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Glycogen synthase kinase-3 inhibitors augment TRAIL-induced apoptotic death in human hepatoma cells

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ABSTRACT

Hepatocellular carcinoma (HCC) displays a striking resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Therefore, the characterization of pharmacological agents that overcome this resistance may provide new therapeutic modalities for HCC. Here, we examined whether glycogen synthase kinase-3 (GSK-3) inhibitors could restore TRAIL sensitivity in hepatoma cells. To this aim, the effects of two GSK-3 inhibitors, lithium and SB-415286, were analyzed on TRAIL apoptotic signaling in human hepatoma cell lines in comparison with normal hepatocytes. We observed that both inhibitors sensitized hepatoma cells, but not normal hepatocytes, to TRAIL-induced apoptosis by enhancing caspase-8 activity and the downstream recruitment of the mitochondrial machinery. GSK-3 inhibitors also stabilized p53 and the down-regulation of p53 by RNA interference abolished the sensitizing effect of lithium on caspase-3 activation. Concomitantly, GSK-3 inhibitors strongly activated c-Jun N-terminal kinases (JNKs). The pharmacological inhibition of JNKs with AS601245 or SP600125 resulted in an earlier and stronger induction of apoptosis indicating that activated JNKs transduced protective signals and provided an anti-apoptotic balance to the pro-apoptotic effects of GSK-3 inhibitors. These findings demonstrate that GSK-3 exerts a negative and complex constraint on TRAIL apoptotic signaling in hepatoma cells, which can be greatly alleviated by GSK-3 inhibitors. Therefore, GSK-3 inhibitors may open new perspectives to enhance the anti-tumor activity of TRAIL in HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer mortality worldwide. Its incidence is continuously rising in western countries, mainly due to the dissemination of hepatitis C virus infection and excessive alcohol consumption. HCC displays a striking resistance to conventional therapies while the only curative therapy, i.e. liver transplantation, is possible in only 5% of the patients [1]. Therefore, the development of innovative and targeted therapies may represent an alternative for the treatment of advanced HCC [2]. Among possible future therapies, drugs that would enhance death receptor signaling in HCC cells would be of particular interest.

HCC is associated with a loss of hepatocyte susceptibility to the apoptotic signals triggered by the death ligands CD95L or

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tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [3,4]. Through binding to their receptors, i.e. CD95, TRAIL-R1 and TRAIL-R2, CD95L and TRAIL cause receptor aggregation, followed by recruitment of the adaptor Fas-associated death domain (FADD) and of procaspase-8 to form the death inducing signaling complex (DISC). The activation of caspase-8 then triggers a cascade of events, which may involve the mitochondrial machinery and culminates in cell death.

The resistance of HCC cells to death ligand-induced apoptosis has been attributed to different mechanisms. First, a decrease in CD95 expression is frequent and it is associated with dedifferentiation, poor prognosis and tumor recurrence [5–7]. In contrast, HCC cells are resistant to TRAIL despite maintained expression of TRAIL receptors [8–10]. Post-receptor resistance can occur at the level of the DISC and results from an overexpression of cellular FLICE/caspase-8-inhibitory protein (cFLIP) [11,12]. Overexpressions of Bcl-xL [13] and survivin [14] have been also proposed to account for TRAIL resistance.

Recently, the serine/threonine kinase glycogen synthase kinase-3 (GSK-3) has been identified as a negative regulator of tumor necrosis factor-α (TNF-α) signaling in hepatocytes. Thus, knocking down the β-isoform of GSK-3 in mice causes embryonic lethality due to hepatocyte hypersensitivity to TNF-α [15]. Likewise, GSK-3 inhibition with the specific inhibitor lithium sensitizes hepatocytes to TNF-α-induced apoptosis [16]. Interestingly, GSK-3 inhibitors have been reported to facilitate CD95- and TRAIL-induced apoptosis in Jurkat, prostate and colon cancer cells [17–19]. However, whether GSK-3 inhibitors may also modulate the sensitivity of untransformed and transformed hepatocytes to the death ligands CD95L and TRAIL is unknown.

The objective of the present study was to examine whether the pharmacological inhibition of GSK-3 restores the sensitivity of human hepatoma cells to CD95 and TRAIL receptor agonists. We could show that GSK-3 inhibitors sensitize HCC cells, but not untransformed hepatocytes, to TRAIL-induced apoptosis. Our findings may open up a therapeutic window for TRAIL in combination with GSK-3 inhibitors in the treatment of HCC.

2. Materials and methods

2.1. Cell culture

Human hepatoma cells (HepG2, HuH6, Hep3B and Mahlavu) were maintained in minimal essential medium (MEM) containing Earle's salts, 1% nonessential amino acids, 1 mM sodium pyruvate and 10% fetal bovine serum (Invitrogen, Carlsbad, CA). HepG2 and Hep3B cells were from the American Type Culture Collection and HuH6 and PLC/PRF5 were provided by Dr Christine Perret (Institut Cochin, Paris, France). Human hepatocytes were isolated from healthy liver tissue obtained from three different subjects undergoing partial liver resection and primary cultures were established as previously described [20] in accordance with the French legislation. After 24 h in serum-free medium, cells were treated with lithium chloride, camptothecin (Sigma–Aldrich, St Louis, MO), SB-415286 (Tocris, Ellisville, MO), recombinant human TRAIL (Bender MedSystems, Burlingame, CA), an anti-CD95 antibody (clone CH-11, Upstate, Charlottesville, VA), the general caspase inhibitor Z-VAD-fmk, the caspase-8 inhibitor Z-IETD-fmk (R&D Systems, Minneapolis, MN), SP600125 (Alexis, San Diego, CA) or AS601245 (Calbiochem, Darmstadt, Germany).

2.2. Flow cytometry analysis

Both adherent and floating cells were collected, washed, fixed in 70% ethanol at −20 °C and stained with 20 g/mL propidium iodide (Sigma–Aldrich) in the presence of 100 μg/mL ribonuclease A (Sigma–Aldrich) for 30 min at 37 °C in the dark. DNA content was analyzed by flow cytometry (FACS Calibur, Becton Dickinson, Mountain View, CA). Apoptotic cells with hypodiploid DNA staining were detected in the sub-G1 peak.

2.3. Western blotting

Whole-cell lysates were prepared as described [21]. Nuclear and cytosolic extracts were prepared by using the NE-PER™ kit (Pierce Biotechnology, Rockford, IL). Lysates containing 40 μg protein were subjected to gel electrophoresis. Proteins were then transferred to nitrocellulose membranes (GE Healthcare, Chalfont St. Giles, UK) by electroblotting for 1.5 h. Blots were blocked in 5% nonfat dry milk (Bio-Rad Laboratories, Inc., Hercules, CA) or BSA (Sigma) for 1 h, and then incubated at 4 °C overnight with antibodies against: phospho-Ser^{9}-GSK-3β, phospho-Ser^{473}-AKT, AKT, phospho-Ser^{641}-GS, cleaved caspase-8, -9 and -3, bid, phospho-Thr^{183}/Tyr^{185}-JNK, JNK, p53 (Cell Signaling Technology, Danvers, MA), GSK-3β (BD Biosciences PharMingen), TRAIL-R1 (Upstate Biotechnology), TRAIL-R2 (Imgenex, San Diego, CA), bax and anti-poly(ADP-ribose) polymerase (PARP) (Santa Cruz, Biotechnology). After incubation with horseradish peroxidase-coupled anti-IgG antibody (Cell Signaling Technology) at room temperature for 1 h, the blots were developed using enhanced chemiluminescent detection reagent (Pierce Biotechnology, Inc., Rockford, IL) and subsequently exposed to Kodak Biomax MR films (Sigma–Aldrich). Membranes were destripped using Re-Blot plus mild solution (Chemicon, Temecula, CA) and reprobed with an anti-β-actin antibody (Sigma–Aldrich) to ensure equivalent loading.

2.4. Caspase-3 assay

The activity of caspase-3 was calculated from the cleavage of a colorimetric substrate (Ac-DEVD-pNA) (BIOMOL Research Laboratories, Plymouth Meeting, PA). Cells were lyzed in 50 mM Tris–HCl (pH 7.5), 0.03% Nonidet P-40, 1 mM dithiotreitol (all from Sigma–Aldrich) and centrifuged. Assays performed in 96-well plates contained 100 μg protein, 0.2 mM Ac-DEVD-pNA in reaction buffer (100 mM HEPES pH 7.5, 10% sucrose, 0.1% CHAPS, 10 mM dithiotreitol, Sigma–Aldrich). Assays were performed at 37 °C and release of pNa was detected by reading absorbance at 405 nm from 0 to 5 h to mark the linearity of the enzymatic reaction in time. Enzyme activities were measured as initial velocities.

2.5. Down-regulation of p53 expression using a siRNA strategy

p53 expression was down-regulated by using a siRNA synthesized by Ambion (Austin, TX) which targets the following sequence 5′-GCAUGAACCG-GAGGCCCAUTT-3′ in the p53 mRNA as previously reported [22]. A siRNA of irrelevant sequence was used a control (Ambion). HepG2 cells plated in 6-well plates (3×10^5 cells/well) were cultured for 24 h to 40% confluency and then transfected with 75 nM siRNA using LipofectAMINE 2000 (InVitrogen Corp.) according to the manufacturer's instructions. An adenovirus Ad5GFPCMV encoding GFP (gift from Dr Bernard Klonjkowski, ENVA, Maisons-Alfort, France), at a moi of 10 pfu/cell, was added 1 h later to the siRNA/LIPOFECTAMINE complexes to enhance transfection efficiency as previously reported in adenofection experiments [23]. 24 h later, cells were washed with PBS and stimulated for a further 24 h with or without TRAIL and/or lithium before being harvested for protein extraction or analyzed for caspase-3 activity.

2.6. Statistical analysis

Blots were quantified using Genetools software (Syngene, Cambridge, UK). Results are means ± S.E.M. Mean values were compared using the Student's *t* test.

3. Results

3.1. Effects of GSK-3 inhibitors on TRAIL- and CH-11-induced apoptosis in human hepatoma cells and primary hepatocytes

The effects of two widely used GSK-3 inhibitors, lithium and SB-415286, were examined in HepG2 cells in which apoptosis was induced by TRAIL or CH-11 (a CD95 agonistic antibody) and evaluated by flow cytometry analysis of sub-G1 cells. Lithium and SB-415286 were used at 20 mM and 25 μM, respectively which are concentrations frequently used for *in vitro* experiments. These concentrations are higher than the IC_{50} (determined in *in vitro* kinase assays using recombinant GSK3) [24,25] to obtain effective intracellular concentrations in intact cells. After 24 h, the combination of lithium or SB-415286 with a low dose of TRAIL (25 ng/mL) massively increased the proportion of sub-G1 cells (from 4.3 ± 0.7% to 61.7 ± 8.2% and 69.6 ± 6%, respectively) while TRAIL alone increased cell death to 26.8 ± 9% (Fig. 1A). With a low concentration of CH-11, only CH-11 combined with SB-415286 induced a significant increase in the number of sub-G1 cells (20.9 ± 1.2%) (Fig. 1A). However, with a higher dose (250 ng/mL), the proportion of sub-G1 cells reached 34.3 ± 5.4% and 43.7 ± 6.5% with lithium and SB-415286, respectively, whereas CH-11 alone increased cell death to 18 ± 3.4% (Fig. 1A). Lithium and SB-415286 had no effect alone. These findings indicated that GSK-3 inhibitors sensitized HepG2 cells to the cytotoxic effects of TRAIL and CH-11. The pan-caspase inhibitor z-VAD-fmk prevented the sensitizing effects of lithium (Fig. 1B) and SB-415286 (data not shown), providing evidence that both responses involved apoptotic mechanisms.

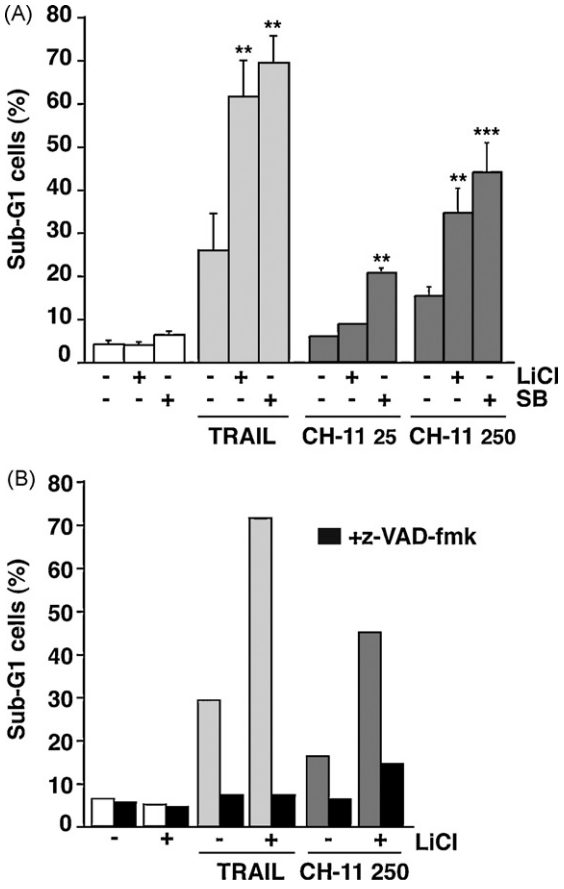

Fig. 1 – Lithium and SB-415286 potentiate TRAIL- and CH-11-induced apoptosis in HepG2 cells. (A) HepG2 cells were treated for 24 h with or without TRAIL (25 ng/mL), an agonistic anti-CD95 antibody (CH-11, 25 and 250 ng/mL), lithium (LiCl, 20 mM) and/or SB-415286 (SB, 25 μM) and analyzed for the sub-G_1 cell population by flow cytometry. Results are means ± S.E.M. of at least three independent experiments. (B) HepG2 cells were treated for 24 h with or without TRAIL (25 ng/mL), CH-11 (250 ng/mL), lithium (20 mM) and/or a pan-caspase inhibitor z-VAD-fmk (20 μM) and analyzed for the sub-G_1 cell population by flow cytometry. Results are means of two independent experiments. **, $p < 0.02$; ***, $p < 0.01$ compared with cells treated with the death receptor agonist alone.

We next examined whether similar effects would be found in other human hepatoma cell lines or in non-tumoral hepatocytes. HuH6, Mahlavu and Hep3B cells were resistant to 25 ng/mL TRAIL but their resistances were overcome by combining TRAIL with lithium or SB-415286 (Fig. 2A). Lithium and SB-415286 also sensitized HuH6 and Mahlavu cells to the cytotoxic effect of 25 ng/mL CH-11 but they were ineffective in Hep3B cells that are deficient in CD95 receptor [26]. In primary human hepatocytes, CH-11 induced apoptosis (from 7.3 ± 0.9% to 19.0 ± 3.2%) while TRAIL had no effect (Fig. 2B) which is consistent with earlier studies [27–29]. Lithium and SB-415286 alone were nontoxic but they significantly increased CH-11-induced apoptosis (by 2.1 and 1.5 fold, respectively) while they did not overcome hepatocyte resistance to TRAIL.

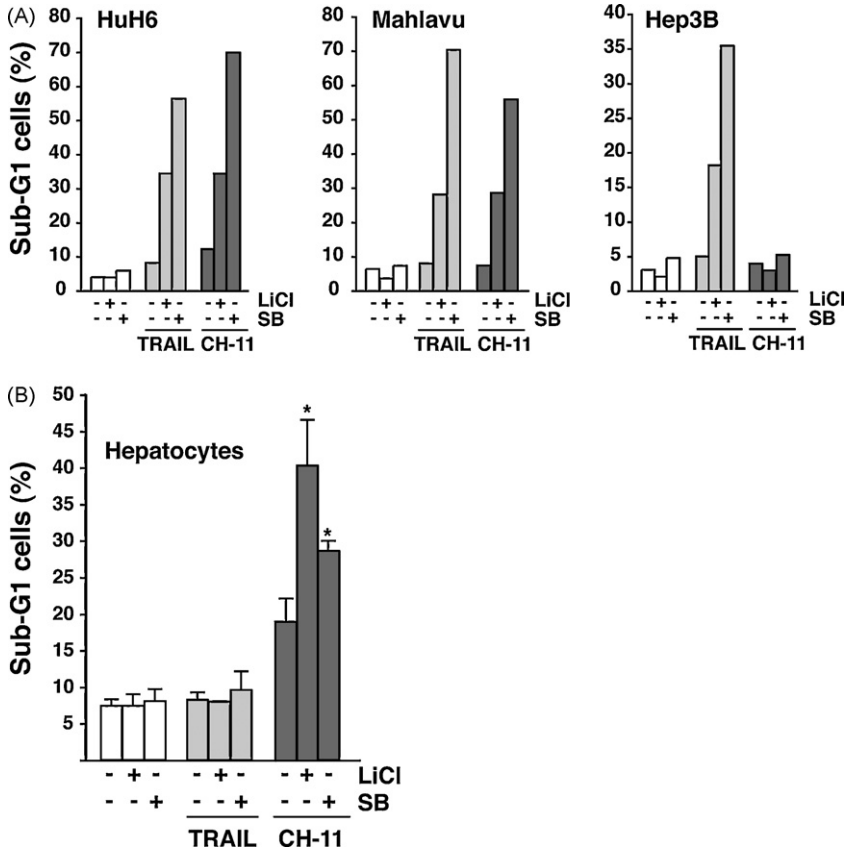

Fig. 2 – Effects of GSK-3 inhibitors on TRAIL- and CH-11-induced apoptosis in hepatoma cell lines and normal human hepatocytes. HuH6, Mahlavu and Hep3B cell lines (A) and normal human hepatocytes (B) were treated for 24 h with or without TRAIL (25 ng/mL), CH-11 (25 ng/mL), lithium (LiCl, 20 mM) and/or SB-415286 (SB, 25 μM) and analyzed for the sub-G_1 cell population by flow cytometry. Results are means of two (A) to three (B) independent experiments. *, $p < 0.05$ compared with cells treated with CH-11 alone.

Taken together, these findings showed that GSK-3 inhibitors enhanced CD95-dependent apoptosis in non-tumoral healthy hepatocytes as well as in hepatoma cell lines while they sensitized HCC cells, but not normal hepatocytes, to TRAIL. This is of particular interest, when considering the possible use of GSK-3 inhibitors in combination with TRAIL for the treatment of HCC.

3.2. *The phosphorylations of AKT and GSK-3 are not modified during TRAIL sensitization by GSK-3 inhibitors*

In order to address whether GSK-3 activity was modulated during the combined treatment of TRAIL with lithium or SB-415286, we examined the phosphorylation level of the inhibitory serine 9 residue of GSK-3β isoform which correlates with the inactivation level of the enzyme as well as the phosphorylation level of glycogen synthase (GS), a direct substrate of GSK-3. Whole-cell extracts were obtained from HepG2 cells treated for 3 and 6 h with lithium, SB-415286 and/or TRAIL. Lithium and SB-415286 act through distinct mechanisms to inhibit GSK-3. Thus, lithium is a Mg^{2+} competitive inhibitor and increases the serine 9 phosphorylation of GSK-3 [30]. SB-415286 acts as an ATP competitive inhibitor and does not affect GSK-3 phosphorylation [25]. Accordingly, we observed that lithium but not SB-415286 increased GSK-3β^{Ser9} phosphorylation in HepG2 cells (Fig. 3). Both GSK-3 inhibitors decreased GS phosphorylation indicating that GSK-3 was effectively inhibited under these conditions. Upon treatment with TRAIL, the phosphorylation states of GSK-3 and of GS were not further modified. Since the down-regulation of AKT phosphorylation and/or expression has been reported to result in increased TRAIL sensitivity in cancer cells [31–33], we also examined the status of AKT. No modification of AKT phosphorylation and expression was observed in any tested conditions (Fig. 3). Altogether, these findings indicated that the potentiation of TRAIL-induced apoptosis by GSK-3 inhibitors occurred without modification of AKT and GSK-3 phosphorylation and expression.

3.3. *The sensitizing effect of GSK-3 inhibitors requires the enhancement of caspase-8 activity*

Next, we examined the molecular mechanisms underlying the effects of GSK-3 inhibitors on TRAIL-induced cell death in HepG2 cells. Because up-regulation of TRAIL receptor expression contributes to TRAIL sensitization by conventional

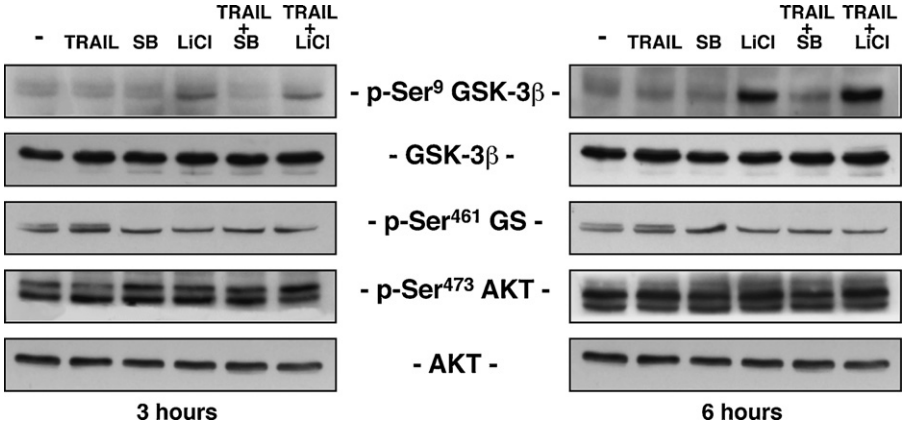

Fig. 3 – The phosphorylation and expression levels of GSK-3β and AKT are not modified during TRAIL sensitization by GSK-3 inhibitors. HepG2 cells were treated for 3 and 6 h with or without TRAIL (25 ng/mL), lithium (LiCl, 20 mM) and/or SB-415286 (SB, 25 μM). Whole-cell extracts were analyzed for GSK-3β, GS, and AKT phosphorylation and/or expression by Western blot analysis. Blots are representative of three independent experiments.

chemotherapeutic drugs, irradiation or proteasome inhibitors [34,35], we tested the effect of GSK-3 inhibitors on TRAIL-R1 and TRAIL-R2 levels. As reported [27], three isoforms of TRAIL-R2 were detected in HepG2 cells: one intracellular p60 form and two membrane p43 and p49 forms (Fig. 4A). Upon treatment with lithium or SB-415286 for 24 h (Fig. 4A), there was no increase in TRAIL-R1 and TRAIL-R2 levels. Similarly, TRAIL alone or combined with GSK-3 inhibitors did not affect the levels of TRAIL receptors (data not shown), indicating that TRAIL sensitization by GSK-3 inhibitors did not involve the up-regulation of TRAIL receptors.

We next focused on the intracellular signaling pathways triggered by TRAIL and examined whether the sensitizing effect of GSK-3 inhibitors routed at a receptor proximal level through the activation of the initiator caspase-8. Pre-treatment with the caspase-8 inhibitor z-IETD-fmk abolished apoptosis induced by TRAIL combined with lithium or SB-415286 (Fig. 4B) indicating the requirement of caspase-8 activation for the sensitizing effect of GSK-3 inhibitors. The kinetics of cleaved caspase-8 showed that caspase-8 cleavage was clearly increased after 3 h of treatment with TRAIL *plus* lithium or *plus* SB-415286 (Fig. 4C) while no cleavage was detected with TRAIL (Fig. 4D), lithium or SB-415286 alone (data not shown). The combined treatments also induced the activation of the effector caspase-3 and the cleavage of its substrate PARP (Fig. 4C). The sensitizing effect of GSK-3 inhibitors involved the mitochondria pathway since the combined treatments promoted bid cleavage, bax disappearance from cytosol (suggesting its translocation to the mitochondria) (Fig. 4E) and caspase-9 activation (Fig. 4C). Consistent with the absence of a pro-apoptotic effect in normal hepatocytes (Fig. 2B), the combined treatments did not promote PARP cleavage in these cells (Fig. 4F). Altogether, these data suggested that GSK-3 exerted a negative constraint on the apoptotic cascade initiated by TRAIL in hepatoma cells and that GSK-3 inhibitors had the potential to release this constraint.

3.4. *p53 participates to the sensitizing effect of GSK-3 inhibitors on TRAIL-induced apoptosis*

The activation of the tumor suppressor p53 is a frequent event of apoptotic cascades and recent evidence indicates that p53 may promote TRAIL-induced apoptosis [36–39]. Since GSK-3 regulates p53 abundance and activity [40–42], we next addressed the contribution of p53 to TRAIL sensitization by GSK-3 inhibitors. Lithium and SB-415286 alone increased total and nuclear levels of p53 and these effects were maintained in the presence of TRAIL (Fig. 4A). TRAIL alone had no effect on p53 expression (Fig. 5A). Altogether, these findings indicated that, under conditions where GSK-3 inhibitors sensitized HepG2 cells to TRAIL-induced apoptosis, the amount of p53 protein was increased.

A siRNA strategy was used to examine p53 contribution in cell sensitization to TRAIL-induced apoptosis by GSK-3 inhibitors. To have a sensitive measurement of apoptosis, caspase-3 activity was evaluated using a colorimetric assay. The transient transfection of a p53 siRNA (but not of an unrelated siRNA) led to efficient down-regulation of p53, in the presence or absence of TRAIL and/or lithium (Fig. 5B). Importantly, while the p53 siRNA had little effect on caspase-3 activity by its own or in the presence of TRAIL, it significantly decreased caspase-3 activation induced by TRAIL combined with lithium (Fig. 5C), indicating that p53 participated to the sensitizing effect of GSK-3 inhibitors on TRAIL-induced apoptosis.

3.5. *c-Jun N-terminal kinases (JNKs) restrict the sensitizing effect of GSK-3 inhibitors on TRAIL-induced apoptosis*

TRAIL sensitization in response to different signals occurs through JNK activation in cancer cells [43–46]. As GSK-3 may regulate JNK activity [47–49], we then asked whether JNKs played a role in the sensitizing effect of GSK-3 inhibitors. TRAIL combined with lithium or SB-415286 massively

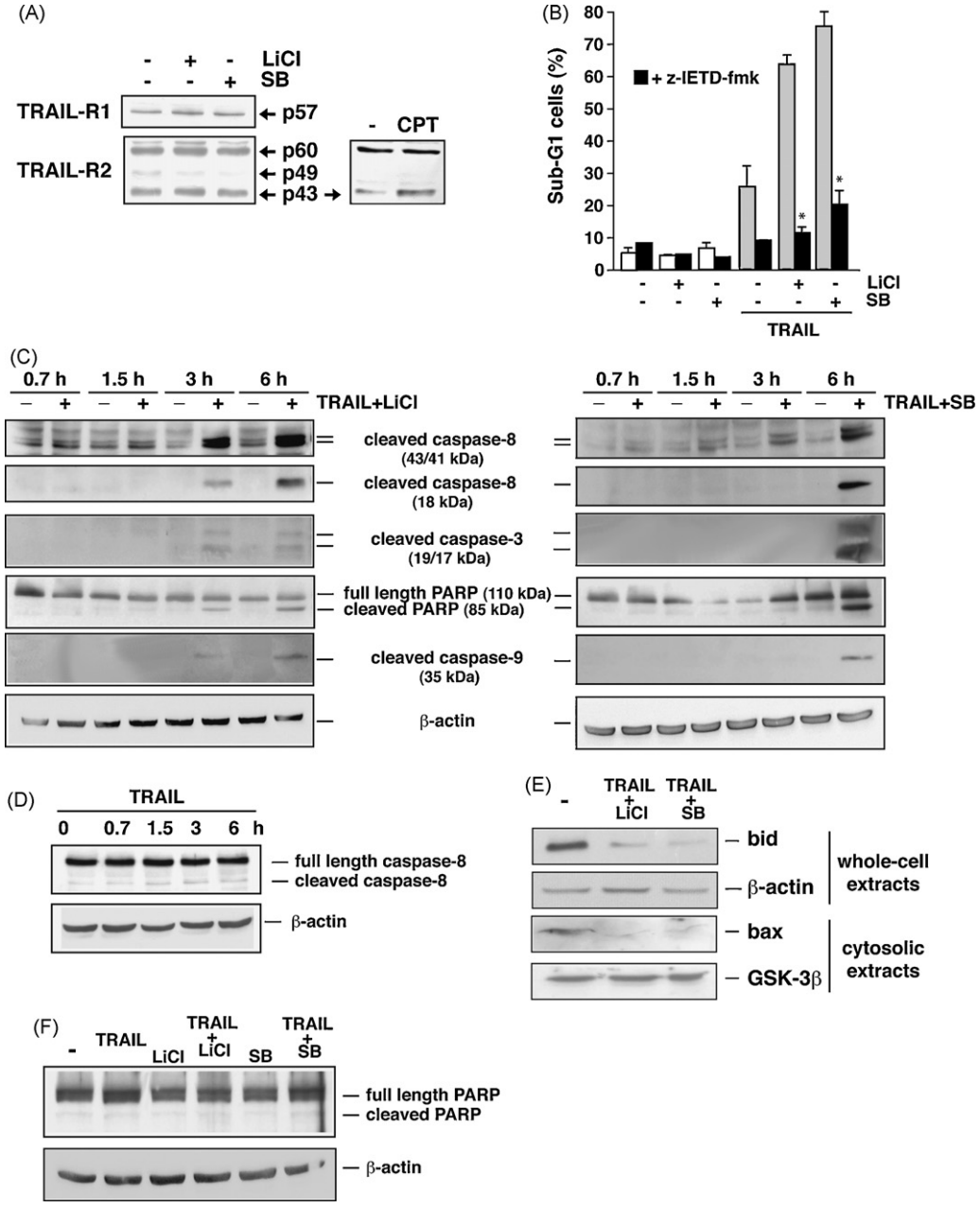

Fig. 4 – The sensitizing effect of GSK-3 inhibitors on TRAIL-induced apoptosis requires enhancement of caspase-8 activity in HepG2 cells. (A) HepG2 cells were treated for 24 h with or without lithium (LiCl, 20 mM) or SB-415286 (SB, 25 μM). Whole-cell extracts (40 μg) were analyzed for TRAIL-R1 and TRAIL-R2 expression by Western blot analysis. Cells treated with camptothecin (CPT, 0.5 μM, 12 h) were used as a positive control for TRAIL-R2 up-regulation [69]. (B) HepG2 cells were treated for 24 h with or without TRAIL (25 ng/mL), lithium (LiCl, 20 mM), SB-415286 (SB, 25 μM) and/or the caspase-8 inhibitor z-IETD-fmk (20 μM) and analyzed for sub-G_1 cells by flow cytometry. (C and D) HepG2 cells were treated for 0.7, 1.5, 3 and 6 h with or without TRAIL (25 ng/mL), lithium (20 mM) and/or SB-415286 (SB, 25 μM). Whole-cell extracts (40 μg) were analyzed for caspase-8, -3, -9 and PARP cleavage by Western blot analysis. (E) HepG2 cells were treated for 6 h with or without TRAIL (25 ng/mL), lithium (LiCl, 20 mM) and/or SB-415286 (SB, 25 μM). Whole-cell extracts were analyzed for bid expression and cytosolic extracts were analyzed for bax expression by Western blot analysis. Reprobing with β-actin and GSK-3β antibodies was performed to ensure equivalent loading. (F) Human normal hepatocytes were treated for 6 h with or without TRAIL (25 ng/mL), lithium (20 mM) and/or SB-415286 (SB, 25 μM). Whole-cell extracts (40 μg) were analyzed for PARP cleavage by Western blot analysis. Blots are representative of three independent experiments and quantitative values are means ± S.E.M. of three independent experiments. *, $p < 0.01$ compared to cells treated with TRAIL/lithium or TRAIL/SB-415286.

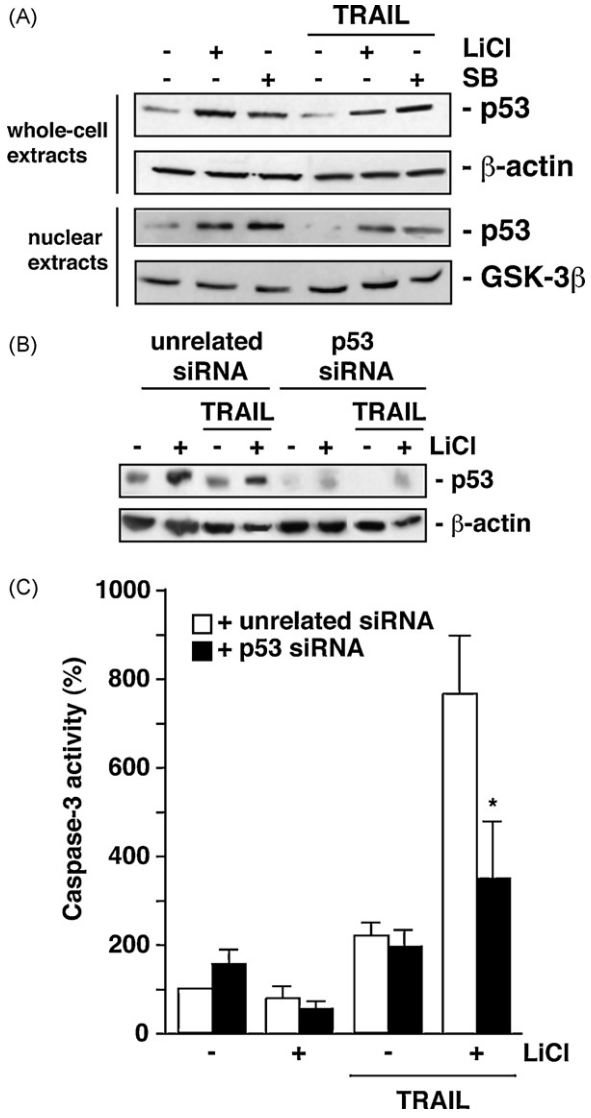

Fig. 5 – p53 is required for the sensitizing effect of GSK-3 inhibitors on TRAIL-induced apoptosis in HepG2 cells. (A) HepG2 cells were treated for 12 h with or without TRAIL (25 ng/mL), lithium (LiCl, 20 mM) and/or SB-415286 (25 μM). Whole-cell (upper) and nuclear (lower) extracts were analyzed for p53 expression by Western blotting. HepG2 cells were transfected with a p53 or an unrelated siRNA and treated for 24 h with or without TRAIL (25 ng/mL) and/or lithium (LiCl, 20 mM) and analyzed for p53 expression by Western blotting (B) and for caspase-3 activity by a colorimetric assay (C). Representative blots of three independent experiments are shown. Results are means ± S.E.M. of three independent experiments. *, $p < 0.05$ compared to cells transfected with the unrelated siRNA. Basal caspase-3 activity was 0.002 OD/min/μg protein.

increased JNK phosphorylation without modifying JNK expression (Fig. 6A). JNK activation was detected at an early time-point (0.7 h) (Fig. 6A, upper) and was maintained for at least 17 h (Fig. 6A, lower). JNKs were not activated by TRAIL alone (Fig. 6A, lower and data not shown). JNK activity was also increased by lithium and SB-415286 alone but JNK activation was greatest with the combinatorial treatments (Fig. 6A, lower and Fig. 6B).

To define the role of JNK activation in the sensitizing effect of GSK-3 inhibitors, two chemical inhibitors of JNKs, AS601245 [50] and SP600125 [51], were used. A massive apoptosis was observed in the presence of AS601245 after a 24-h treatment with lithium/TRAIL or SB-415286/TRAIL (Fig. 7A, left) which was significantly more important than in cells treated with lithium/TRAIL or SB-415286/TRAIL. At an earlier time-point (17 h), AS601245 or SP600125 treatment also resulted in a significant and strongest induction of apoptosis. JNK inhibitors alone had no effect on apoptosis and did not modify cell response to lithium or SB-415286. Evaluation of PARP cleavage after a 6-h treatment confirmed that JNK inhibitors enhanced apoptotic signaling induced by lithium/TRAIL or SB-415286/TRAIL (Fig. 7B). Altogether, these findings showed that GSK-3 inhibitors activated JNKs and that this activation limited the initiation and magnitude of apoptosis induced by lithium/TRAIL or SB-415286/TRAIL.

4. Discussion

We show in the present study that GSK-3 inhibitors overcome the inherent resistance of human hepatoma cells towards TRAIL-induced apoptosis. In HepG2 cells, the sensitizing effect of GSK-3 inhibitors was complex since it involved the promotion of apoptotic signals (caspase-8, caspase-3 and p53 activation) and of protective signals (JNK activation), which limited the magnitude of apoptosis. This suggests that hepatoma cells present compensatory anti-apoptotic signaling pathways, functioning in a network that may contribute to TRAIL resistance (Fig. 6C).

TRAIL has emerged as a promising anti-cancer agent since it selectively induces apoptosis in a wide range of tumor cells without causing toxicity to normal cells [52]. Accordingly, soluble untagged recombinant TRAIL is without any toxicity for hepatocytes [27,28,29 and this study] and agonistic monoclonal antibodies to the TRAIL receptors which are in phase II clinical trials are without hepatotoxicity [53]. However, TRAIL therapy is limited in HCC since transformed hepatocytes acquire multiple mechanisms to avoid TRAIL-induced apoptosis, including up-regulation of cFLIP [11,12], Bcl-xL [13] and survivin [14]. Therefore, promoting the efficiency of TRAIL in HCC could offer a new therapeutic perspective for this cancer.

We show that GSK-3 inhibitors strongly enhance TRAIL-induced apoptosis in hepatoma cells (even in HepG2 and Hep3B cells which overexpressed c-FLIP [11,12], survivin [14] and/or Bcl-xL [13]) without affecting primary hepatocytes. The sensitizing effect of GSK-3 inhibitors was not restricted to TRAIL signaling as these drugs also potentiated CD95 agonist signaling. The therapeutic potential of GSK-3 inhibitors in HCC may be tempered with this observation. Indeed, in HCC patients presenting with chronic inflammation (i.e., with chronic hepatitis), increased expression of FasL by activated cytotoxic T lymphocytes and natural killer cells has been reported [54]. Therefore GSK-3 inhibitors could enhance Fas

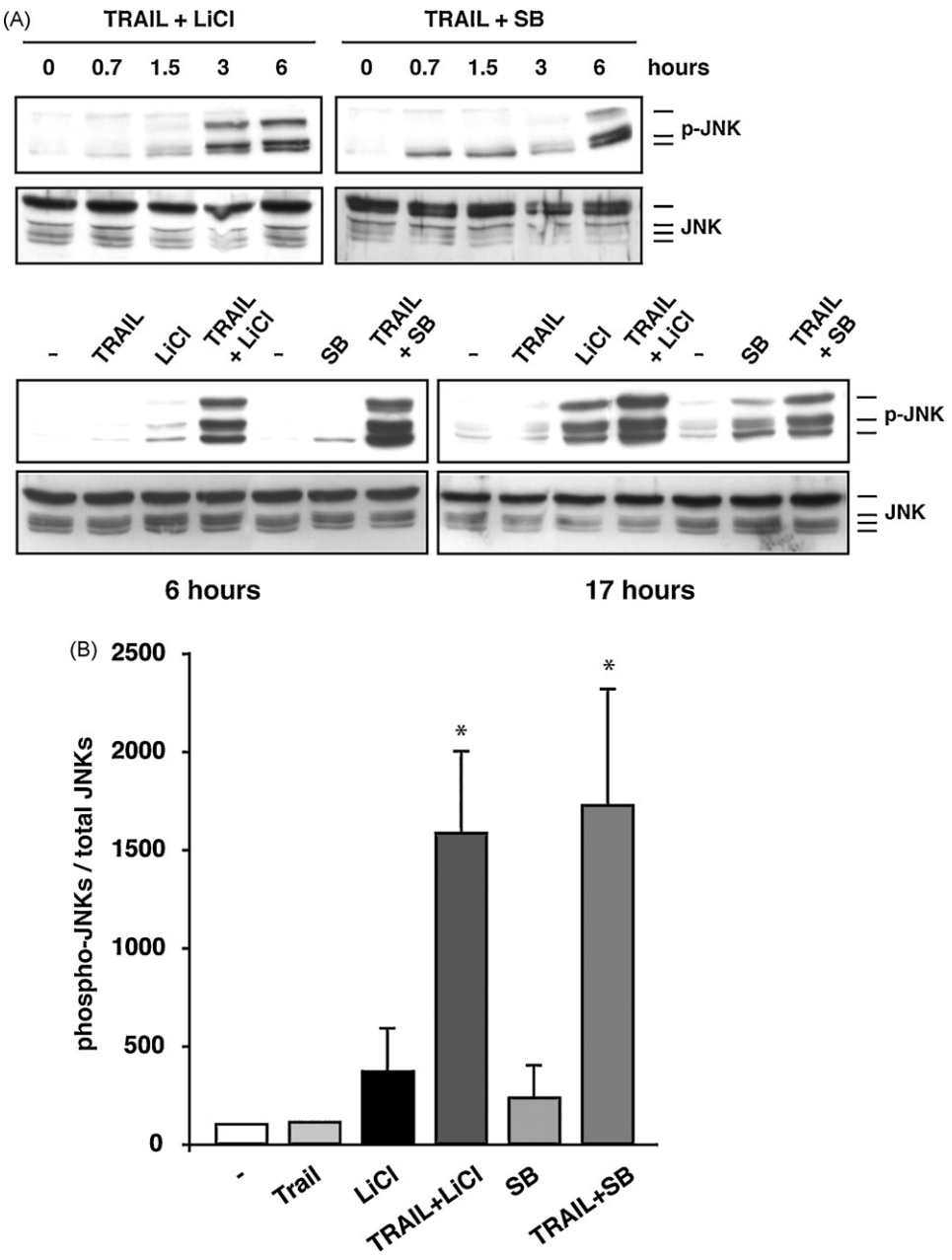

Fig. 6 – JNKs are activated during TRAIL sensitization by GSK-3 inhibitors in HepG2 cells. (A) HepG2 cells were treated up to 17 h with or without TRAIL (25 ng/mL), lithium (LiCl, 20 mM), and/or SB-415286 (SB, 25 μM). Whole-cell extracts (40 μg) were analyzed for phosphorylated and total levels of JNKs by Western blot analysis. (B) A densitometric analysis of the blots obtained after a 6-h treatment was performed and the ratio phosphorylated form/total form of JNKs was calculated. Quantitative values are means ± S.E.M. of three independent experiments. *, $p < 0.02$ compared with cells treated with a GSK-3 inhibitor alone.

signaling in such patients. However, it is not excluded that some benefits could be also obtained from GSK-3 inhibitors since anti-inflammatory properties of GSK-3 inhibitors have been described [55,56]. Pre-clinical *in vivo* studies are currently undertaken to address this important issue.

Some studies [34,35] but not all [11,57] have reported an up-regulation of TRAIL receptors as a mechanism for increasing TRAIL cytotoxicity. Our results exclude such a mechanism during TRAIL sensitization by GSK-3 inhibitors in HCC cells. Rather, they point towards a mechanism of sensitization that takes place intracellularly. Indeed, a caspase-8 inhibitor abolished the potentiation of TRAIL-induced apoptosis by GSK-3 inhibitors and GSK-3 inhibitors enhanced TRAIL-induced caspase-8 activation, which is consistent with a previous report [18] showing that caspase-8 activity increased after co-treatment of prostate cancer cells with TRAIL and SB-216763, another GSK-3 inhibitor. Hepatocyte-derived cells are type II cells in which TRAIL-induced DISC formation is limited

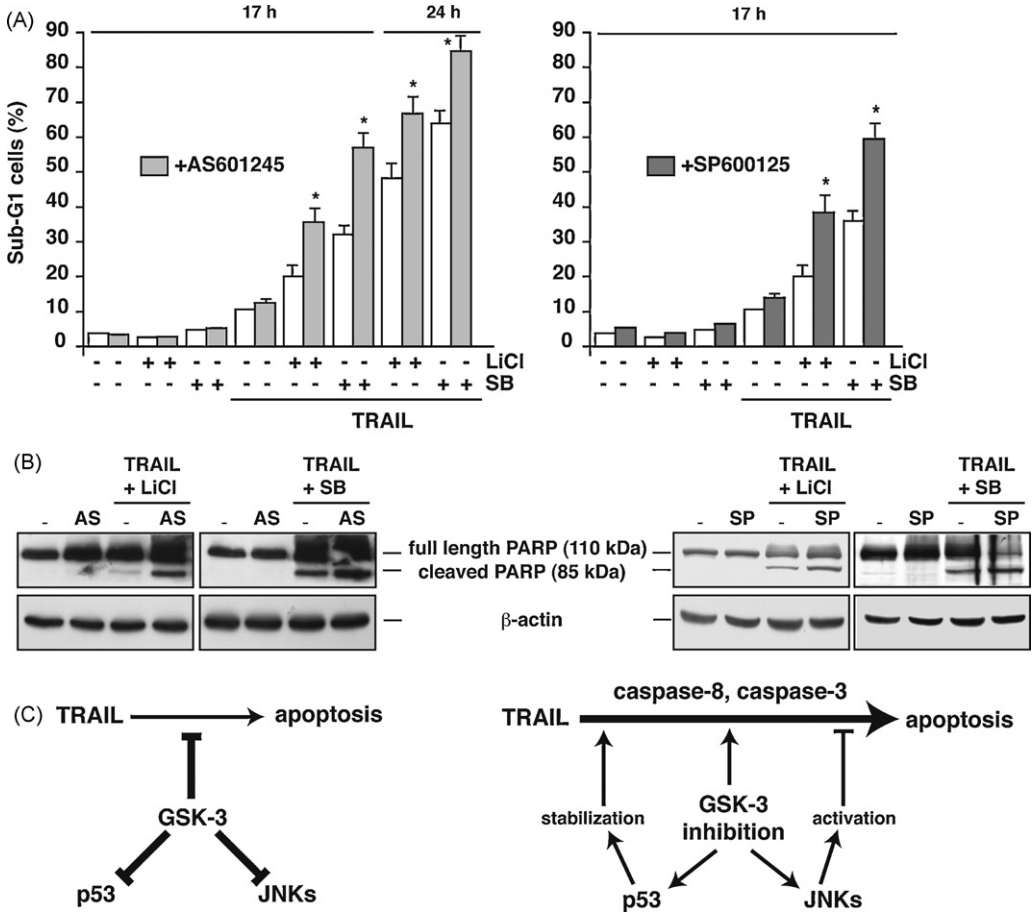

Fig. 7 – JNK inhibition increases the sensitizing effect of GSK-3 inhibitors on TRAIL-induced apoptosis in HepG2 cells. HepG2 cells were treated for 6, 17 and 24 h with or without TRAIL (25 ng/mL), lithium (LiCl, 20 mM), SB-415286 (SB, 25 μM) and/or a JNK inhibitor AS601245 (AS, 2 μM) or SP600125 (SP, 5 μM) and analyzed for sub-G_1 cells by flow cytometry (A) and for PARP cleavage by Western blot analysis (B). Blots are representative of two independent experiments and results are means ± S.E.M. of three independent experiments. *, $p < 0.05$ compared with cells treated with TRAIL and a GSK-3 inhibitor. (C) Schematic representation of the mechanisms by which GSK-3 may modulate TRAIL apoptotic signaling in HepG2 cells. GSK-3 exerts a negative constraint on TRAIL signaling by limiting caspase-8 activation and downstream signaling. GSK-3 also represses p53 expression and JNK activity. In the presence of a GSK-3 inhibitor, these constraints are alleviated; p53 stabilization may participate to TRAIL sensitization while JNKs exert an anti-apoptotic balance to the pro-apoptotic effect.

and the mitochondria-based apoptosis system is recruited to amplify the death signal [58]. Accordingly, GSK-3 inhibitors exacerbated mitochondrial recruitment after TRAIL treatment, by amplifying bid cleavage, bax translocation to the mitochondria and caspase-9 activation.

Our study as well as those previously conducted in other cancer cell types [17–19] well-establish that GSK-3 inhibition has robust pro-apoptotic actions on the extrinsic signaling pathways initiated by death receptors. However, the molecular components initiating this potentiation remain to be elucidated. It seems that the pro-apoptotic effect of GSK-3 inhibition occurs at least at a very early stage in death-receptor-induced signaling, at the level of caspase-8. In this way, GSK-3 inhibitors could increase DISC formation and/or down-regulate the expression of c-FLIP. It seems also that the balance between GSK-3 isoforms is important in regulating the extrinsic apoptosis signaling pathway. Thus, the selective knock-down of GSK-3β, but not of GSK-3α, by RNA interference potentiated TRAIL-induced apoptosis in prostate cancer cells [18,19]. While the involvement of NF-κB in the sensitizing effects of GSK-3 inhibitors on TRAIL signaling has been excluded by others [18], we show that this process may implicated the tumor suppressor p53. This finding reinforces the notion that p53 may contribute to the sensitization of cancer cells to TRAIL-mediated apoptosis [36–39]. This also confirms the close relationship between GSK-3 and p53 showing that GSK-3 regulates p53 abundance [40,41]. However, p53 recruitment is not an absolute requisite for TRAIL sensitization since TRAIL-resistant cells which lack a functional p53 (i.e., Hep3B and Mahlavu cells) could be primed to TRAIL-induced apoptosis by GSK-3 inhibitors.

JNKs are central transducers of apoptotic death in both non-transformed and transformed hepatocyte in response to TNF-α, bile acids, troglitazone and acetaminophen [59–62].

However, JNKs may also mediate anti-apoptotic signals in these cells. For example, mice lacking JNK kinase SEK1 exhibit liver defects with apoptosis during embryonal development [63]. In addition, JNK inhibition increases apoptosis induced by TNF-α *plus* cycloheximide or by the tumor suppressor WWOX in hepatoma cells [64,65]. Consistent with these later studies, we observed that GSK-3 inhibitors activated JNKs in hepatoma cells which negatively regulated TRAIL-induced apoptosis by modulating the strength and the time-course and thus provided an anti-apoptotic balance to the pro-apoptotic effect of GSK-3 inhibitors on TRAIL signaling. These findings also indicate that JNKs are under the negative control of GSK-3. These findings are in line with previous reports showing JNK activation by lithium in neurons [48] or in GSK-3β knockout embryonic fibroblasts [49]. In hepatoma cells, JNK activation was not related to caspase activation since it occurred at an earlier time-point. In addition, modulation of JNK activity was not cell line specific, as similar results were observed in Hep3B cells (data not shown). Altogether, our findings reinforce the concept that GSK-3 sets the threshold for apoptosis induction by regulating concomitantly pro- and anti-apoptotic signals [66].

In summary, we demonstrate that GSK-3 inhibitors sensitize hepatoma cells but not normal hepatocytes to TRAIL-induced apoptosis through the induction of caspase-8 activity in cooperation with an enhancement of the mitochondrial pathway. The difference between sensitization of HCC cells, on one hand, and resistance of normal hepatocytes, on the other hand, may open up a new therapeutic window for TRAIL in combination with GSK-3 inhibitors in the treatment of HCC. The applicability of such a combination to preclinical or even clinical settings is simplified by the fact that small GSK-3 inhibitors are currently under development for other indications, including diabetes, neurodegenerative diseases, and bipolar disorder [67,68].

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