatocellular carcinoma (HCC) is globally the third cause of cancer death, with the majority of patients dying within 1 year of diagnosis (1). Recently Sorafenib, a multikinase inhibitor of the Raf/MEK/ERK pathway and of tyrosine kinase receptors, was shown to induce apoptosis as well as inhibiting tumor cell proliferation and angiogenesis in a variety of tumors (2). The molecular involvement of the Raf-1 and tyrosine kinase signaling pathways is well established in the pathogenesis of HCC and provided a rationale for the use of Sorafenib in HCC treatment. Indeed, Sorafenib was the first targeted therapy to improve the outcome of HCC patients (3) and has become a standard treatment for such disease. In pre-clinical experiments, Sorafenib reduced tumor angiogenesis and increased tumor apoptosis in a mouse xenograft model of human HCC (4). However, the precise mechanisms through which Sorafenib induces cell death in HCC remain to be identified.

Reports have indicated that Sorafenib treatment may induce cell death signaling pathways through the activation of endoplasmic reticulum (ER) stress (5-10). The ER is the first compartment of the secretory pathway and is composed of a system of interconnected membranous tubules and vesicles in which proteins and lipids are synthesized and intracellular calcium levels are regulated. In addition, the ER functions in the maintenance of protein homeostasis (proteostasis) of secreted and transmembrane proteins. Misfolded or toxic proteins present in the ER are retrotranslocated to the cytosol and are subsequently degraded by the proteasome, in a process known as ER-associated degradation (ERAD). When the levels of misfolded protein overwhelms the ER folding and degrading capacity, an evolutionarily conserved cellular adaptive response, named the unfolded protein response (UPR), is triggered through the activation of three ER stress sensors PERK, ATF6α and IRE1α. The UPR initially compensates for damage and restores ER homeostasis, however when ER stress is not alleviated it can activate apoptosis (11, 12). Induction of apoptosis is a prominent mode of cytotoxicity for many chemotherapeutic drugs, of which some use ER stress-mediated cell death pathways.

In the present study, we found that Sorafenib treatment selectively activates the PERK and IRE1α arms of the UPR but prevents activation of the ATF6α arm in liver cancer-derived cells. We demonstrate that Sorafenib alters critical organelles of the
secretory pathway exemplified by the fragmentation of the Golgi complex. We identify that tyrosine phosphorylation of the AAA+ ATPase p97/VCP is prevented upon Sorafenib treatment, in turn leading to the disruption of the secretory pathway. Finally we show that treatment with the VCP inhibitor DBeQ enhances Sorafenib toxicity. Our results highlight for the first time an unanticipated impact of Sorafenib on the secretory pathway in HCC cells and point towards p97/VCP as a potential therapeutic target.
Materials and Methods

Materials – Antibodies to Calnexin (CANX) were described previously (13). Antibodies against ERK1, CHOP and JNK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-alpha-1 antitrypsin (α1AT) antibodies were purchased from DAKO (Glostrup, Denmark) and Invitrogen (Carlsbad, CA, USA). Mouse monoclonal anti-ATF6α was purchased from BioAcademia (Osaka, Japan). Anti-LC3 antibody was from Novus Biologicals (Littleton, CO, USA). Anti phospho-ERK, anti-phospho-JNK (thr183/tyr185) and anti-eIF2α were from Cell Signaling Technology (Dancers, MA, USA). Anti LC3 antibodies were from Abnova (Taipei, Taiwan). Rabbit polyclonal anti-Giantin and mouse monoclonal anti-p97 antibodies were purchased from Abcam (Cambridge, MA, USA). Mouse monoclonal anti-FLAG M2 and DTT were obtained from Sigma (St. Louis, MO, USA). Fluorescently-conjugated secondary antibodies were from Molecular Probes (Invitrogen, Carlsbad, CA, USA). Tunicamycin (Tun) was from Calbiochem (EMD Biosciences Inc., Darmstadt, Germany). GFP-LC3 was kindly given by Dr P. Codogno (Paris, France). Autophagy was monitored as recommended by Klionsky and colleagues (14). Small interfering RNA (siRNA) to ATG5 was from Qiagen (Darmstadt, Germany) and used at 20 nM final concentration in all experiment. NH₄Cl was from Sigma (StLouis, MO, USA) and used at 40 mM final concentration. BpVPhen was from Enzo Life Sciences (Farmingdale, NY, USA) and used at 5 μM final concentration. HepG2 and HuH7 cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and antibiotics in a 37°C incubator containing 5% CO₂.

Semi-quantitative and quantitative RT-PCR - Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Semi-quantitative RT-PCR analysis was carried out as previously described (13). PCR products were applied to 1-3% agarose gels. For quantitative RT-PCR (q-PCR) analysis, all reactions were performed using the SYBR Green PCR Core reagents kit (Bio-Rad, Marnes-la-Coquette, France) using Stratagene X4000 thermocycler (Stratagene, Amsterdam, The Netherlands). Each sample was normalized to Gapdh mRNA. The primers used are listed in Table S1.
Cell lines - Characterization of the cell lines was achieved by genotyping specific polymorphic markers in the past 4 months.
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Results

*Sorafenib induces apoptosis and prevents MAPK activity.*

Sorafenib (Figure 1A) has previously been described to induce cell death in various cellular systems, and so we first tested for a similar response in liver tumor-derived HepG2 cells. Following 24 h of treatment with 10 μM Sorafenib, cells showed toxicity. We quantified this toxicity by analyzing cellular viability using Sulphorhodamine B staining. As the incubation time with Sorafenib increased, the percentage of viable cells decreased to 60% at 24 h and to approximately 30% at 48 h, thereby confirming our visual observations (Figure 1B). To investigate whether the cell death observed in our system occurred via apoptosis, we incubated HepG2 cells with 10 μM Sorafenib for 24 h or 48 h followed by staining with Annexin V/Propidium Iodide (PI). As observed in Figure 1B, the proportion of apoptotic cells increased to 20% at 24 h and then to 60% at 48 h of treatment. Finally, and similar to what was previously demonstrated (15), Sorafenib-treatment of serum-starved cells largely prevented ERK1/2 phosphorylation upon exposure to medium containing 10% FBS (Figure 1C). Together these results confirmed that Sorafenib-mediated cell death in hepatocellular cells correlated with apoptosis induction and inhibition of the ERK/MAPK pathway. However the molecular mechanisms underlying this phenomenon still remained poorly characterized.

*Both IRE1 and PERK arms of the UPR are activated by Sorafenib.*

Besides the inhibition of the ERK/MAPK pathway, Sorafenib-mediated cell death has been linked to the induction of apoptosis through ER stress (9), and so we sought to investigate whether this mechanism was occurring in the Sorafenib-exposed HepG2 cells. We first analyzed whether the IRE1 arm of the UPR was activated. IRE1 is a transmembrane protein that serves as an ER stress sensor and can mediate the UPR. Activation of IRE1 leads to the unconventional splicing of the Xbp-1 mRNA, a target for the IRE1 endoribonuclease (16). Treatment of HepG2 cells with Sorafenib led to a detectable splicing of Xbp-1 after 16 h of incubation. This splicing was significantly reduced in HepG2 cells expressing a dominant negative form of IRE1 (IRE1-DN; (17)) (Figures 2A), indicating that Sorafenib treatment induced Xbp-1 splicing through an IRE1-mediated mechanism. Similar results were obtained with Tunicamycin (Tun), a
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known inducer of ER stress (Figures 2A, S1A). IRE1 has been implicated in apoptotic signaling through the activation of JNK via TRAF2, which leads to the phosphorylation of different cellular targets culminating in apoptosis (16). Since HepG2 cells were shown to undergo apoptosis upon incubation with Sorafenib (Figure 1C), we investigated whether JNK phosphorylation occurred after treatment with Sorafenib. In wild-type cells, JNK phosphorylation was clearly detectable after 2 h of treatment and increased with time. In contrast, no detectable JNK phosphorylation was observed in response to Sorafenib in IRE-DN cells (Figure 2B). Another potential link between ER stress and apoptosis is mediated by PERK, a transmembrane protein that senses ER stress, in turn phosphorylating and thereby inactivating eIF2α, eventually leading to a reduction in protein synthesis (12). In wild-type HepG2 cells Sorafenib stimulated eIF2α phosphorylation, which was significantly stronger in the IRE1-DN cells (Figure 2C, Table S2). This indicated that the PERK arm is activated in HepG2 cells upon treatment with Sorafenib. To further study the induction of ER stress, we analyzed the expression of other UPR-associated genes, namely Chop and Gadd34. The expression of Chop, a potent transcription factor involved in the promotion of apoptosis upon ER stress, and which accumulates in the cells after eIF2α phosphorylation (12), increased in wild-type HepG2 cells after 8 h of treatment with Sorafenib (Figure 2D). Incubation with Imatinib, another tyrosine kinase inhibitor, did not affect Chop expression. Moreover, in IRE1-DN cells, there was also an increase in the expression of Chop, but to a lesser extent than that seen in wild-type cells, thereby confirming the contribution of IRE1 signaling to Chop mRNA induction. The expression levels of Gadd34, whose protein product contributes to eIF2α dephosphorylation, underwent a marked increase in its expression upon Sorafenib treatment (Figure 2D). Gadd34 induction ultimately mediates translation recovery and is a downstream target of ATF4, whose translation is triggered upon eIF2α phosphorylation (12). Gadd34 mRNA expression increased followed both Chop mRNA expression pattern and eIF2α phosphorylation (Figure 2D). Incubation with Imatinib did not lead to any discernible increase in Gadd34 expression.

Sorafenib induces disruption of the secretory pathway and autophagy.
The involvement of the third arm of the UPR in HepG2 cells exposed to Sorafenib was also investigated. ATF6 is a transmembrane protein that serves as a proximal ER stress sensor, and that upon ER stress induction migrates from the ER to the Golgi complex where it becomes activated by proteolysis. The cytosolic portion of this protein translocates to the nucleus where it functions as a transcription factor, which induces the expression of various chaperones (such as BiP and GRP94), CHOP and XBP1 (18, 19). To investigate the role of ATF6α, we transfected HepG2 cells with a FLAG-ATF6α construct and performed immunocytochemistry followed by fluorescence microscopy. Transfected cells treated for 2 h with the ER stress inducer DTT displayed a nuclear localization of ATF6α (Figure 3A, upper panels). However, when we treated the cells for 2 h with Sorafenib prior to DTT treatment, ATF6α remained in the ER (Figure 3A, bottom panels). Quantification of the images revealed that 15 ± 3% of the DTT-treated cells contained nuclear-localized ATF6α, compared with only 2 ± 0.1% of the cells incubated with Sorafenib only (Figure 3B), indicating that Sorafenib treatment prevented the activation of the ATF6α pathway. This inhibition of ATF6α activation was further confirmed by measurement of the expression of Orp150, Herpud1 and BiP, which did not vary when compared to untreated cells (Figure 3C), in contrast to the sharp increase in expression seen in cells treated with DTT. Interestingly, when we co-incubated HepG2 cells with Sorafenib and DTT, Herpud1 and BiP mRNA showed expression levels similar to those observed in the untreated cells, seemingly as if ATF6α was not activated (Figure 3C). Activation of the ATF6α pathway was independent of IRE1 activity as illustrated by the results obtained in IRE1-DN cells (Figure 3B). Together this indicates that although the PERK and IRE1α arms of the UPR are activated upon Sorafenib treatment, the ATF6α arm is impaired. This further suggests that the secretory pathway might be affected in Sorafenib-treated cells. In order to test the functionality of the secretory pathway, we examined alpha-1 antitrypsin (α1AT), a serpin family protease inhibitor secreted by hepatocytes. By monitoring the α1AT content in the culture medium and in the cell lysate by immunoblotting, we found that α1AT secretion was reduced by more than 60% upon Sorafenib treatment whereas the intracellular content remained identical.
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(Figures 3D). Imatinib treatment had only a modest effect on inhibition of secretion (Figures 3D).

We next sought to establish whether these effects correlated with phenotypic changes in secretory pathway organelles. We therefore studied the morphology of the Golgi complex using immunofluorescence. We observed that Sorafenib-treated cells displayed a time-dependent increase in Golgi fragmentation (Figures 4A). A more pronounced effect was observed in IRE1-DN cells, with a higher proportion of cells presenting fragmented Golgi. This was not associated to defects at the level of the cytoskeleton, as demonstrated by the presence of intact microtubule and actin networks (Figure S1B, C). To analyze whether the observed Golgi fragmentation could be reversed upon Sorafenib withdrawal, we incubated HepG2 cells for 4 h with Sorafenib, which was then removed and incubation continued for a further 2 h in the presence of complete medium. Incubation with Sorafenib led to approximately 60% and 100% of wild-type displaying fragmented Golgi (Figure 4B). Incubation with Sorafenib led to approximately 100% of IRE1-DN cells displaying fragmented Golgi (Figure S2). In wild-type cells, when Sorafenib was removed the majority of the cells were able to recover, resulting in only 10% of the cells displaying fragmented Golgi (Figures 4B). In the IRE1-DN cells, the effect of Sorafenib on Golgi morphology could also be reversed, albeit to a lesser extent than in the wild-type cells, with approximately 60% of the cells still displaying a fragmented organelle (Figure S2). This was most likely due to the fact that the absence of IRE1 in the IRE-DN cells results in a lower capacity of the secretory pathway to restore its homeostasis.

Taking into account the results presented above regarding the disruption of the secretory pathway and the induction of ER stress, we investigated whether autophagy was occurring in the Sorafenib-treated HepG2 cells as a clearance mechanism for damaged organelles. Sorafenib, in conjunction with the histone deacetylase inhibitor vorinostat, has previously been shown to induce autophagy, a cellular adaptive response to stress (7). We transfected HepG2 cells with a GFP-tagged LC3 construct and analyzed its subcellular localization upon Sorafenib treatment. GFP-LC3 was evenly distributed throughout the cell under basal conditions (Figure 4C). However, when cells were incubated for 8 h with Sorafenib, GFP-LC3 distribution became punctate with many of
the punctate structures co-localizing with the Golgi complex (Figure 4C, lower panel). This phenomenon was further amplified in IRE1-DN cells (not shown). Finally, HepG2 (or HuH7, not shown) cells were transfected with a siRNA to ATG5 to prevent autophagosome maturation or with NH₄Cl to attenuate phagolysosomal fusion in the presence of Sorafenib. The presence of the lipidated form of LC3 (LC3-II) was monitored using immunoblot. This revealed that ATG5 silencing prevented Sorafenib-induced LC3 maturation whereas preventing phagolysosomal fusion led to the accumulation of LC3-II, thereby showing an increased autophagic flux upon Sorafenib treatment (Figure 4D).

Sorafenib targets p97/VCP phosphorylation

So far our experiments have established that Sorafenib, a molecule defined as a multikinase inhibitor, acts on HepG2 (or in HuH7) cells by disrupting the secretory pathway thereby promoting the induction of ER stress and autophagy. In addition, treatment with Sorafenib induces the cells to undergo apoptosis. We hypothesized that the Sorafenib-induced effects on the secretory pathway may be mediated by the altered phosphorylation of a target protein normally involved in one the above cellular processes. To identify such a candidate an in-depth literature search was carried out, revealing that p97/VCP, an AAA+ ATPase involved in vesicle transport and fusion, presented these characteristics (20, 21). Furthermore, we have previously shown that the status of p97/VCP tyrosine phosphorylation is important for the assembly of the ER thereby serving as a regulatory step in the early secretory pathway (20, 21). We first analyzed the phosphorylation status of p97/VCP in cells treated with Sorafenib for 4 h. We found that p97/VCP was tyrosine-phosphorylated in the untreated controls, but this phosphorylation was abolished in Sorafenib-treated cells (Figure 5A). As a control, we incubated HepG2 cells with BpVphen, a tyrosine phosphatase inhibitor, and observed that under these conditions p97/VCP phosphorylation increased when compared to controls. When cells were co-incubated with Sorafenib and BpVphen, p97/VCP phosphorylation was greatly reduced compared to the negative control (Figure 5A). This suggests that Sorafenib acts on p97/VCP by preventing its tyrosine phosphorylation. Using a cell-free system, we have previously shown that non-phosphorylated p97/VCP associates with membranes, where it participates in transitional ER membrane remodeling (22). Since we observed
that Sorafenib impaired the functionality of the early secretory pathway by promoting Golgi fragmentation, we analyzed the localization of p97/VCP in our system. To this end, we prepared membrane fractions from control or drug-treated HepG2 cells and performed immunoblotting against p97/VCP. In the presence of Sorafenib, the membrane association of p97/VCP was increased, while in the presence of BpVphen it showed only residual membrane association (Figure 5B), consistent with our previous report (22). When both inhibitors were combined, p97/VCP displayed lower membrane association when compared to the untreated control, but membrane association was greater than when only BpVphen was used (Figure 5B). One explanation for this would be the higher degree of p97/VCP phosphorylation that was observed under these conditions, as shown in Figure 5A. To test whether p97/VCP tyrosine phosphorylation was altered upon inhibition of its activity, the same experiment as in Figure 5A was carried out but BpVphen was replaced by the p97/VCP reversible inhibitor DBeQ (23) and revealed that DBeQ did not alter the phosphorylation of this protein (Figure 5C). We then further characterized the impact of these molecules on Golgi fragmentation. HuH7 cells treated with 1 or 10 μM Sorafenib showed an incremental fragmentation of the Golgi apparatus (Figure 5D, 4, 7). BpVphen did not impact significantly on the fragmentation and DBeQ treatment led to the perinuclear concentration of the Golgi apparatus (Figure 5D, 2, 3). The co-treatment of HuH7 with both Sorafenib and BpVPhen partially reversed the Sorafenib effect (compare Figure 5D, 4-5 and 7-8). Incubation with the combination with Sorafenib and DBeQ led to the formation of large intracellular puncta (Figure 5D, 6), likely to constitute autophagosomes as illustrated in Figure 4.

**Sorafenib-mediated toxicity occurs through the regulation of p97/VCP**

Since Sorafenib is toxic to HepG2 cells (Figure 1), and since it affects the phosphorylation and the localization/function of p97/VCP, we postulated that targeting p97/VCP activity might enhance Sorafenib cellular effects. To this end, HepG2 cells were treated with the recently described p97/VCP reversible inhibitor, DBeQ (23, 24), either in the presence or absence of Sorafenib. Incubation of HepG2 or HuH7 cells for 48 h with increasing concentrations of Sorafenib demonstrated that cell death increased with the concentration of the inhibitor, resulting in calculated IC50 of 4.3 ± 0.22 and 2.7 ±
0.11 μM (Figure 6A). DBeQ alone was not toxic to HepG2 cells at the concentrations tested in this study (Figure 6A). However, when Sorafenib and DBeQ (20 μM) were combined, a synergistic effect was observed, leading to a statistically significant decrease in the Sorafenib IC50 to 0.6 ± 0.12 μM and 0.4 ± 0.07, p < 0.03 (Figure 6A). These results were also supported by the quantification of Sorafenib-induced apoptosis in HepG2 (Figure 6B, left) and HuH7 (Figure 6B, right) cells, which was increased when cells were incubated with the combination of Sorafenib (2 μM) and DBeQ (20 μM). Finally, LC3 maturation was assessed using immunoblot in HuH7 cells treated with Sorafenib (2 μM) or DBeQ (20 μM) or a combination of both (Figure 6C). This revealed that as previously shown DBeQ induced an accumulation of LC3-II (23), phenomenon that was amplified by co-treatment with low dose of Sorafenib, thereby indicating the amplification of the autophagic process.

Altogether, our data indicate that Sorafenib-mediated cell death occurs in part through the disruption of the secretory pathway, which could due to the altered tyrosine phosphorylation of p97/VCP. Moreover, we show that this phenomenon can be further enhanced when cultured cells are exposed to a reversible inhibitor of p97/VCP.
Discussion

In this study we demonstrate that Sorafenib induces cell death in a HCC model cell line, at least in part through the activation of the UPR, which results from Sorafenib-mediated impairment of secretory pathway function. In addition to disrupting the secretory pathway, Sorafenib treatment promotes autophagy, most likely as a compensatory mechanism. We also show that upon Sorafenib treatment, tyrosine phosphorylation of the AAA+ ATPase p97/VCP is prevented, thereby stabilizing its membrane association. Together, these results led us to propose that inhibition of p97/VCP might lead to enhance Sorafenib-mediated toxicity. This hypothesis was validated using the reversible pharmacological inhibitor of p97/VCP, DBeQ, which further sensitized the cells to Sorafenib.

Sorafenib primarily inhibits VEGFR, PDGFR and Raf kinases, although other kinases are also potential targets (4, 5, 25). It was previously reported that p97/VCP is phosphorylated (26), and that its phosphorylation is regulated by the tyrosine phosphatase PTPH1 in cultured cells (27) and the tyrosine kinases JAK2 in vitro systems (22), or upon ligation of the TCR in T cells (28). Other tyrosine kinases may also be involved, as p97/VCP presents conserved tyrosine phosphorylation sites for ABL and LCK kinases (data not shown). Sorafenib has been shown to inhibit JAK2 activation (29), which could explain its effect on p97/VCP phosphorylation. However, the effects of Sorafenib on JAK2 remain controversial (30), and more work is needed to identify the kinases responsible for p97/VCP tyrosine phosphorylation in the liver.

The functions of p97/VCP in the liver have been described in both physiological and pathological mechanisms. Proteomics studies have suggested that p97/VCP might play some role in liver regeneration and in fetal liver development (31), and it has also been shown that p97/VCP contributes to the formation of Mallory bodies (32). Moreover, p97/VCP autoantibodies have been found in cases of autoimmune hepatitis (33), primary biliary cirrhosis (34, 35) and in HCC (36). Elevated expression of p97/VCP in HCC has also been associated with increased incidence of tumor recurrence (37). Our results show that in the context of HCC, p97/VCP might play an important role in maintaining protein secretion homeostasis, through a tightly regulated tyrosine phosphorylation-dependent mechanism (Figure 6D). Together, this information suggests that p97/VCP might play an
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instrumental role in liver physiology and pathophysiology and consequently represents an interesting potential therapeutic target.

In our study we also show that Sorafenib treatment leads to the disruption of the secretory pathway, thereby uncoupling the PERK/IRE1 branches of the UPR from the ATF6 branch, which requires functional ER-to-Golgi transport. This relatively unique observation also highlights the role of Sorafenib-inhibited kinases in the maintenance of the secretory pathway and might provide novel insights into a kinase-mediated control of protein secretion. Moreover, we identify p97/VCP as a central player coordinating the balance between protein secretion and clearance/autophagy through phosphorylation-dependent mechanisms. This study therefore demonstrates that p97/VCP could represent a relevant therapeutic target to improve HCC treatment through alteration of proteostasis as previously suggested for other models (38). As a consequence in HCC, alteration of the secretory pathway by Sorafenib, leading to partial UPR activation would be greatly enhanced by inhibition of p97/VCP, thus leading to proteostasis imbalance and ultimately to cell death. As cancer cells are particularly sensitive to such alteration (38), this type of strategy would be particularly adapted for HCC, which arise in most cases on cirrhotic livers.

In conclusion, we propose that in HCC Sorafenib prevents p97/VCP tyrosine phosphorylation, thereby leading to the disruption of the secretory pathway and ER stress-mediated cell death. This mechanism represents a novel aspect of Sorafenib-mediated cell death in HCC and points towards p97/VCP as a potential therapeutic target in this disease.
Acknowledgments

Sorafenib was kindly provided by Bayer. DBeQ was kindly provided by Dr Franck Schoenen (MLPCN Specialized Chemistry Center, KS, USA). We are indebted to Gaelle Cubel for technical help.

Grant support

This work was funded by a grant from Avenir (Inserm) – Institut National du Cancer (INCa), Ligue contre le Cancer, a Programme d’Action Intégré de Recherche sur le CHC to EC and a Ulysses/IRCSET grant to EC and JCS. EC was supported by a contrat d’interface from Inserm – Centre Hospitalo-Universitaire (CHU) Pellegrin, Bordeaux. PY was funded by a « poste vert » Inserm, EM was funded by a grant from the Association de Recherche contre le Cancer (ARC). MGB was funded by an ESF grant.
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References

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Figure Legends

Figure 1. Sorafenib induces cell death in HepG2 cells and prevents MAPK activation. (A) Structure of Sorafenib. (B) HepG2 cells were either untreated or treated with 10 μM Sorafenib for 24 or 48 h and then analyzed for viability and apoptosis using Sulforhodamine B and Annexin V/Propidium iodide staining, respectively. The results are shown as mean fold increase compared to untreated cells and are displayed as means of three independent experiments ± SD. Student’s t-test: * p-value < 0.05, ** p-value < 0.01, ## p-value < 0.005. (C) HepG2 cells were pre-starved for 16 h after which they were treated in the presence of 10% FBS with 10 μM Sorafenib or medium alone for increasing amounts of time (0, 2, 4, 8 and 16 h). The ratios of phosphorylated ERK (P-ERK)/ERK were calculated by densitometry from immunoblots against ERK and P-ERK obtained from total protein extracts. Results are shown as means of three independent experiments ± SD. Student’s t-test: * p-value < 0.05.

Figure 2. Sorafenib induces ER stress. (A) Total RNA was isolated from HepG2 cells and the splicing of XBP1 (uXBP-1, unspliced XBP-1; sXBP-1, spliced XBP-1) was analyzed by semi-quantitative RT-PCR. (B) The P-JNK/JNK ratios were calculated by densitometry from immunoblots against JNK and P-JNK obtained from total protein extracts of cells treated as above. Results are shown as means ± SD. (C) Immunoblot of eIF2α and phosphorylated eIF2α (P-eIF2α). Results are representative of 4 independent experiments. (D) Analysis of Chop and Gadd34 mRNA expression was performed by q-PCR. Results shown are the means ± SD of 3 experiments each with 2 replicates. Student’s t-test *: p < 0.05, compared to control, #: p < 0.03 HepG2 mock transfected compared to IRE1-DN transfected cells.

Figure 3. ATF6α activation upon ER stress is prevented by Sorafenib. (A) the subcellular localization of FLAG-ATF6α was analyzed in HepG2 cells by immunofluorescence followed by wide-field fluorescence microscopy. Calnexin (CANX) is used as a marker for the ER. Scale bar corresponds to 25 μm. (B) The number of cells containing nuclear-localized FLAG-ATF6α was quantified. Between 300 and 500 cells were analyzed for each condition and results are represented as the means ± SD.
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Student’s t-test *: p < 0.01. (C) Quantification of the mRNA expression of ATF6 responsive genes – Orp150, Herpud1 and BiP. Results shown are the means ± SD of 3 experiments each with 2 replicates. Student’s t-test *: p < 0.05, compared to control. (D) Secretion of α1 antitrypsin (α1AT) in HepG2 cells was analyzed by immunoblotting (top). The amount of the corresponding intracellular α1AT was monitored in total cell lysate (bottom). Quantification of secreted α1AT was performed on the immunoblots by densitometry. Results are shown as a percentage of CTL and are the means ± S.D. of 4 independent experiments. Student’s t-test *: p < 0.05, ***: p < 0.01 compared to control.

Figure 4. Sorafenib induces reversible Golgi complex fragmentation and autophagy in HepG2 cells. (A) Golgi complex staining using anti-Giantin antibodies (green), and Hoechst 33342 as a nuclear marker (blue). Images were acquired by wide-field fluorescence microscopy. Scale bars correspond to 25 μm (right). The number of cells containing fragmented Golgi complex was quantified. Between 250 and 400 cells were analyzed for each condition and results are represented as the means ± SD (left). (B) Same as in A but following Sorafenib withdrawal. Student’s t-test *: p < 0.03, **: p < 0.01. (C) HepG2 cells were transfected with a GFP-LC3 (green) construct for 24 h and were then either left untreated (CTL) or treated with 10 μM Sorafenib for 8 h. Cells were then analyzed by confocal microscopy. Scale bars represent 25 μm. (D) Total protein extracts of HepG2 cells transfected with a siRNA to ATG5 (for 48 h) or treated with 40 mM NH4Cl in the presence or not of 10 μM Sorafenib for 8 h were subjected to immunoblotting against LC3. Tubulin is shown as a loading control. Results are representative of 3 experiments.

Figure 5. Tyrosine phosphorylation of p97/VCP is negatively regulated by Sorafenib. (A) HepG2 cells were either untreated (CTL) or treated with 10 μM Sorafenib, 5 μM BpVphen or 10 μM Sorafenib combined with 5 μM BpVphen for 4 h. p97/VCP was immunoprecipitated from total cell lysates and immunoblots against phospho-tyrosine (pY) and p97/VCP were performed. The amount of pY in each sample is shown at the bottom of the immunoblots as a percentage of CTL. A representative experiment out of 4 is shown (left). Quantification of the 4 experiments by densitometry represented as the
mean ± SD. Statistical significance is shown *: p < 0.03, **: p < 0.01 (right). (B) Cells were treated as above and the presence of p97/VCP in the total membrane fraction was analyzed by immunoblotting. CANX is shown as a loading control. A representative experiment out of 3 is shown (left). Quantification of the 4 experiments by densitometry represented as the mean±SD. Statistical significance is shown *: p < 0.04, **: p < 0.01 (right). (C) Same as in (A) but BpVphen was replaced by 20 μM DBeQ. (D) HuH7 cells were either not treated (1) or treated with 1 μM (4) or 10 μM (7) Sorafenib for 2 h, 5 μM BpVphen (2), 20 μM DBeQ (3) for 3 h or the combination of 1 μM Sorafenib + 5 μM BpVphen (5), 10 μM Sorafenib + 5 μM BpVphen (8) and 1 μM Sorafenib + 20 μM DBeQ (6). Cells were fixed and stained for Giantin (green) and Hoechst 33342 (blue). Images were acquired by confocal microscopy. Scale bars correspond to 10 μm.

**Figure 6.** Sorafenib-mediated cell toxicity occurs in part through p97/VCP. (A) HepG2 (left) and HuH7 (right) cells were incubated for 48 h with increasing concentrations of Sorafenib or DBeQ or a combination of an increasing concentration of Sorafenib and 20 μM DBeQ, and the cellular viability was analyzed using Sulphorhodamine B staining. (B) HepG2 (left) and HuH7 (right) cells were treated with 2 μM Sorafenib and 20 μM DBeQ for 36 h. Apoptosis was determined using Annexin V staining and is represented as a mean percentage ± SD representative of 3 independent experiments. Statistical significance is shown *: p < 0.05, **: p < 0.01. (C) HuH7 cells were treated with 2 μM Sorafenib and 20 μM DBeQ or the combination of both for 16 h. LC3 maturation was assessed using immunoblot with anti-LC3 antibodies. A blot representative of 3 independent experiments is shown. (D) Schematic representation of sorafenib-mediated regulation of cell death through p97/VCP and the secretory pathway.
Figure 2
Figure 3
Figure 4
Figure 5
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Mol Cancer Ther Published OnlineFirst October 5, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-12-0516

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