Salirasib sensitizes hepatocarcinoma cells to TRAIL-induced apoptosis through DR5 and survivin-dependent mechanisms

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Ras activation is a frequent event in human hepatocarcinoma that may contribute to resistance towards apoptosis. Salirasib is a ras and mTOR inhibitor that induces a pro-apoptotic phenotype in human hepatocarcinoma cell lines. In this work, we evaluate whether salirasib sensitizes those cells to TRAIL-induced apoptosis. Cell viability, cell death and apoptosis were evaluated in vitro in HepG2, Hep3B and Huh7 cells treated with DMSO, salirasib and YM155 (a survivin inhibitor), alone or in combination with recombinant TRAIL. Our results show that pretreatment with salirasib sensitized human hepatocarcinoma cell lines, but not normal human hepatocytes, to TRAIL-induced apoptosis. Indeed, FACS analysis showed that 25% (Huh7) to 50% (HepG2 and Hep3B) percent of the cells treated with both drugs were apoptotic. This occurred through activation of the extrinsic and the intrinsic pathways, as evidenced by a marked increase in caspase 3/7 (five to ninefold), caspase 8 (four to sevenfold) and caspase 9 (eight to 12-fold) activities in cells treated with salirasib and TRAIL compared with control. Survivin inhibition had an important role in this process and was sufficient to sensitize hepatocarcinoma cells to apoptosis. Furthermore, TRAIL-induced apoptosis in HCC cells pretreated with salirasib was dependent on activation of death receptor (DR) 5. In conclusion, salirasib sensitizes hepatocarcinoma cells to TRAIL-induced apoptosis by a mechanism involving the DR5 receptor and survivin inhibition. These results in human hepatocarcinoma cell lines and primary hepatocytes provide a rationale for testing the combination of salirasib and TRAIL agonists in human hepatocarcinoma.

Cell Death and Disease (2013) 4, e471; doi:10.1038/cddis.2012.200; published online 24 January 2013

Subject Category: Cancer

Hepatocellular carcinoma (HCC) is the third leading cause of cancer related mortality, and its incidence is rising. Only a minority of the patients can be offered a curative treatment, and recurrence affects the majority of those patients within a few years. Accordingly, new therapeutic approaches for this dreadful disease are needed.

Loss of sensitivity to apoptosis is a hallmark of cancer. In HCC, this occurs through several mechanisms including resistance to apoptosis mediated by death receptors (DR’s). Among the DR’s ligands, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is considered as a promising candidate for cancer treatment, as it is able to selectively induce apoptosis in transformed cells without affecting normal cells. This has led to the development and clinical evaluation of recombinant human TRAIL and of monoclonal agonist antibodies directed against DR4 and DR5, the two receptors responsible for TRAIL-induced cell death. However, tumors cells, including HCC cells, may be or become resistant to TRAIL-induced apoptosis. This occurs through downregulation of DR’s and adapter molecules responsible for downstream caspase activation, or through increased expression of antiapoptotic factors.

Ras activation has been shown to be an ubiquitous and early event in human HCC, whereas mTOR activation is present in half of the cases. Activation of both ras and mTOR has been implicated in resistance of HCC cells to apoptosis. Furthermore, a recent study suggests an association between an activating K-ras mutation and resistance to TRAIL-induced apoptosis in human pancreatic and lung cancer cell lines.

Salirasib (farnesylthiosalicylic acid, FTS) is an S-farnesyl cysteine analog that interfere with docking of active GTP-bound ras at the cell membrane. FTS competes with active ras for its membrane anchorage sites thereby impairing ras down-stream signaling leading to inhibition of ras-dependent cell growth. It exhibits antitumoral effects in several cancer cell lines and has recently been evaluated in a phase 1 study in patients with solid tumors, showing that it was well tolerated.

Previous work of our team has shown that high dose salirasib blocks hepatocytes proliferation in vivo in rats after partial hepatectomy. We have also shown that its administration model of diethylnitosamine-induced hepatocarcinogenesis in rats prevents liver tumor development by...
apoptosis induction in preneoplastic foci, predominantly through the DR’s pathway while it redirects the proliferation balance from transformed hepatocytes to non-transformed cells. More recently, we have found that salirasib reduces the growth of human HCC cell lines both in vitro and in a xenograft model. The growth inhibitory effect was mainly linked to an inhibition of cell proliferation. However, salirasib also induced a proapoptotic drift, with an increased expression of DR’s and a reduced expression of the apoptosis inhibitors survivin and cFLIP. Hypothesizing that salirasib does not only inhibit cell proliferation but also prepares cells to undergo apoptosis we determined whether salirasib would sensitize human HCC cell lines to TRAIL-induced apoptosis. We further attempted to better understand the molecular mechanism involved in the observed effect.

Results

Salirasib sensitizes HCC cells to TRAIL-induced cell death

Concomitant administration of TRAIL and salirasib. In a first set of experiments, cells were incubated in culture medium supplemented with DMSO, 75 μM Salirasib, or 150 μM Salirasib, with or without TRAIL (0, 10, 50 or 100 ng/ml) for 24 h. Cell viability was assessed by a WST-1 assay (Figure 1a). TRAIL alone induced a dose-dependent decrease in cell viability in HepG2 cells, while it had no impact on Hep3B and HuH7 cells viability. Salirasib alone reduced cell viability in all three cell lines at a dose of 150 μM, but not at 75 μM. Concomitant treatment of TRAIL and salirasib did not result in a significant further reduction in cell viability in the three tested cell lines.

Administration of TRAIL in salirasib pretreated cells. In the next set of experiments, cells were preincubated with DMSO or salirasib alone for 24 h and then TRAIL was added or not for an additional 24 h (Figure 1b). These experiments confirmed that TRAIL alone induced a dose-dependent reduction of cell viability in HepG2 cells, whereas it had no effect on Hep3B and HuH7 cells.

Treatment of cells with salirasib alone for 48 h reduced cell viability in the three tested cell lines in a dose-dependent manner, dropping to 50% for 150 μM compared with untreated controls (DMSO).

Importantly, in HepG2 cells, addition of TRAIL to cells pretreated with salirasib for 24 h further reduced cell viability, and the resulting dose-response curve was steeper than the curve obtained with TRAIL alone, suggesting a synergistic effect of the combination of salirasib and TRAIL. Furthermore, pretreatment with salirasib 150 μM sensitized Hep3B and HuH7 cells to TRAIL: indeed, addition of TRAIL to salirasib-conditioned cells further reduced cell viability, and this in a dose-dependent manner. By contrast, addition of TRAIL to cells pretreated with 75 μM of salirasib for 24 h did not further reduce cell viability in any of the tested cell lines.

On the basis of these experiments, we chose the experimental conditions where no or 50 ng/ml of TRAIL were added to the culture medium after 24 h of preincubation with DMSO or 150 μM salirasib for all additional experiments, because a sensitization to TRAIL was observed in all the three cell lines under these conditions.

As TRAIL is supposed to mainly act through induction of cell death, we studied LDH released into the culture medium to assess cell death induction in our experimental setting (Figure 1c). We have previously reported that salirasib inhibits cell proliferation in the three tested cell lines. Consistently with the antiproliferation effect of salirasib, treatment with salirasib alone in the current experiments did not induce a significant LDH release in any of the tested cell lines. In accordance with the results of the cell viability assays, TRAIL alone only induced a relevant LDH release in HepG2. A small (less than 3%) statistically significant difference in LDH release in HuH7 and Hep3B cells treated with TRAIL compared with DMSO was also observed. The LDH release assay is known to have a low signal–to–noise ratio and a high variability especially in the lower range. The measured values were close to the detection limit of the assay and thus likely reflect background noise. Addition of TRAIL to cells pretreated with salirasib, induced a marked LDH release in Hep3B and HuH7 cells reaching 23 and 16% of the maximum LDH release obtained with a lysis buffer, respectively, and further increased the TRAIL-induced LDH release in HepG2 cells to 49% of the maximum LDH release. These results show that TRAIL induces cell death on top of the antiproliferation effect of salirasib reported in our previous work.

TRAIL-induced apoptosis in salirasib pretreated cells involves both the DR and the mitochondrial apoptotic pathways. We next assessed whether TRAIL induced apoptosis in salirasib pretreated cells by measuring the subG0 cell population by FACS (Figure 2a). Salirasib alone induced a slight but statistically significant increase in subG0 events in Hep3B and HuH7 cells, but not in HepG2 cells. TRAIL alone induced apoptosis in HepG2 cells, but not in the two other cell lines, consistent with Hep3B and HuH7 cells being resistant to TRAIL-induced apoptosis. Addition of TRAIL to salirasib-pretreated cells induced massive apoptotic response. Indeed, subG0 events accounted for 53, 50 and 26% of HepG2, Hep3B and HuH7 cell populations, respectively.

Measurement of caspase 3/7 activity, the principal effector caspases committing cells to apoptosis, confirmed TRAIL-induced apoptosis in salirasib-pretreated cells (Figure 2b). In agreement with FACS data, caspase activation was not seen in salirasib treated cells, while TRAIL alone increased caspase activity in HepG2 cells only. Addition of TRAIL to cells pretreated with salirasib induced a dramatic increase in caspase-3/7 activity in all three cell lines (9-fold in HepG2, 8.5-fold in Hep3B and 5.5-fold in HuH7).

We next evaluated the implication of the DR and the mitochondrial pathways of apoptosis by assessing caspase-8 (Figure 2c) and caspase-9 (Figure 2d) activation, respectively. TRAIL alone induced a modest increase in caspase-8 activity and caspase-9 activity in HepG2 cells, but not in HuH7 and Hep3B cells. Salirasib alone had no effect on the activity of caspase 8 or 9. By contrast, addition of TRAIL to cells pretreated with salirasib, induced a marked increase of caspase-8 activity (fourfold in HepG2 and HuH7 cells, sevenfold in Hep3B cells) and an even more pronounced
Salirasib sensitizes HCC cells to TRAIL-induced cell death. (a) HepG2 (left column), Huh7 (central column) and Hep3B (right column) cells were seeded in 96-wells plates and incubated for 24 h with DMSO or the indicated concentration of salirasib and the indicated concentration of human recombinant TRAIL (n = 8–16 for each group) for 24 h. Cell viability was assessed by a WST-1 assay. (b) HepG2, Huh7 and Hep3B cells were seeded in 96-wells plates and incubated with DMSO or the indicated concentration of salirasib for 24 h. The culture medium was then replaced by a medium containing the same concentration of salirasib and the indicated concentration of human recombinant TRAIL (n = 12–16 per group) for further 24 h and cell viability was assessed by a WST-1 assay. (c) Cells were seeded in 96-wells plates and incubated with DMSO or 150 µM salirasib for 24 h. TRAIL (50 ng/ml) was added for further 24 h. LDH release was then assessed. Results are expressed as percentage of the maximum LDH release obtained with a lysis buffer. Data are presented as mean ± S.E.M. **P<0.001, ***P<0.0001 in treated groups versus control group.
increase in caspase-9 activity (8.6-fold in HepG2 and Huh7 cells, 12.8-fold in Hep3B cells). These data suggest that both pathways contribute to apoptosis induction.

**Salirasib-induced sensitization to TRAIL-induced apoptosis is associated with survivin downregulation.** Survivin is a caspase-9 inhibitor, which is downregulated by salirasib, an event that has been shown to promote apoptosis in glioblastoma cells. As our results show an activation of caspase-9 in salirasib-pretreated cells exposed to TRAIL, we studied the expression of survivin both by PCR and western blotting in cells treated with salirasib alone for 24 or 48 h.

At the mRNA (Figure 3a) level, salirasib reduced survivin expression in a time- and dose-dependent fashion. After 48 h of incubation with 150 μM salirasib, a drop of mRNA levels by 60, 70 and 45% was observed in HepG2, Hep3B and Huh7, respectively. In parallel, exposure to 150 μM salirasib for 48 h reduced survivin protein levels by 40 to 55% in all three cell lines (Figure 3b).

Survivin inhibition sensitizes HCC cells to TRAIL-induced apoptosis. The above results suggest that survivin repression might be at least a part of the mechanism responsible for salirasib sensitization of cells to TRAIL-induced apoptosis. To test this possibility, we assessed whether inhibition of survivin by YM155, a small molecule known to suppress survivin protein and mRNA expression currently in clinical development, is sufficient to reproduce the observed sensitization of HCC cells to TRAIL-induced apoptosis.

First, we measured cell viability upon increasing doses of YM155 ranging from 1 to 1000 nM and demonstrated a dose-dependent reduction with an IC50 of 64 nM (95% CI 51 to 81 nM) in HepG2 cells, 107 nM (88 to 130 nM) in Huh7 cells and 197 nM (173 to 224 nM) in Hep3B cells (Figure 4a).

Second, we incubated cells with 150 nM of YM155 for 48 h and observed a marked downregulation of survivin protein expression in all the three cell lines (Figures 4b and 4c) in accordance with the predicted mechanism of action of the compound.

Similar to our experiments performed with salirasib, we then evaluated apoptosis in cells exposed to DMSO or 150 nM of YM155 for 48 h with or without TRAIL for the last 24 h. Determination of the subG0 cell fraction by FACS (Figure 4d)
revealed that YM155 alone elicited a 2.5-fold increase in apoptosis in Hep3B and Huh7 cells, but not in HepG2 cells. Addition of TRAIL to YM155 pretreated cells, caused a further increase of apoptosis exceeding apoptosis induced by TRAIL alone in HepG2 cells, or apoptosis induced by YM155 alone in Huh7 and Hep3B. Indeed, subG0 events accounted for 39, 47 and 22% of HepG2, Hep3B and Huh7 cells, respectively. These results show that elective inhibition of survivin is sufficient to reproduce the TRAIL-sensitization effect observed with salirasib suggesting that downregulation of survivin significantly contributes to salirasib-induced sensitization to TRAIL.

**TRAIL-induced apoptosis in salirasib pretreated cells is mediated by DR5.** TRAIL may elicit apoptosis upon binding to either DR4 or DR5. Several agonist antibodies currently in clinical development target either DR4 or DR5. This prompted us to study the respective role of these receptors in apoptosis induced by the combination of salirasib and TRAIL. We first examined whether salirasib pretreatment for 24 h upregulated DR4 and DR5 transcript levels in our cell lines. Salirasib induced DR4 mRNA expression in HepG2 and Hep3B but not in Huh7 cells whereas induction of DR5 mRNA expression was observed in all the three cell lines (Figure 5a). Next, we assessed the impact of antagonistic antibodies directed against DR4 or DR5 (Figure 5b) on the viability of cells pretreated with salirasib for 24 h and followed by administration of TRAIL. In all three tested cell lines, DR4 inhibition only partially reversed the reduction in cell viability induced by the combination of salirasib and TRAIL. By contrast, DR5 inhibition almost completely abrogated the effect of TRAIL in HepG2, and totally reversed it in Huh7 and Hep3B. Inhibition of both DR4 and DR5 did not further enhance the effect observed with inhibition of DR5 alone in Huh7 and Hep3B, while it induced a slight but statistically significant additional increase in HepG2 cell viability. In parallel with the upregulation of DR5 transcript levels, these data strongly suggest that sensitization to TRAIL induced cell death in salirasib pretreated cells principally depends upon DR5.

To further confirm the involvement of DR5 in this process, we evaluated the effect of a DR5 agonistic antibody on cell viability (Figure 5c). Mimicking results obtained with TRAIL, the DR5 agonist had no or little effect on Hep3B and Huh7 cells viability, while it induced a dose-dependent decrease of viability in HepG2 cells. More importantly, the DR5 agonist...
reduced cell viability in a dose-dependent manner in salirasib-pretreated Hep3B and Huh7 cells. Altogether, these data clearly show that sensitization to TRAIL induced cell death in salirasib pretreated cells principally involves activation of DR5.

Concomitant treatment with salirasib and TRAIL is not toxic for human hepatocytes. It is widely accepted that TRAIL is not toxic for normal cells. However, this may not be the case when TRAIL is used in combination with other treatments because the same molecular mechanisms may be involved in TRAIL-resistance of both normal and tumor cells, resulting in sensitization of otherwise resistant normal cells. In order to address this issue, primary normal human hepatocytes were pretreated with DMSO or 150 μM salirasib for 24 h before addition of 50 or 100 ng/ml of human recombinant TRAIL. Cell viability and cell death (LDH release) were then determined. No significant difference in cell viability nor any measurable LDH release was observed in cells treated with salirasib and TRAIL, either alone or in combination, compared with controls (DMSO) (Figure 6). These results confirm that combination of salirasib and TRAIL is not toxic for primary human hepatocytes.

Discussion

The ability of TRAIL to selectively trigger apoptosis in cancer cells has led to the clinical development of recombinant TRAIL and TRAIL-receptor agonists as anticancer therapy. To date, stable disease is the best response obtained in the majority of patients treated with such agents, and the best clinical use of this new therapeutic class seems to be in combination with conventional chemotherapy or targeted agents to overcome resistance.

Our results showed for the first time that pretreatment with salirasib sensitizes human HCC cell lines to TRAIL-induced
apoptosis. Both the extrinsic and the intrinsic pathways of apoptosis are involved in this process, as evidenced by a strong induction of caspase-8 and caspase-9 activity. This finding suggests that HCC cells are type 2 cells capable of amplifying apoptotic signaling initiated by DRs through the recruitment of the mitochondrial pathway, as observed in other cell types.\(^4\) Moreover, TRAIL-induced reduction in cell viability was strongly attenuated when cells were incubated with an antagonist antibody against DR5, but not DR4. Consistently, an agonist antibody specific for DR5 also reduced cell viability in cells pretreated with salirasib. These data are in line with the finding of a greater contribution of DR5 than DR4 to apoptosis induction in cancer cells harboring both receptors.\(^21\) As both DR4 and DR5 agonists are under clinical development\(^22,23\), our results provide a rationale for the use of DR5 in the setting of HCC in clinical practice.

Liver toxicity is an important concern regarding the use of TRAIL and DR agonists in humans.\(^5\) It is therefore crucial to ensure that a treatment used to sensitize tumor cells to TRAIL-induced apoptosis does not induce toxicity in normal hepatocytes. As mice only harbors one apoptosis-inducing TRAIL receptor, equally homologous to both human apoptosis-inducing TRAIL receptors, DR4 and DR5,\(^24\) classical murine models might not be suited to evaluate the toxicity of TRAIL-based treatments. Therefore, we evaluated the effect of TRAIL or salirasib alone or in combination in vitro in primary human hepatocytes. Importantly, we observed no reduction in hepatocytes viability and no significant cytotoxicity in any of the treatment groups compared with untreated controls, suggesting that normal hepatocytes are not affected by either or both drugs. This point is relevant for translation to clinical settings.

We show here that salirasib sensitized HCC cells to TRAIL-induced apoptosis only when cells were pretreated with salirasib before the exposure to TRAIL but not when the two drugs were applied simultaneously for 24 h from the beginning. Thus, cells need to be primed by salirasib to undergo apoptosis. As shown in our previous work, multiple

**Figure 5** TRAIL-induced apoptosis in salirasib pretreated cells is mediated by DR5. (a) Quantification of DR4 and DR5 mRNA by quantitative PCR after 24 h of treatment with 150 μM of salirasib. (b) HepG2, Huh7 and Hep3B cells were seeded in 96-wells plates and incubated for 24 h with DMSO or 150 μM salirasib with or without anti-DR4 and/or anti-DR5 antibodies. Cell viability was then assessed by a WST-1 assay. (c) Cells were seeded in 96-wells plates and cultured with DMSO or the indicated concentration salirasib for 24 h. They were then further incubated for 24 h in a medium containing DMSO or salirasib supplemented with the indicated concentration of agonistic anti-DR5 antibody. Data are presented as relative change compared with untreated controls (mean ± S.E.M.). *P < 0.05, **P < 0.001, ***P < 0.0001 in treated groups versus control group (salirasib + TRAIL group in b), *P < 0.05, **P < 0.001, ***P < 0.0001 compared to the salirasib alone group in b.
Correlates with a reduced overall survival.26 Previous work of roughly 24 h to occur,17 which likely defines a transition period and cell death was assessed by a LDH release assay (b). Data are presented as relative change compared with untreated controls (mean ± S.E.M.)

Figure 6 Treatment with salirasib and TRAIL is not toxic for human hepatocytes. Primary human hepatocytes seeded in collagen-coated 96-wells plates were incubated in a culture medium containing DMSO or 150 μM salirasib for 24 h. The culture medium was then replaced by a medium containing the same concentration of salirasib with or without 100 ng/ml TRAIL (n = 8 per group) for 24 h. Cell viability was then assessed using a WST-1 assay (a) and cell death was assessed by a LDH release assay (b). Data are presented as relative change compared with untreated controls (mean ± S.E.M.)

Our team showed that salirasib reduces survivin mRNA expression in the three tested HCC cell lines.17 In the current study, protein expression of survivin markedly decreased after 48 h of treatment with 150 μM of salirasib, coinciding with the sensitization effect to TRAIL. It is therefore tempting to speculate that salirasib-induced inhibition of survivin has an important role in this process. This concept is also supported by data in the literature showing that downregulation of survivin by an antisense compound induced apoptosis in HepG2 cells.27 Furthermore, salirasib also reduced survivin expression in glioblastoma cell lines, promoting caspase-dependent cell death.19 We have previously shown that salirasib abrogates ras activation in our HCC cell lines that persists even after EGF stimulation known to strongly induce ras activity.17 Several lines of evidence suggest that Ras could directly modulate survivin expression in different cancer cell lines based on approaches such as the use of dominant-negative Ras as well as Ras inhibitors like salirasib. Thus downregulation of survivin mRNA and protein levels in our cells might at least in part be imputable to ras inhibition.19,28–32

In mitochondria-dependent type 2 cells, IAPs such as survivin are major determinants of TRAIL-induced apoptosis,6 and it is thus plausible that inhibition of survivin would sensitize them to TRAIL-induced apoptosis by suppressing a brake on the mitochondrial pathway of apoptosis. It is likely that a similar effect occurs in our three HCC cell lines, as caspase-9 activation (mitochondrial pathway) upon TRAIL exposure in cells pretreated with salirasib is stronger than caspase-8 activation (DR pathway) under the same conditions. Moreover, sensitization to TRAIL-induced apoptosis by salirasib parallels a reduction of survivin expression. In addition, we showed that selective inhibition of survivin by YM-155 sensitizes HCC cells to TRAIL-induced apoptosis to a similar extent than salirasib treatment. This is consistent with the finding by others that survivin inhibition by a siRNA sensitizes HCC cells to TRAIL-induced apoptosis.33 Altogether, these data indicate that survivin also has an important role in the sensitization process to TRAIL-induced cell death in our HCC cell lines.

Conclusion

Our results show for the first time that salirasib sensitizes human HCC cell lines to TRAIL-induced cell death by activation of both the intrinsic and the extrinsic pathways of apoptosis, while this combination is not toxic for primary human hepatocytes. Apoptosis induction in cells treated with this combination is mediated by the DR5, but not by the DR4 receptor. Furthermore, we show that survivin inhibition by salirasib has an important role in this sensitization process. As salirasib and TRAIL-agonists are currently under clinical development, these results in human HCC cell lines and primary hepatocytes provide a rationale for testing the combination of salirasib and TRAIL agonists in human HCC.

Materials and methods

Compounds. Salirasib was kindly provided by Concordia Pharmaceuticals (Fort Lauderdale, FL, USA). Unless stated otherwise, all cell culture reagents were purchased from Invitrogen (Brussels, Belgium). Human recombinant TRAIL was obtained from Millipore (Billerica, MA, USA), antagonist anti-DR4 and anti-DR5
antibodies from Diacalone (Besançon, France) and agonist anti-DR5 antibody from R&D (Abingdon, UK). YM155 was purchased from Selleck Chemicals (Houston, TX, USA).

**Cell culture**

Culture conditions. HepG2, HuH7 and Hep3B were obtained from the European Collection of Cell Culture (Salisbury, UK) and cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% streptomycin and penicillin, 1% non essential amino-acid, in 5% CO2 at 37 °C. Cells were allowed to adhere overnight. Thereafter, they were incubated in medium supplemented with 0.1% dimethylsulfoxide (DMSO, vehicle) or salirasib, with or without TRAIL. Culture medium was renewed once a day. Cells were seeded in 6-well plates for cell cycle analysis or in 96-well plates for WST-1, LDH, and caspase assays, at a density of 5 × 10^3 and 5 × 10^4 cells per well, respectively. For protein and RNA preparation, cells were plated at a density of 1.5 × 10^5 per 10 cm petri dish.

Primary human hepatocytes were provided by Lonza (Verviers, Belgium) plated as constant monolayers in a 96-wells collagen-coated plate, and cultured in human blood medium supplemented with hepatocyte basal medium Singlequots (Lonza) and 10% FBS.

**Experimental design.** For cell viability studies, the cells were incubated with DMSO or salirasib (75 or 150 μM) for up to 48 h. TRAIL (25, 50 or 100 ng/ml) was added either immediately to the cell culture medium or after 24 h preincubation with salirasib. Cell viability was also studied in cells cultured with various concentration of YM155, a survivin inhibitor, in order to determine its IC50 value, defined as the value at which 50% of the cell growth is inhibited compared with the DMSO control. IC50 was calculated by nonlinear regression analysis using GraphPad Prism software (San Diego, CA, USA).

Analyses of apoptosis, LDH release and caspases activity were performed in cells exposed to DMSO or 150 μM salirasib during 48 h, and 50 ng/ml of TRAIL were added after 24 h preincubation with salirasib. Apoptosis was also analyzed in cells exposed to 150 nM of YM155 instead of salirasib. Protein and RNA extraction was performed in cells exposed to DMSO, 150 μM salirasib or 150 nM of YM155, during 24 or 48 h. All experiments were repeated at least twice on separate days.

**Determination of cell viability and LDH release.** Cell viability was determined using a colorimetric WST-1 assay (Roche, Vilvoorde, Belgium) according to the manufacturer’s instructions. LDH release was assessed using the Cytotoxicity Detection KitPlus (Roche, Vilvoorde, Belgium) following the manufacturer’s instructions.

**Determination of apoptosis.** Cells were harvested by trypsination and washed with PBS. They were fixed in ice-cold ethanol, washed, resuspended in PBS and treated with RNase A. Finally, cells were stained with propidium iodide (Sigma-Aldrich, Bornem, Belgium). The stained cells were analyzed by flow cytometry (BD FACSCalibur, BD Biosciences, Erembodegem, Belgium) and DNA content was quantified using CellQuest Pro software (BD Biosciences, Erembodegem, Belgium). Apoptosis was assessed by assessing sub-G0 events in the euploid cell fraction.

Caspases activity was assessed using the Caspase-Glo-3/7, – 8, and –9 assays (Promega, Leiden, Netherlands) according to the manufacturer’s instructions.

**Western blotting.** Cells were harvested in ice-cold lysis buffer (0.1% Triton X-100, 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol, 1.5 mM MgCl2, 1 mM dithiothreitol, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin and aprotinin). Equal amounts of protein, determined by a BCA protein assay (Pierce, Rockford, IL, USA), were separated by SDS-polyacrylamide gel electrophoresis and transferred on to polyvinylidene fluoride membranes according to standard techniques. Membranes were incubated overnight with an anti-actin antibody (Cell Signalling, Danvers, MA, USA) and revealed with the ‘Western Lightning Chemiluminescent Reagent Plus’ (Perkin Elmer, Boston, MA, USA) detection system. Immunoreactive proteins were quantified by densitometry analysis using the Gel DocXR System 170-8170 device and software (Bio-Rad, Nazareth, Belgium). β-actin was used as loading control.

**Reverse transcription and quantitative PCR.** Cells were harvested in Trizol Reagent (Invirogen, Brussels, Belgium) for RNA extraction. RNA was reverse transcribed and subjected to quantitative PCR with the StepOnePlus real-time PCR System (Applied Biosystems, Lithick, Belgium) using SYBRgreen. Primers were designed using the Primer Express design software (Applied Biosystems, Lithick, Belgium). The following primers were used: survivin 5'-CG AGGCCTGTTTCAATCCA (forward) and 5'-CAACCGAGAAGTCTTCTT (reverse); DR4 5'-GCGCTGAGAATCTCACA (forward) and 5'-TGTG CATGTCAGAGAGAATG (reverse); DR5 5'-GACTCGAGACAGTGCTCAG (forward) and 5'-CCATGAGGGACCACTCCTC (reverse); RPL19, 5'-CAGCGGATTCTCATGGAACA (forward) and 5'-TTGCTGCCAGCGAGGCTTCT (reverse). Quantification was obtained according to the ΔΔCt method.34 The final result of each sample was normalized to its respective ribosomal protein L19 (RPL19, internal standard) value.

**Statistical analysis.** Results are expressed as relative change compared with DMSO controls and are given as the mean ± S.E.M. The statistical differences between groups were tested using a two-tailed Student’s t-test. Statistical significance was assumed for P-values < 0.05.

**Conflict of Interest**

The authors declare no conflict of interest.

**Acknowledgements.** This work was supported by University FSR grants to PS. NC is the recipient of a FSR fellowship. IL is a FRS-FNRS research associate.
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