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SORAFENIB SENSITIZES HEPATOCELLULAR CARCINOMA CELLS TO PHYSIOLOGICAL APOPTOTIC STIMULI

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Abstract

Sorafenib increases survival rate of patients with advanced hepatocellular carcinoma (HCC). The mechanism underlying this effect is not completely understood. In this work we have analyzed the effects of sorafenib on autocrine proliferation and survival of different human HCC cell lines. Our results indicate that Sorafenib *in vitro* counteracts autocrine growth of different tumor cells (Hep3B, HepG2, PLC-PRF-5, SK-Hep1). Arrest in S/G2/M cell cycle phases were observed coincident with cyclin D1 down-regulation. However, sorafenib's main anti-tumor activity seems to occur through cell death induction which correlated with caspase activation, increase in the percentage of hypodiploid cells, activation of BAX and BAK and cytochrome c release from mitochondria to cytosol. In addition, we observed a rise in mRNA and protein levels of the pro-apoptotic "BH3-domain only" PUMA and BIM, as well as decreased protein levels of the anti-apoptotic MCL1 and survivin. PUMA targeting knockdown, by using specific siRNAs, inhibited sorafenib-induced apoptotic features. Moreover, we obtained evidence suggesting that sorafenib also sensitizes HCC cells to the apoptotic activity of Transforming Growth Factor-β (TGF-β) through the intrinsic pathway and to Tumor Necrosis Factor-α (TNF) through the extrinsic pathway. Interestingly, sensitization to sorafenib-induced apoptosis is characteristic of liver tumor cells, since untransformed hepatocytes did not respond to sorafenib inducing apoptosis, either alone or in combination with TGF-β or TNF. Indeed, sorafenib effectiveness in delaying HCC late progression might be partly related to a selectively sensitization of HCC cells to apoptosis by disrupting autocrine signals that protect them from adverse conditions and pro-apoptotic physiological cytokines.

Keywords

TNF; TGF-beta; hepatocytes; PUMA; cancer

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Introduction

Hepatocellular carcinoma (HCC) is the fifth cause of cancer-associated mortality in the West and one of the leading worldwide causes of death. New biomarkers for early detection of at-risk patients and more efficacious and selective tumor-targeted agents are needed (Andrisani et al., 2010; Worns and Galle, 2010). Successful development of such agents will be linked to the ability to appropriately select patients for more individualized treatments according to potential predictive markers of response (Finn, 2010). This approach will allow tailoring therapies based on the individual molecular background of the tumor and its adjacent tissue (Luo et al., 2006; Villanueva et al., 2010). Therefore, it is extremely important to better identifythe molecular mechanisms of action of drugs with proved efficacy in this malignancy.

Sorafenib has recently shown to provide a modest but significant increase of survival rate of patients with advanced HCC (Llovet et al., 2008). The mechanisms underlying these effects remain incompletely understood. This drug has an anti-angiogenic action through direct effects on vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) receptors (Wilhelm et al., 2008). Its capacity to impair the Raf/mitogen activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway (Liu et al., 2006), or inhibit the BCR/ABL kinase activity (Kurosu et al., 2009), as well as its demonstrated effects on STAT3 (Signal transducer and activator of transcription) (Blechacz et al., 2009; Chen et al., 2010; Tai et al., 2011; Ulivi et al., 2009) have been linked to its anti-proliferative effects in different tumors, which might include both growth arrest and cell death. A recent study has suggested that induction of the growth arrest DNA damage-inducible gene 45β (GADD45β) might also contribute to sorafenib-induced apoptosis in HCC cells (Ou et al., 2010).

In spite of the extensive literature of the last years around sorafenib effects, the specific molecular pathways involved in sorafenib-induced tumor cell death remain unclear and caspase-dependent and -independent mechanisms, as well as endoplasmic reticulum stress have been proposed (Liu et al., 2006; Panka et al., 2006; Rahmani et al., 2007). *In vitro* studies have shown that under baseline culture conditions, sorafenib is a poor apoptotic inducer in HCC cells unless this drug is used at high doses. However, it greatly potentiates the apoptotic effects of other therapeutic drugs, such as TRAIL, BCL-XL inhibitors, rapamycin, or MEK/ERK inhibitors (Chen et al., 2010; Ganten et al., 2004; Hikita et al., 2010; Newell et al., 2009; Ou et al., 2009). HCC cells show overactivation of survival signals that confer them resistance to unfavorable *milieu* and to the pro-apoptotic stimuli present in the liver tumor (Fabregat, 2009). It might be hypothesized that sorafenib could sensitize cells to extracellular apoptotic agents by counteracting autocrine survival pathways. However, whether sorafenib might potentiate the HCC response to stress or pro-apoptotic physiological stimuli present in the liver tumor has not been explored yet.

In this work we show results that support the role of an apoptotic-mediated event occurring upon *in vitro* sorafenib treatment of different HCC cell lines, through up-regulation of the “BH3-domain only” PUMA. Interestingly, sorafenib *in vitro* also facilitated the pro-apoptotic activity of physiological inducers, such as the Transforming Growth Factor-beta (TGF-β) and the Tumor Necrosis Factor-alpha (TNF) through both intrinsic and extrinsic mechanisms. These effects are specific of liver tumor cells, since untranformed human hepatocytes do not undergo apoptosis in response to sorafenib.

Materials and Methods

Cell culture conditions

Hep3B, HepG2, SK-Hep1, and PLC/PRF/5 human cell lines were obtained from the European Collection of Cell Cultures (ECACC). This cell bank performed cell line characterizations and cells were passaged in the laboratory for fewer than six months after receipt or resuscitation. HH4 non-transformed human hepatocyte cell line was established as described (Tang et al., 2007) and human fetal hepatocytes (HFH) were isolated and plated as previously published (Lazaro et al., 2003). For cell culture, the following media were used: MEM for Hep3B and HepG2, 1mM pyruvate-supplemented MEM for SK-Hep1, DMEM for PLC/PRF/5, William's E medium supplemented with seeding media in collagen-coated plates for HH4 and HFH. Cells were grown in medium supplemented with 10% fetal bovine serum and maintained in a humidified atmosphere of 37°C, 5% CO_2. Sorafenib tosylate was kindly provided by Bayer Schering Pharma AG (Berlin, Germany) and used at the concentration indicated in each figure. Human recombinant TGF-β1 was from Calbiochem (La Jolla, CA, USA) and TNF was from Peprotech (Rocky Hill NJ, USA). SP600125 and ZVAD-fmk were from Calbiochem (La Jolla, CA, USA). In all the experiments, cells were serum deprived for 16 hours and treated with Sorafenib tosylate (concentrations indicated in each figure). Human recombinant TGF-β1 and TNF were used at 2ng/mL and 20 ng/mL, respectively, and added 30 min after sorafenib treatment. SP600125 (30 μM) and ZVAD-fmk (20μM) were added 30 min before sorafenib addition.

Analysis of cell number

Cell number was assessed with crystal violet staining, as described (Sanchez et al., 1996).

Proliferation measurement by [^{3}H]-thymidine incorporation

Cells were treated during 48 hours in presence of 1 μCi/ml, 1μM thymidine (GE Healthcare, Barcelona, Spain). At the end of the incubation period, radioactivity present in acid precipitated material was measured by using a scintillation counter 1209 Rackbeta (Wallac, Turku, Finland), as previously described (de Juan et al., 1992).

Analysis of gene expression

RNeasy Mini Kit (Qiagen, Valencia, CA) was used for total RNA isolation. RNA was analyzed by reverse transcriptase multiplex ligation–dependent probe amplification (RT-MLPA) using the SALSA MLPA KIT R011 Apoptosis mRNA from MRC-Holland (Amsterdam, The Netherlands) (Caja et al., 2009). Relative expression of each gene was determined as arbitrary units by the ratio of each measured fluorescent peak area to the peak area of the reference probe, in this caseβ-2-microglobulin.

Knock-down assays

Cells were transfected using TransIT-siQuest (Mirus, Madison, USA) according to the manufacturer's recommendation. After incubation, cells were trypsinized and seeded for experiments. Oligos were obtained from Sigma-Genosys (Suffolk, UK). Sequences were as follow: human PUMA1: 5′CCUGGAGGGUCCUGUACAA3′; human PUMA2: 5′ UUGUACAGGACCCUCCAGG3′. The unsilencing (scrambled) siRNA used was selected from previous works (Caja et al., 2009).

Analysis of apoptotic features

Appearance of hypodiploid cells (as a feature of DNA fragmentation related to the apoptotic process) was determined by flow cytometry, as described (Valdes et al., 2002). Caspase-3 activity was analyzed fluorimetricallyas described (Caja et al., 2009). Determination of

Active BAX and BAK forms by flow cytometry was developed as described (Ortiz et al., 2008). During treatment, cell pictures were taken by an Olympus c-5060 camera attached to an Olympus IX70 microscope.

Western blot analysis

Total protein extracts and Western Blot procedure were carried out as previously described (Caja et al., 2009). Rabbit anti-BID, rabbit anti-BIM and mouse anti-cytochrome c were from BD Pharmigen (San Diego CA, USA); rabbit anti-PUMA was from Abcam (Cambridge, UK); mouse anti-β-actin, used as loading control (clone AC-15), was from Sigma (Madrid, Spain); rabbit anti-MCL1, rabbit anti-BCL-XL and rabbit anti-cyclin D1 were from Santa Cruz Biotechnology (CA, USA); rabbit anti-Survivin was from Novus Biologicals, INC (Littleton CO, USA); mouse anti-Caspase-8 (FLICE) was from MBL CO, LTD. (Naka-ku Nagoga, Japan); mouse anti-FLIP was from ENZO life Sciences (USA); mouse anti-HSP60, used to analyze the purity and loading of mitochondrial extracts, was from Stressgen Protein (USA). Protein concentration was measured with BCA™ Protein Assay kit (Pierce, Rockford, USA).

Statistical analyses

Statistical analyses were performed as an estimation of the associated probability to a student's t test (95% confidence interval) or as a one-way ANOVA by Fisher's Least Significant Difference (LSD) method, depending on the involved conditions. In general, experiments were carried out at least 3 independent times with 2–3 technical replicates. Data are mean +/− standard deviation (SD). In all cases statistical calculation was developed using Statgraphics 5.1 software.

Results

Sorafenib neutralizes autocrine proliferative signals in HCC cells

Liver tumor cells are able to grow in the absence of serum due to autocrine signals, among them, epidermal growth factor receptor (EGFR) ligands, which confer them proliferative properties and resistance to apoptotic stimuli (Caja et al., 2010). In order to know whether sorafenib might counteract the effects of these stimuli, we submitted four different HCC cell lines (Hep3B, HepG2, PLC/PRF/5 and SK-Hep1) to serum depleted medium to analyze the effect of sorafenib. As shown in Supplementary Fig. 1, the four cell lines showed autocrine growth that was inhibited by sorafenib in a dose- and time-dependent manner. Experiments of [^{3}H]-Thymidine incorporation demonstrated that DNA synthesis was clearly attenuated when sorafenib was present (Fig. 1A). Cell cycle analysis did not reveal the expected arrest in G0/G1, but accumulation of cells in S/G2/M (Fig. 1B). However, although down-regulation of cyclin D1 was clearly observed in sorafenib treated cells (Fig. 1C), no effect was found in other S/G2/M cyclins (results not shown) or in cyclin-dependent kinase (CDK) inhibitors, such as *CDKN1A* (Fig. 1C). This kind of response led us to hypothesize that HCC cells might be responding to sorafenib inducing cell death.

Sorafenib induces apoptosis through the intrinsic pathway. Role of PUMA

In agreement with this formulated hypothesis, sorafenib-treated HCC cells showed: 1) activation of caspase-3 and 2) increase in the percentage of hypodiploid cells, which was blocked in the presence of a general caspase inhibitor (ZVAD-fmk) (Fig. 2A and B). Preliminary results indicated that sorafenib did not activate caspase-8 (results not shown), which led us to analyze whether sorafenib induced apoptosis through a mitochondrial-dependent intrinsic pathway. Results indicated that this was the case. Indeed, sorafenib

treatment induced BAX and BAK activation and the release of cytochrome c from the mitochondria to the cytosol (Fig. 2C and D).

To better understand the mechanism of sorafenib-induced apoptosis, we analyzed the effects on the expression of a set of genes related to apoptosis by RT-MLPA in HepG2 and Hep3B cells (see Supplementary Fig. 2). Results indicated a significant effect of sorafenib on the transcript levels of two pro-apoptotic members of the Bcl-2 (BH3-domain only) family: *BIM* and *PUMA*, which correlated with increased protein levels analyzed by Western blots (Fig. 3A and Supplementary Fig. 2). Furthermore, we observed a post-transcriptional regulation of the anti-apoptotic protein MCL1, whereas BCL-XL levels were not affected. The effect on survivin, a member of the inhibitor of apoptosis (IAP) family, appeared to depend on the length of drug treatment. Early after sorafenib treatment, protein levels decreased, while with longer times of drug exposure, transcription appeared to be attenuated (Fig. 3A).

Due to the strong effect observed on PUMA and BIM levels, we decided to target knock-down both genes to better know their role in sorafenib-induced apoptosis in HCC cells. Whereas we could not find a clear effect when BIM was down-regulated (results not shown), HepG2 and Hep3B cells transfected with specific PUMA siRNAs showed a significant attenuation of the sorafenib-induced apoptotic response both in terms of caspase-3 activation and appearance of hypodiploid cells. Identical effects were observed with two different siRNAs (results for siRNA1 are shown in Fig. 3B–D). Recent results had indicated that c-Jun-N-terminal kinase (JNK) activation occurs in response to sorafenib in tumor cells (Ou et al., 2010; Wei et al., 2010), contributing to its apoptotic effect. For this, we checked the effect of JNK inhibitors on sorafenib-induced PUMA up-regulation. As shown in Fig. 3E, JNK inhibition completely blocked increase in PUMA levels caused by sorafenib.

All these results together indicate that sorafenib induces apoptosis through an intrinsic mechanism where JNK-mediated PUMA up-regulation plays a relevant role.

Sorafenib selectively sensitizes HCC cells to respond to TGF-β and TNF in terms of apoptosis

TGF-β induces apoptosis in hepatocytes, through a mitochondrial-dependent mechanism (Herrera et al., 2001). Although it is up-regulated in a great percentage of HCC patients (Ito et al., 1991), many HCC cells are refractory to its pro-apoptotic effects due to intrinsic overactivation of survival signals, such as Ras/MEK/ERKs pathway(Caja et al., 2009). Indeed, we wondered whether sorafenib might sensitize HCC resistant cells to TGF-β-induced effects. As shown in Fig. 4, HepG2 cells, which are clearly resistant to TGF-β in terms of apoptosis, responded to this cytokine when sorafenib was present, as evidenced in the higher activation of caspase-3 and the increase in hypodiploid cells observed with the combination of sorafenib and TGF-β as compared to sorafenib alone (Fig. 4A–B). Analysis of apoptosis regulatory genes (Supplementary Fig. 3A and Fig. 4C) revealed that co-treatment with sorafenib and TGF-β induced a higher increase in both PUMA and BIM at the protein level, when compared with the treatment with sorafenib alone. Interestingly, the presence of sorafenib impaired the increase in MCL1, characteristic of the anti-apoptotic response of HCC cells to TGF-β (Χαφα ετ αλ., 2009), and amplified the post-transcriptional effects on survivin levels. All together these results suggest that sorafenib would sensitize HCC cells to the mitochondrial-dependent apoptosis induced by TGF-β. Interestingly, in Hep3B, which are TGF-β responsive cells, sorafenib also significantly amplified the apoptotic response to this cytokine (Supplementary Fig. 3B).

The proinflammatory cytokine TNF has the ability to induce apoptosis through the extrinsic pathway. However, in most cells it also activates antiapoptotic signals, such as the nuclear

factor kappaB (NF-kappaB) pathway, which offset its pro-apoptotic effects (Wullaert et al., 2007). When Hep3B cells, which do not respond to TNF in terms of cell death, were treated with the combination of sorafenib and TNF a cooperative effect was observed and almost all the cells died in 24–36 h (Fig. 5A). This effect correlated with an early and high activation of caspase-3 and a marked increase in the percentage of hypodiploid cells (Fig. 5B–C). A similar effect was observed in HepG2 cells (results not shown). Combination of sorafenib and TNF produced time-dependent increase in caspase-8 activation, which was not observed in either of the individual treatments (Fig. 6A). Previous reports suggested that the capacity of sorafenib to sensitize tumor cells to the CD95 extrinsic apoptosis pathway correlated with diminished expression of c-FLIP-s levels (Zhang et al., 2008a). However, we could not observe any effect of the co-treatment of sorafenib and TNF on FLIP either at mRNA or protein levels (Fig. 6A). As a consequence of caspase-8 activation, BID was cleaved and the intrinsic pathway of apoptosis activated, as evidenced by a significant increase in the percentage of cells showing active BAX (Fig. 6B). Interestingly, sorafenib impaired the increase of MCL1 induced by TNF in Hep3B cells (Fig. 6C). However, in contrast to the results observed in the combination with TGF-β, sorafenib effects on PUMA and BIM were identical in the absence or presence of TNF (Fig. 6C). Therefore, sorafenib sensitized HCC cells to respond to TNF in terms of apoptosis through the extrinsic pathway, which later activates the intrinsic mechanism through caspase-8-mediated cleavage of BID.

Finally, a relevant point was whether the capacity of sorafenib to induce apoptosis and sensitize HCC cells to physiological apoptotic stimuli was also observed in normal, untransformed, hepatocytes. In an immortalized adult human hepatocyte cell line (HH4) and primary cultures of fetal human hepatocytes, sorafenib effects on cell viability were modest and correlated with inhibition of DNA synthesis. Caspase activation was not observed in response to sorafenib and we could not observe sensitization of cells to respond to TNF or TGF-β-induced apoptosis in a significant way (Fig. 7). These results indicate that sorafenib preferentially sensitizes liver tumor cells to die by apoptosis.

Discussion

Sorafenib is the first drug of proved clinical efficacy on HCC (Llovet et al., 2008). A better knowledge of the mechanisms underlying these effects would allow to understand the reasons for its efficacy and to assist in predicting synergistic effects with other drugs. Here we show that sorafenib blunts autocrine proliferative and survival signals in HCC cells. This effect was coincident with cyclin D1 (*CCND1*) down-regulation, which was previously reported to be associated to *in vitro* sorafenib effects (Liu et al., 2006) and recently corroborated in *in vivo* orthotopic models of human HCC (Huynh et al., 2010). However, the cell cycle profile did not indicate a classical arrest in G1, but in S/G2/M phases. This profile is frequently observed in cells undergoing apoptosis, since cells in G1 are more susceptible to die. Indeed, we indicate that sorafenib sensitizes cells to a mitochondrial-dependent apoptotic pathway. Previous results had indicated that sorafenib might induce apoptosis through MCL1 down-regulation and up-regulation of BIM (Yu et al., 2005; Zhang et al., 2008b), which mediate activation of the intrinsic pathway in tumor cells. However, we show for the first time an essential role for PUMA in this process. Two different HCC cell lines used in this study up-regulate PUMA in response to sorafenib and apoptotic features are clearly attenuated when PUMA is targeted knock-down with specific siRNAs. PUMA is a general sensor of cell death stimuli and has been proposed as a promising drug target for cancer therapy and tissue damage. It can be induced through p53-dependent and -independent pathways (Yu and Zhang, 2008). Interestingly, our results indicate that sorafenib-mediated PUMA up-regulation is not p53-dependent, since Hep3B cells show p53 deletion, whereas HepG2 are p53 wild type. Recent studies have suggested that sorafenib activates JNK in HCC and pancreatic cancer cells, which are necessary for its apoptotic

effects (Ou et al., 2010; Wei et al., 2010). PUMA can be up-regulated by JNK in hepatocytes lipoapoptosis (Cazanave et al., 2009). Here we propose that this could be the underlying effect of sorafenib on HCC cells, since a JNK inhibitor completely blocked sorafenib-induced PUMA up-regulation. Although traditionally involved in the tumor cell response to conventional cytotoxic agents that induce DNA damage, recent results indicate that PUMA could be also involved in the apoptotic response to targeted therapies, such as kinase inhibitors (Sun et al., 2009). All together, our results suggest that PUMA induction through JNK represents a novel mechanism of sorafenib to induce apoptosis in HCC cells and provide potential ways to enhance or predict the sensitivity to sorafenib therapies. Furthermore, we clearly observe a sorafenib-mediated regulation of survivin, an inhibitor of apoptosis protein with dual role on HCC cell proliferation and survival (Ito et al., 2000), regulation that occurs both at transcriptional and posttranscriptional levels.

During hepatocarcinogenesis both stroma and hepatocarcinoma cells produce pro-apoptotic cytokines. TGF-β1 is an important regulatory suppressor factor in hepatocytes, inhibiting proliferation and inducing cell death (Carr et al., 1986; Oberhammer et al., 1992). Expression of TGF-β is up-regulated in a great proportion of HCC patients (Ito et al., 1991), however, overactivation of survival signals in HCC cells help to protect them from its effects (Fabregat, 2009). Here we show that presence of sorafenib sensitizes resistant cells (HepG2) to TGF-β-induced apoptosis and amplifies the response of cells, such as Hep3B, that are poorly responsive to this cytokine. Although significant effects are observed on the regulation of BIM and PUMA, probably the most interesting effect of sorafenib is that it impairs up-regulation of the anti-apoptotic genes BCL-XL and MCL1 by TGF-β. These effects might be linked to the capacity of sorafenib to block the Raf/MEK/ERK pathway (Liu et al., 2006), since we have recently described that inhibition of the MEK/ERK pathway in HCC cells sensitizes them to TGF-β-induced cell death through a mitochondrial-dependent mechanism, coincident with decreased levels of BCL-XL and MCL1, and BAX/BAK activation (Caja et al., 2009). The proinflammatory cytokine TNF plays a pivotal role in liver pathophysiology because it has the capacity to induce both hepatocyte proliferation and cell death (Wullaert et al., 2007). Many HCC develop through inflammatory conditions, coincident with enhanced production of cytokines, such as IL-6 and TNF (Park et al., 2010). This causes hepatic inflammation and activation of the transcription factors STAT3 and NF-kappaB, which in turn contribute to prevent TNF-induced cell death (Park et al., 2010; Wullaert et al., 2007). In this work we show that HCC cells regain the capacity to respond to TNF when treated with sorafenib, through an extrinsic mechanism involving caspase-8 activation and BID cleavage. We do not find sorafenib-induced regulation of FLIP, which is in agreement with recent results where the effects of sorafenib on TRAIL-induced apoptosis were evaluated (Chen et al., 2010). Sensitization of HCC cells to TNF-induced cell death might be related to sorafenib-induced STAT-3 inhibition and reduction of TNF-induced MCL1, as has been proposed in previous works that explored the capacity of sorafenib to sensitize cells to TRAIL (Chen et al., 2010; Ricci et al., 2007). Interestingly, we also show that sensitization to sorafenib-induced apoptosis is a characteristic of liver tumor cells, since untransformed hepatocytes do not respond to sorafenib by inducing apoptosis, either alone or in combination with TGF-β or TNF.

In summary, here we show that the effectiveness of sorafenib in delaying HCC late progression might be related to its capacity to selectively sensitize HCC cells to apoptosis, through impairing autocrine signals that protect them from adverse situations and pro-apoptotic physiological cytokines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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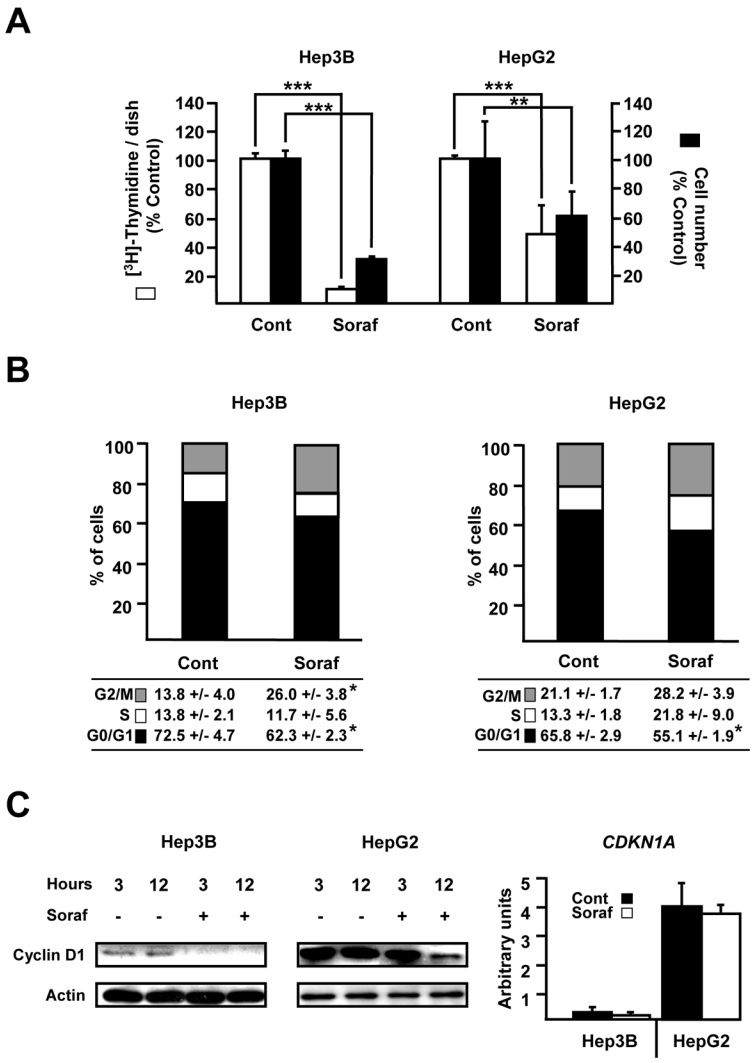

Figure 1. Sorafenib inhibits autocrine cell proliferation, but does not promote a cell cycle arrest in G0/G1

Serum depleted HepG2 and Hep3B cells were treated with 2μM sorafenib. **A.** [^{3}H]-Thymidine incorporation (left axis, white bar) and cell number (right axis, black bar) after 36 h of treatment. Results expressed as percentage of control (untreated) cells. **B.** Percentage of cells in each phase of the cell cycle. **C.** Left, Western blot of cyclin D1 (3 and 12h treatment). Right, *CDKN1A* gene expression analyzed by RT-MLPA (12h treatment). In A, B-bottom and C-right, mean±SD is shown (n=3). Student's t test versus untreated cells: *p<0.05; **p<0.01; ***p<0.001. Representative experiments are shown in B-top (n=3) and C-left (n=2).

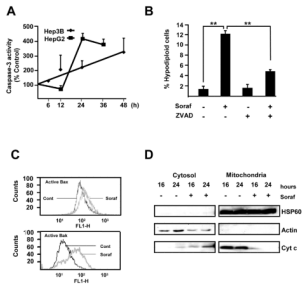

Figure 2. Sorafenib promotes mitochondrial-dependent apoptosis

Hep3B and HepG2 cells were treated with sorafenib (2μM). **A.** Caspase-3 activity. Time response. Results expressed as percentage of control (untreated) cells**. B.** Analysis of the percentage of hypodiploid cells in Hep3B cells (36h). **C.** Cells showing BAX or BAK active forms analyzed by flow cytometry in HepG2 cells (24h). **D.** Cytochrome c release to the cytosolic compartment. Mean±SD is shown (n=3). One-way ANOVA (LSD method): *p<0.05; **p<0.01; ***p<0.001. Representative experiments are shown in C (n=4) and D (n=2).

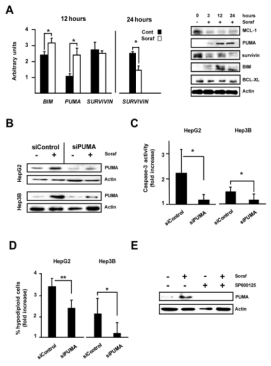

Figure 3. Targeting knock-down of PUMA attenuates sorafenib-induced apoptotic features

A. Changes in apoptosis-related gene expression in HepG2 cells after sorafenib treatment (2μM). Left: *BIM (BCL2L11), PUMA (BBC3), SURVIVIN (BIRC5A)* transcript levels, analyzed by RT-MLPA (12 and 24h). Right: Protein levels, analyzed by Western blot, at the indicated times. **B–D.** Unspecific siRNA (siControl) and specific PUMA siRNA1 were transfected in HepG2 or Hep3B cells, which were cultured in the absence of serum and with or without 2μM sorafenib. PUMA protein levels by Western blot (16h) are shown in B. Caspase-3 activity (24h) is shown in C. Percentage of hypodiploid cells (36h) is shown in D. In C and D, data are calculated as fold increase in sorafenib-treated cells versus untreated cells. **E**. PUMA protein levels in Hep3B cells with or without sorafenib (2μM) and/or the JNK inhibitor SP600125 (30μM) (16h). In graphs, mean±SD (n=3) is shown. Student's t test versus untreated cells: *p<0.05; **p<0.01; ***p<0.001. Representative experiments are shown in A-right (n=3), B (n=4) and E (n=2).

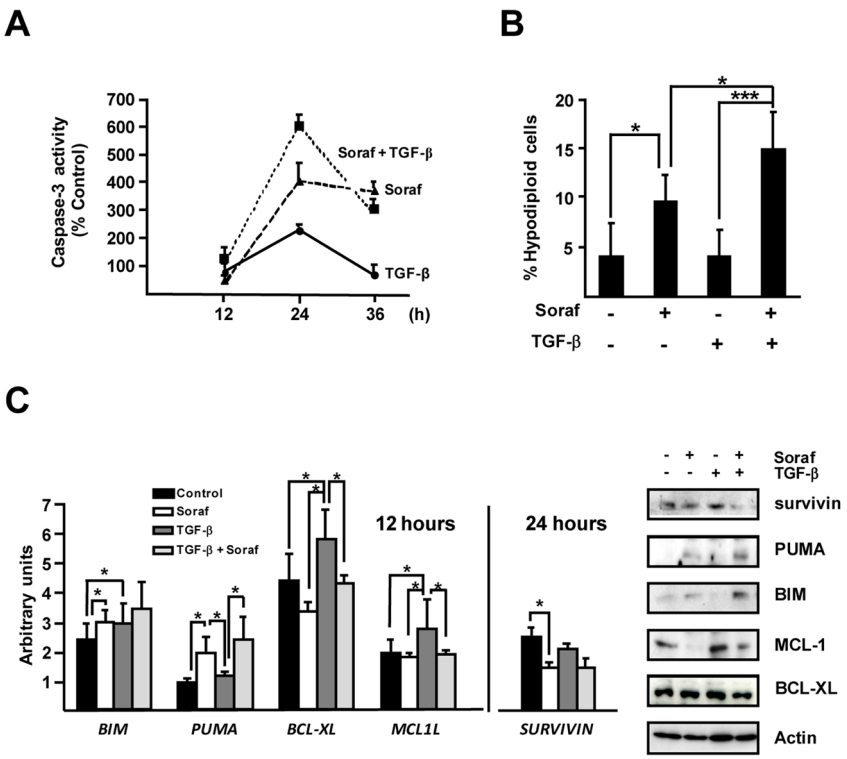

Figure 4. Sorafenib sensitizes HepG2 cells to TGF-β-induced apoptosis

HepG2 cells were treated with or without sorafenib (2μM) and with or without TGF-β (2ng/mL). **A.** Caspase-3 activity. Time response. Results expressed as percentage of control (untreated) cells **B.** Percentage of hypodiploid cells (36h). **C.** Left*: BIM (BCL2L11), PUMA (BBC3), BCL-XL (BCL2L1), MCL1, SURVIVIN (BIRC5A)* transcript levels, analyzed by RT-MLPA (12 and 24h). Right. Protein levels, analyzed by Western blot (24h). Mean±SD is shown (n=3). One-way ANOVA (LSD method): *p<0.05; **p<0.01; ***p<0.001. In A-right and C-right, representative experiments are shown (n=3).

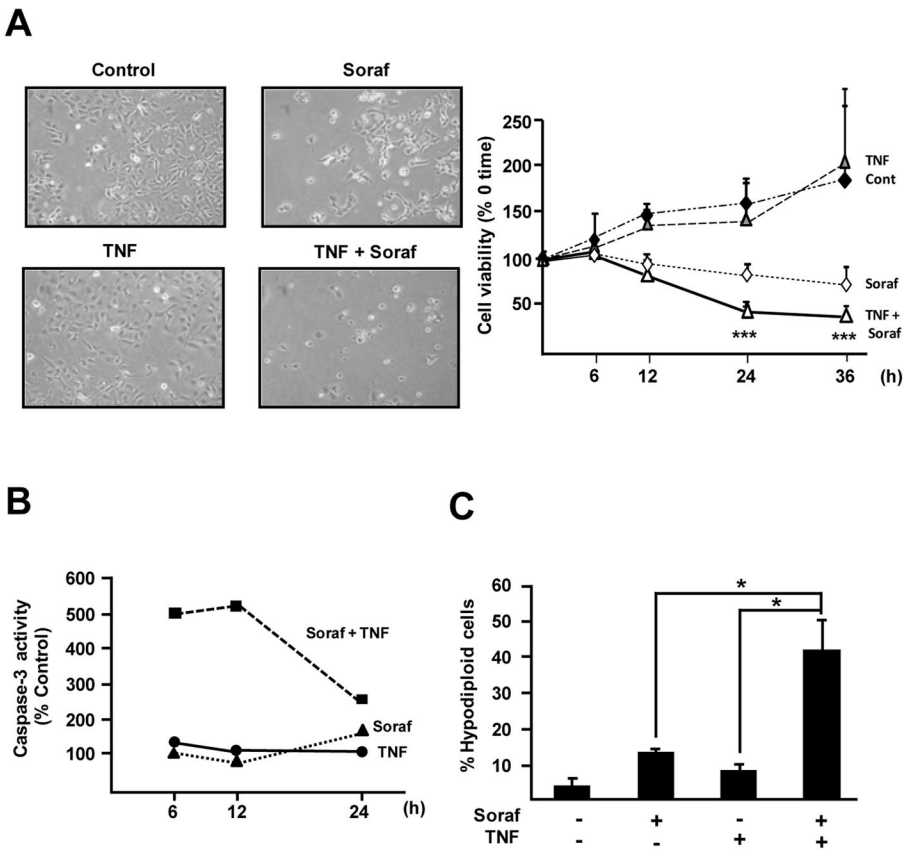

Figure 5. Sorafenib and TNF effects on apoptosis in Hep3B cells

Hep3B cells treated with or without sorafenib at 2μM and with or without TNF at 20ng/mL. **A.** Cell culture pictures at 36h (left). Cell viability (right). **B.** Caspase-3 activity. Time response. Results expressed as percentage of control (untreated) cells**. C.** Percentage of hypodiploid cells (36h). In A-left and B a representative experiment is shown (n=3). In A-right and C, Mean±SD is shown (n=3). One-way ANOVA (LSD method): *p<0.05; **p<0.01; ***p<0.001.

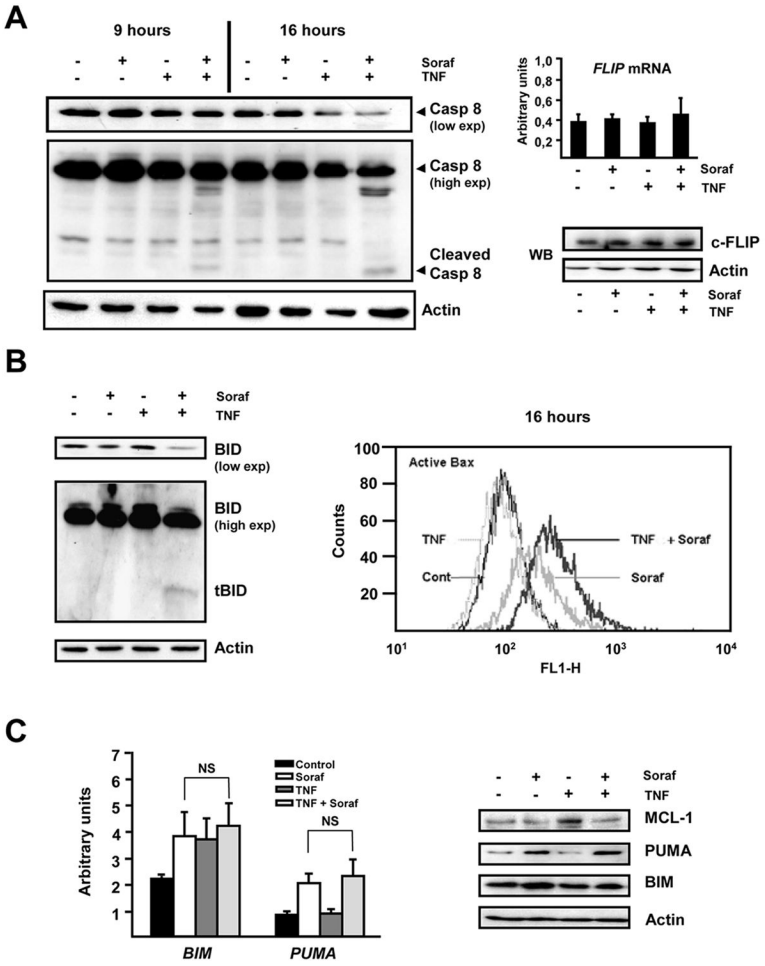

Figure 6. Sorafenib sensitizes HCC cells to respond to TNF byinducing apoptosis through both extrinsinc and intrinsic pathways

Hep3B cells treated as in Figure 5. **A.** Left: Caspase-8 cleavage at the indicated times of treatment. Right: *FLIP* mRNA levels (top, 6h treatment) analyzed by RT-MLPA and c-FLIP protein levels (bottom, 3h treatment), analyzed by Western blot. **B.** BID cleavage (left) analyzed by Western blot; BAX active form labelling (right) analyzed by flow cytometry (16 h treatment in both cases) **C**. *BIM (BCL2L11), PUMA (BBC3),* mRNA levels (12h, left) analyzed by RT-MLPA. BIM, PUMA protein levels (6h, right) analyzed by Western blot. Mean±SD is shown (n=3). One-way ANOVA (LSD method): *p<0.05; **p<0.01; ***p<0.001. Representative experiments are shown in A-left (n=2), Aright-top (n= 3), B-left (n= 2) and C-right (n=2).In A-left and B-left two different film expositions are shown to highlight changes in the levels of pro-caspase 8 and BID, as well as their cleaved fragments.

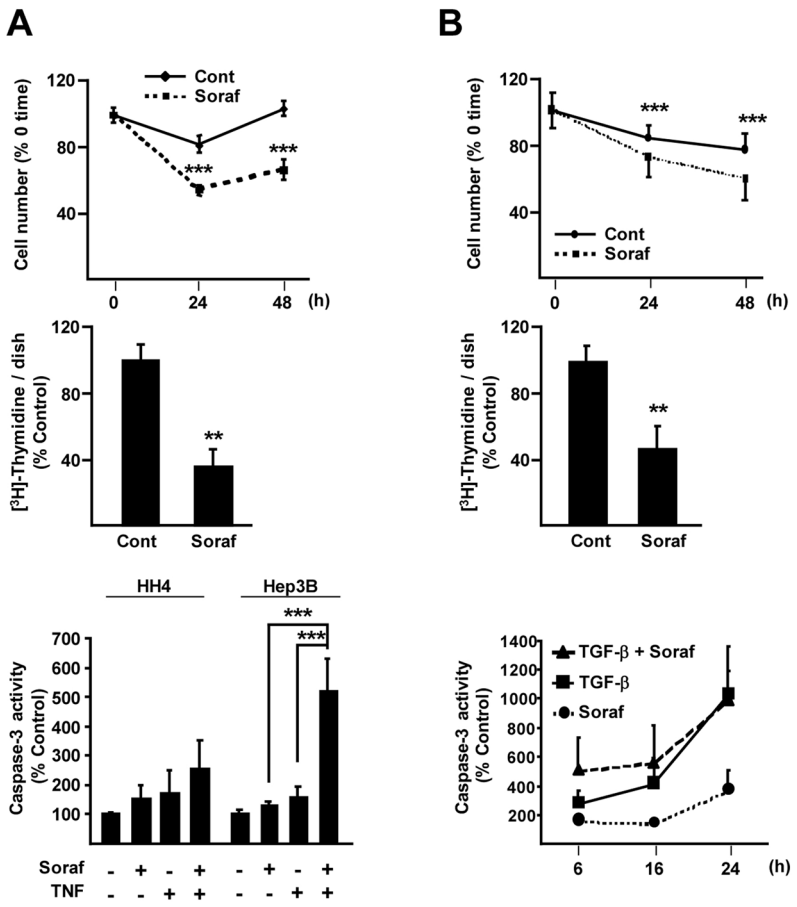

Figure 7. Effects of sorafenib in HH4 and HFH cells

HH4 cell line, primary cultures of fetal human hepatocytes (HFH) and Hep3B cells were serum depleted and treated with or without sorafenib at 2μM, with or without TGF-β (2ng/mL) and with or without TNF at 20ng/mL. **A.** HH4 cells: Top: Cell viabilityin response to sorafenib (2 μM). Time response. Middle: [^{3}H]-Thymidine incorporation (48h). Bottom: Caspase-3 activity (6h), as compared to the response observed in Hep3B cells. **B.** HFH cells: Top: Cell viabilityin response to sorafenib (2 μM). Time response. Middle: [^{3}H]-Thymidine incorporation (48h). Bottom: Caspase-3 activity at the indicated times. In cell viability analysis, results are expressed as percentage of cells at the zero time. In [^{3}H]-Thymidine incorporation and caspase-3 analyses, results are expressed as percentage of control (untreated) cells. Means±SD are shown (n=3). Student's t test against untreated cells: *p<0.05; **p<0.01; ***p<0.001. In bottom graphs (caspase-3 activity), one-way ANOVA (LSD method): *p<0.05; **p<0.01; ***p<0.001. A representative experiment (n=3) is shown in B bottom.