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The Ras inhibitor farnesylthiosalicyclic acid (FTS) prevents nodule formation and development of preneoplastic foci of altered hepatocytes in rats

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ABSTRACT

Background: Aberrant activation of oncogenes, such as Ras, likely contributes to the development of hepatocarcinoma (HCC).
Aims/methods: We evaluated *in vivo* the effect of intraperitoneal injections of the Ras inhibitor S-trans, trans-farnesylthiosalicyclic acid (FTS) on Ras activation and the development of preneoplastic liver lesions in rats receiving weekly diethylnitrosamine (DEN) injections for 16 weeks. Western blotting, quantitative PCR, immunohistochemistry, Tunel and caspase activity assays were used.
Results: FTS prevents liver nodule formation and reduces foci expressing the tumour marker GSTp. FTS abrogates DEN-induced Ras membrane activity, increases Tunel positive cells in transformed, GSTp-expressing hepatocytes, up-regulates caspase 3 and 8 activity, induces Fas, Fas ligand and JNK phosphorylation that occurs independently of TNFα and Trail. Cytochrome C release, Bax, Bcl2, Bcl-xl, Ki67 and nuclear cyclin D expression is not affected by FTS.
Conclusions: FTS inhibits Ras activation and prevents preneoplastic liver nodule development by inducing apoptosis in transformed hepatocytes through activation of the Fas/Fas ligand system. FTS might be new molecule for HCC treatment.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most frequent cancer worldwide arising from hepatocytes undergoing malignant transformation in response to various stimuli. Survival remains poor for intermediate and advanced stage HCC, because of the aggressiveness of lesions at the time of diagnosis and the lack of curative therapy.[1] A strong correlation exists between cirrhosis and hepatocarcinogenesis since most patients with HCC have pre-existing cirrhosis.[1] Continuous and irregular proliferation associated with inflammation is believed to produce genetic abnormalities leading to the development of HCCs.[2] Multistep tumour genesis includes mutational activation of genes of the Ras family.[3] The Ras gene products are monomeric membrane-localised oncoproteins belonging to a superfamily of small GTPases. The G-proteins function as molecular switches linking receptor and non-receptor tyrosine kinase activation

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to downstream cytoplasmic or nuclear events resulting in various cellular responses, like proliferation, differentiation and/or apoptosis.[4] Ras regulates molecular events by cycling between inactive GDP-bound and active GTP-bound forms that bind specifically to the Ras-binding domain of Raf1 controlling downstream signalling cascades. Membrane anchorage is important for the biological activity of Ras proteins and depends on their carboxy-terminal farnesylcysteine group.[4,5]

S-trans-trans-farnesylthiosalicyclic acid (FTS) structurally resembles the carboxyl-terminal farnesylcysteine group common to all Ras proteins and has been shown to act as a functional Ras antagonist in cells. FTS dislodges the protein from its anchorage domains and facilitates its degradation, thus reducing cellular Ras content.[5–7] FTS acts predominantly on the active, GTP-bound forms of Ras proteins. It principally competes with Ras-GTP for binding to specific binding sites in the plasma membrane preventing active Ras from activating intracellular downstream signalling pathways.[8] Acting this way, FTS was found to be a potent growth inhibitor of non-hepatic Ras expressing cancer cell lines in culture,[9–11] of non-hepatic tumour cells xeno-grafted to nude mice and of hepatic stellate cells *in vivo*.[8,10–13] Ras mutations in human HCC [14,15] or in human hepatoma cell lines [16] have been reported. Recently, it has been outlined that Ras might be a potential target in human HCC.[17,18] Nevertheless, to date clinical studies in HCC patients, *in vitro* data in hepatic tumour cell lines, or *in vivo* studies in animal models of hepatocarcinogenesis with Ras as a potential interventional target have not been reported. Our previous work shows that high dose FTS blocks hepatocyte proliferation *in vivo* in rats after partial hepatectomy.[19] We also demonstrated an inhibitory effect of FTS on proliferation of the hepatic tumour cell line HepG2 *in vitro*.[19] Given this strong inhibitory effect on proliferation of normal and transformed hepatocytes *in vivo* and *in vitro*, we were prompted to investigate *in vivo* the consequences of FTS treatment on development of focal liver lesions in rats induced by repeated injections of diethylnitrosamine (DEN). DEN has been shown to induce predominantly liver cancer and is frequently used to study hepatocarcinogenic processes.[20] In addition, the DEN model also best fits the changes observed in human HCC on functional genomics.[21] We used the model described by Schiffer et al.[22] where weekly DEN injections mimic the sequence of fibrosis and cirrhosis encountered in human hepatocarcinogenesis and usually not observed in genetically engineered HCC models.[20]

2. Methods

2.1. Animals

Male *Wistar* rats (body weight 180–190 g) were obtained from the rat breeding facilities of the Université Catholique de Louvain Medical School, Brussels, Belgium. Animals were kept in a temperature and humidity-controlled environment in a 12 h light–dark cycle. They were allowed free access to water and standard food pellet diet (Usine d'Alimentation Rationnelle, Villemoisson-sur-Orges, France). Animals were handled according to the guidelines established by the Université Catholique de Louvain.

2.2. Synthesis of farnesylthiosalicylic acid (FTS)

FTS was synthesised as described previously.[23] Purified FTS was dissolved in DMSO (10 mg FTS/10 ml DMSO) for injection.

2.3. Experimental design

Rats received weekly intraperitoneal injections of diethylnitrosamine (DEN) at a dose of 50 mg/kg body weight over 16 weeks. FTS was administered intraperitoneally at a dose of 5 mg/kg body weight three times per week starting after 6 weeks of DEN induction. Two groups of eight animals each were treated as follows: one group received DEN only and one group was administered DEN and FTS. Animals of both groups were sacrificed one week after the last DEN injection (week 16) a time point where according to Schiffer et al.[22] diffuse focal liver lesions resembling HCC do arise. Naïve, non-treated animals ($n = 5$) were used as controls. At the time of sacrifice, blood was drawn, the number of tumours, defined as dyschromic, >3 mm nodules at the liver surface, were assessed macroscopically by two independent investigators and livers were removed, snap frozen or fixed in formalin for histopathology.

2.4. Preparation of liver cell fractions

Frozen liver tissue samples were homogenised in ice-cold lysis buffer I or II (Supplementary Table 1) using a Potter–Wheaton teflon homogeniser. The homogenates were centrifuged at 10,000*g* for 5–10 min at 4 °C and the supernatant was stored at –80 °C.

Membrane extracts were prepared as previously described with minor modifications.[16] Liver homogenates were cleared by centrifugation (100,000*g*) for 60 min at 4 °C. The resulting pellet was solubilised in 1 ml of lysis buffer III (Supplementary Table 1), centrifuged and the supernatant (membrane fraction) was stored at –80 °C.

Nuclear extracts were prepared using a commercially available nuclear extract kit according to the manufacturer's instructions (Active Motif, Rixensart, Belgium).

2.5. Histology and immunohistochemistry

Five micrometres serial sections of formalin-fixed, paraffin-embedded livers were stained with haematoxylin–eosin for conventional histology. Ki67 and glutathione-S-transferase (GSTp) expression was assessed by immunohistochemistry and quantified by quantitative morphometry as described by our group.[24]

2.6. Western blotting and immunodetection

Western blotting was performed using standard techniques. Primary, secondary antibodies and working conditions for immunodetection are depicted in Table 1. Membranes were revealed with the 'Western Lightning Chemiluminescence Reagent Plus' (PerkinElmer, Boston, MA, USA) detection system and immunoreactive protein was quantified by densitometry using the Gel Doc 2000 device and software (Bio-Rad, Nazareth, Belgium).

Table 1 – Antibodies and working conditions for Western blotting.

Antibody	Working conditions	Source
Rabbit anti-GSTp	1:100, over night, 4 °C	MBL, Woburn, MA, USA
Mouse monoclonal anti-Fas	1:1000, over night, 4 °C	BD Transduction Laboratories, Franklin Lakes, NJ, USA
Mouse monoclonal anti-Hsp90	1:2500, 1 h, 20 °C	BD Transduction Laboratories
Mouse monoclonal anti-Bax	1:300, over night, 4 °C	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Mouse monoclonal anti-Bcl2	1:500, over night, 4 °C	Santa Cruz
Mouse monoclonal anti-Bcl-xl	1:500, over night, 4 °C	Santa Cruz
Mouse monoclonal anti-cyclin D	1:4000, 2 h, 20 °C	Santa Cruz
Mouse monoclonal anti-cytochrome C	1:1000, 2 h, 20 °C	Santa Cruz
Rabbit polyclonal anti-JNK	1:5000, over night, 4 °C	Santa Cruz
Mouse monoclonal anti-pJNK	1:2000, over night, 4 °C	Cell Signalling, Danvers, MA, USA
Rabbit anti-Ras	1:1000, over night, 4 °C	Cell Signalling
Peroxidase-conjugated mouse anti-rabbit and goat anti-mouse IgG	1:5000 to 1:60,000; 1 h, 20 °C	Dianova, Hamburg, Germany

2.7. *Quantitative polymerase chain reaction (PCR)*

Total RNA was prepared from frozen liver tissue using TriPure Isolation Reagent (Roche Diagnostics, Mannheim, Germany). RNA was reverse transcribed and subjected to PCR with the GeneAmp® 5700 Sequence Detection System and software (Applied Biosystems, Den Ijssel, Netherlands) using Cybergreen fluorogenic probes. Primers were designed using the Primer Express™ design software (Applied Biosystems) and sequences are presented in Table 2. Quantification was obtained according to the ΔΔCT method as specified by the manufacturer. The final result of each sample was normalised to its respective Ribosomal protein L19 (RPL19) value (invariable internal standard).

2.8. *Ras pull-down assays*

Frozen liver tissue samples were homogenised in ice-cold precipitation buffer (Supplementary Table 1) using a Potter–Wheaton teflon homogeniser. Homogenates were centrifuged at 4 °C and supernatant containing 1 mg of protein was mixed with 10 μl of Raf1-RBD (Ras-binding domain) agarose beads (Upstate, Lake Placid, NY, USA) and rotated overnight at 4 °C. Immunocomplexes were washed three times with precipitation buffer and disrupted by boiling in Laemmli buffer.

2.9. *Caspase 3 and 8 activity assays*

Caspase activities were assessed using commercially available Caspase 3 colorimetric assay (Alexis Biochemicals, Lausen, Switzerland) and Caspase-Glo® 8 assay kits (Promega, Madison, WI, USA) following the manufacturer's instructions.

2.10. *Tunel assay*

Apoptosis was determined on paraffin-embedded liver sections using the In Situ cell Death Detection Kit (Roche-applied-science, Vilvoorde, Belgium) following the manufacturer's instructions.

2.11. *Statistical analysis*

Results are expressed as means ± SEM. Statistical differences were assessed using the Student's *t*-test and significance was assumed for p values < 0.05.

3. Results

3.1. *DEN-induced macroscopic nodule formation is significantly suppressed by FTS treatment*

DEN-administration induces hepatomegaly and a significant increase in AST and ALT levels (Tables 3 and 4). Compared to their respective DEN-counterparts, a strong reduction of liver weight and of the liver to body weight ratio is found in DEN + FTS-treated animals close to the values observed in untreated control animals (Table 3). In parallel, FTS treatment leads to a significant reduction by at least 50% of AST and ALT levels suggesting reduced liver damage in these animals (Table 4). No mortality or signs suggestive of toxicity of the compound are observed in the FTS-treated group.

Macroscopically, administration of DEN alone over 16 weeks leads to a nodular formation with numerous whitish nodules distributed over the surface of the liver (Fig. 1A)

Table 2 – Primers used for PCR amplifications.

	Forward (3′–5′)	Reverse (5′–3′)
Fas	CGGAAAGCAAGATCGATGAGA	AATCCAGCTGCTCCAGTGCT
FasL	GCCCGTGAATTACCCATGTC	TGGAGGAGCCCAAGGAGAA
TNFα	CCACCACGCTCTTCTGTCTAC	AGTGACAAGCCTGTAGCCCA
Trail	CGGAGAGGGCATTCATTTCTC	CAGGCCCTCCTCCTGGAT
Rpl19	CAAGCGGATTCTCATGGAACA	TGGTCAGCCAGGAGCTTCTT

Table 3 – Effect of DEN- and FTS-treatment on body and liver weight.

Group treatment	Body weight (g)[A]	Liver weight (g)[A]	Ratio (%) of liver to body weight[A]
Untreated controls	340 ± 26	12.5 ± 2.0	3.7 ± 0.4
DEN 16 weeks	410 ± 34[a,**]	18.3 ± 3.0[a,*]	4.4 ± 0.6[a,*]
DEN + FTS 16 weeks	305 ± 60[b,***]	8.7 ± 2.4[b,***]	2.8 ± 0.5[b,***]

A Mean ± SD; $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$ from untreated controls (a) or DEN 16 weeks (b).

Table 4 – AST and ALT activities in DEN-induced animals compared to DEN + FTS treated animals.

	AST (IU/l)[A]	ALT (IU/l)[A]	LDH (IU/l)[A]
Control (untreated)	75 ± 6	76 ± 11	ND
Chronic administration			
DEN 16 weeks	430 ± 37[***]	222 ± 68[*]	ND
DEN-FTS 16 weeks	202 ± 41[b,**]	111 ± 11[b,*]	ND
Acute concomitant administration			
DEN alone (48 h)	146 ± 51	74 ± 12	219 ± 61
DEN-FTS (48 h)	125 ± 8	56 ± 22	216 ± 8

ND = not done
A Mean ± SD; $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$ compared to non-treated control group or DEN 16 weeks (b).

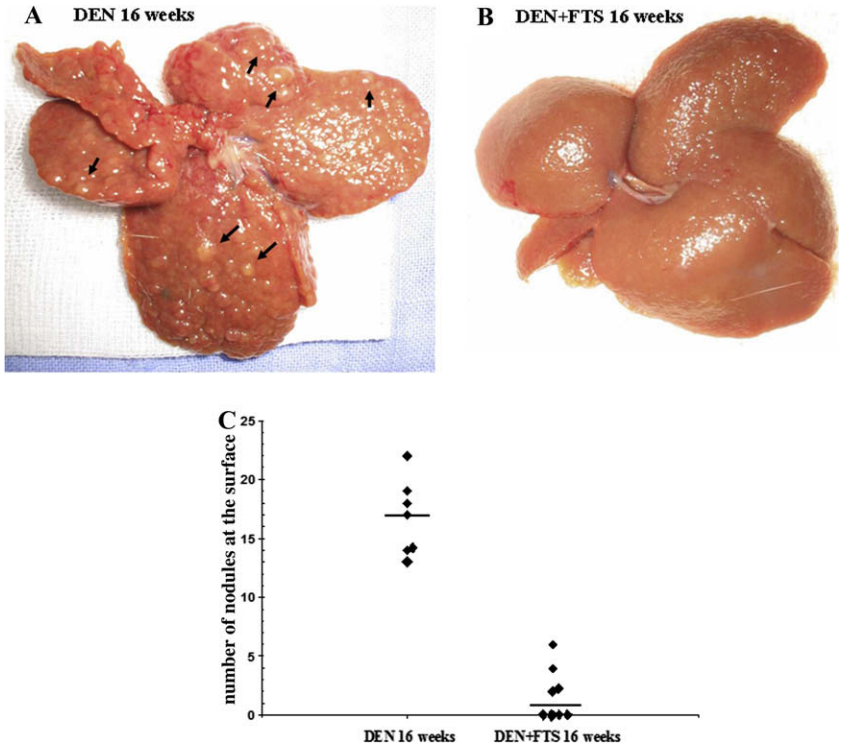

Fig. 1 – Macroscopic nodule formation after 16 weeks of DEN or DEN + FTS. Macroscopic appearance of livers showing multiple whitish, dyschromic nodules (arrows) at the liver surface of DEN-induced animals (A) that is prevented by FTS treatment (B). Overall nodule count in individual animals (C) showing inhibition of surface nodule formation in DEN + FTS-treated animals.

as well as within the liver (not shown). The nodular appearance of the liver and whitish nodules formation is almost completely prevented by FTS treatment (Fig. 1B) with a more than 80% reduction in the mean number of macroscopic nodules at the liver surface of DEN + FTS-treated rats (Fig. 1C). Indeed, no nodules, whether within the liver or at the liver surface, are found in four out of eight animals that have been administered DEN + FTS.

3.2. *FTS treatment suppresses DEN-induced formation of preneoplastic liver lesions and significantly decreases GSTp expression, a marker of cell transformation*

Careful histological examination shows diffusely nodular livers in animals administered DEN alone (Fig. 2). The nodules contain acidophilic or mixed acidophilic and basophilic hepatocytes (Fig. 2) consistent with the histological criteria of preneoplastic foci of altered hepatocytes (FAH).[25] Some small nodules composed small strongly basophilic hepatocytes are also observed. By contrast, the nodular architecture on histology was completely suppressed by FTS treatment (Fig. 2). At best, occasional small clusters of hepatocytes adopting a pseudonodular shape can be identified on high magnification in FTS-treated livers (Fig. 2).

We next examined expression of GSTp that is overexpressed in hepatocytes within dysplastic hepatocellular foci or HCCs, but not in surrounding 'normal' hepatocytes.[26] GSTp is not expressed in normal livers (not shown). After 16 weeks of DEN administration, we observed an extended GSTp positive staining, occupying 75% of the liver parenchyma (Fig. 3A and C). In particular, all acidophilic and mixed acidophilic/basophilic nodules on histology stain diffusely positive for GSTp whereas the small strongly basophilic nodules are only partially positive for GSTp (Fig. 2). By contrast in animals that were administered DEN + FTS, overall GSTp expression is strongly reduced on Western blotting (Fig. 3D) and areas of GSTp positive hepatocytes are significantly diminished (<25% of the liver parenchyma) (Fig. 3B and C). The rare pseudonodules identified on histology stained positive for GSTp (Fig. 2). However, most of the small clusters of GSTp positive hepatocytes in DEN + FTS-treated animals do not show any particular abnormalities on standard histology.

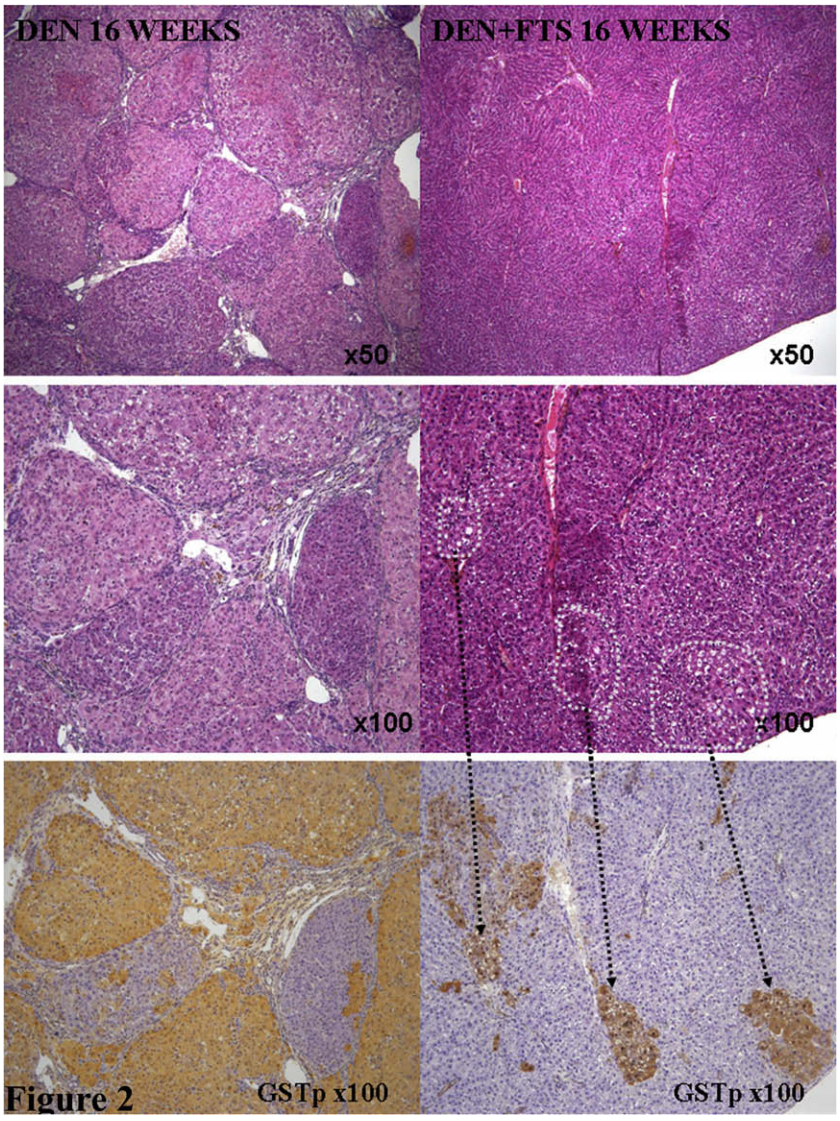

Fig. 2 – Liver histology after 16 weeks of DEN or DEN + FTS. Representative haematoxylin–eosin sections showing diffuse acidophilic or mixed acidophilic/basophilic liver nodules that stained GSTp positive on serial sections in DEN-treated animals. The nodular pattern was completely abolished in animals treated with DEN + FTS with occasional small GSTp positive pseudonodules on high magnification (dotted lines).

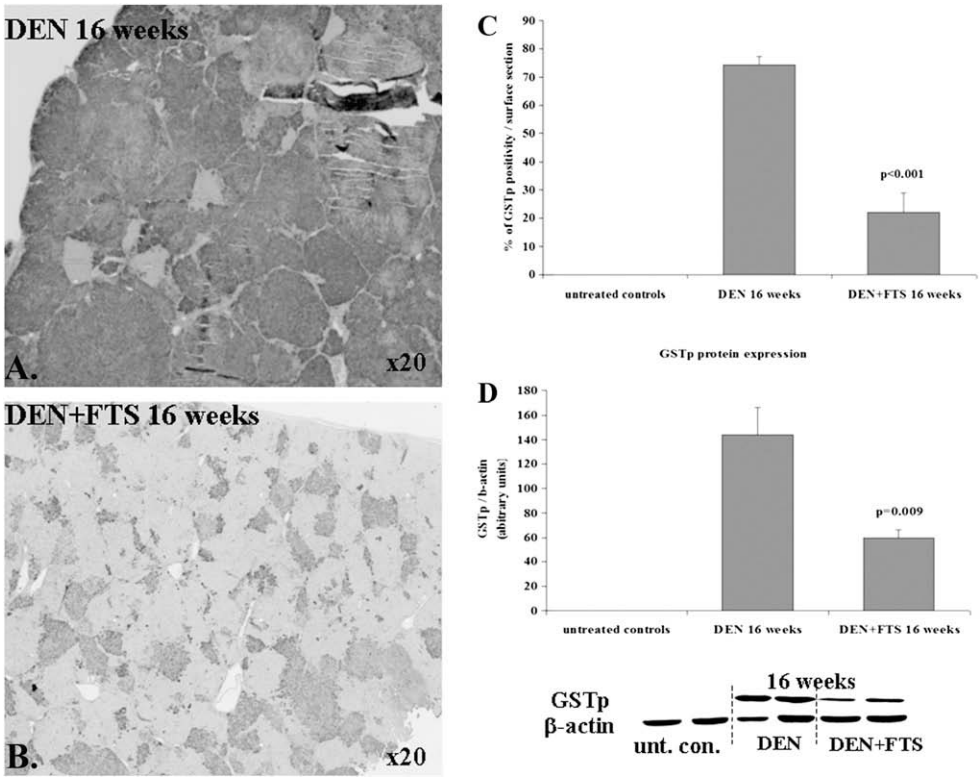

Fig. 3 – GSTp expression after 16 weeks of DEN or DEN + FTS. (A, B) Representative immunohistochemistry of GSTp expression. (C) Quantitative morphometry of GSTp positive areas normalised to the section surface showing strongly reduced GSTp expression in DEN + FTS-treated animals. (D) Representative Western blot and quantification of GSTp expression in liver homogenates confirming the immunohistochemistry findings.

The macroscopic and microscopic data as well as reduced GSTp expression demonstrate that FTS strongly decreases formation of preneoplastic liver lesions.

3.3. *FTS treatment does not interfere with DEN-induced liver toxicity*

To exclude the possibility that FTS might interfere with DEN uptake and thus lower DEN toxicity, we compared transaminase and LDH levels 48 h after single concomitant ip injections of DEN + FTS or DEN alone. This time point has been chosen for peak levels being observed in rats 48 h after a single ip DEN dose.[27] AST and LDH levels do increase similarly in animals treated with DEN + FTS and with DEN alone (Table 4). In addition, the extent of histological damage is also similar in both groups and principally restricted to the areas of the central veins (liver zone 3) that are surrounded by marked eosinophilic and necrotic hepatocytes (Fig. 4). Both observations suggest that FTS does not hinder DEN-induced damage from developing.

3.4. *FTS inhibits Ras expression and activity in DEN-treated livers*

Ras immune reactive protein in membrane fractions and Ras activity are not detected in untreated control animals. Strong Ras recruitment to cell membranes and high Ras activity are observed after 16 weeks of DEN-treatment (Fig. 5). By contrast, DEN-induced membrane expression and Ras activity is almost completely abrogated in response to FTS treatment (Fig. 5).

3.5. *Reduction in preneoplastic lesions is associated with increased apoptosis in DEN + FTS-treated rats*

No apoptotic cells are found in untreated control animals. Using the Tunel assay, rare isolated apoptotic bodies are found on liver sections suggesting a low apoptotic index in animals administered DEN alone (Fig. 6A). In DEN + FTS-treated animals, the number of apoptotic bodies per section strongly increased, assembling in multiples small groups that after comparison with serial sections stained for GSTp co-localise within or immediately adjacent to areas of GSTp positive hepatocytes (Fig. 6B and C). In addition, overall liver caspase 3 activity significantly increased in DEN + FTS-treated animals confirming up-regulation of apoptosis by FTS administration (Fig. 6D). These results suggest that FTS induces apoptosis principally in transformed, GSTp positive hepatocytes in DEN-induced animals.

3.6. *FTS induced apoptosis is associated with activation of the Fas/Fas ligand system and JNK overactivation*

Compared with untreated controls and DEN-induced animals, DEN + FTS treatment significantly up-regulates expression of Fas mRNA and protein (Fig. 7A and C). Interestingly, FasL

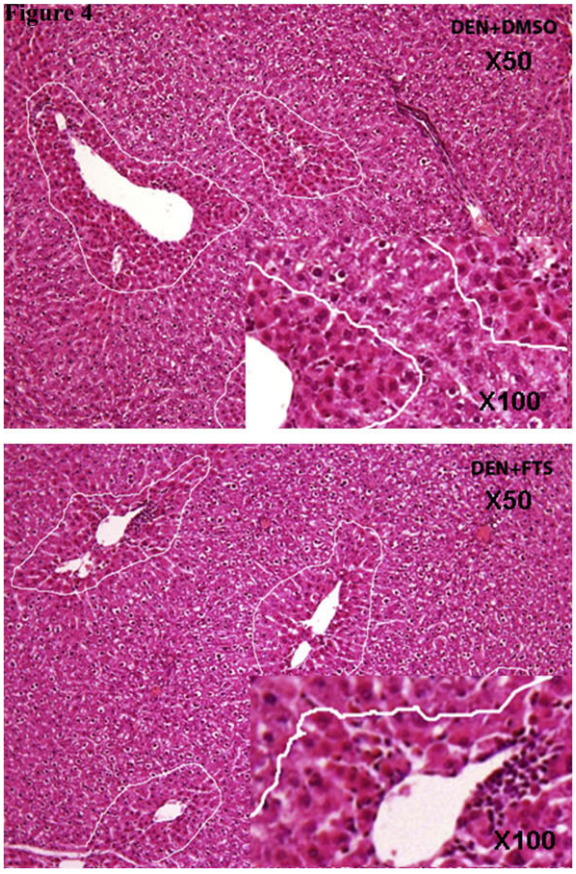

Fig. 4 – Histological damage induced by a single intraperitoneal injection of DEN. Representative haematoxylin–eosin sections 48 h after a single DEN injection alone or concomitantly with FTS. Similar histological damage (hypereosinophilic, necrotic hepatocytes) restricted to liver zone 3 (central vein) is observed in both groups. The white lines delimit the extension of the damaged zone.

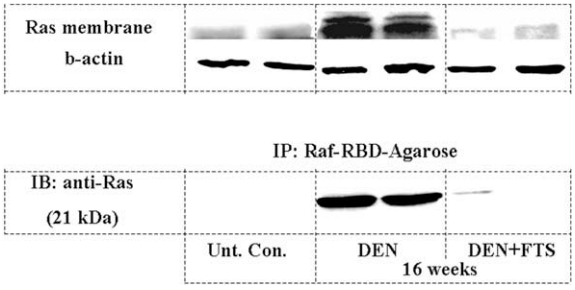

Fig. 5 – Ras membrane expression and Ras activity after 16 weeks of DEN or DEN + FTS. Representative Western blot Ras expression in membrane fractions (upper panel) and evaluation of hepatic Ras activity by Ras pull-down assays (lower panel) showing a strong induction of Ras after DEN administration that is almost completely abolished in DEN + FTS-treated animals.

mRNA expression is down-regulated in DEN-induced animals compared to untreated controls whereas FTS treatment at least partially restored FasL mRNA levels (Fig. 7A). Consistently with activation the Fas/FasL system by FTS, we also observe an increase in liver caspase 8 activity in FTS-treated animals compared with their DEN-induced counterparts (Fig. 7D).

In parallel to Fas up-regulation, FTS treatment elicits a strong increase in JNK phosphorylation (Fig. 7C) suggesting activation SAPK/JNK signalling cascade by FTS. Additional factors known to activate JNK and induce apoptosis such as TNFα and Trail do not show significant changes of their mRNA levels upon FTS treatment (Fig. 7B) indicating that activation of both, JNK and the extrinsic pathway of apoptosis, occur independently from TNFα and Trail. Cytochrome C release slightly increases in DEN-induced animals compared with untreated controls but is not further enhanced upon FTS treatment (Fig. 8). In addition, analyses of factors associated with the mitochondrial pathway of apoptosis show that FTS treatment does neither affect pro-apoptotic Bax, nor anti-apoptotic Bcl-2 and Bcl-xl expression (Fig. 8). Taken together these observations suggest that FTS primarily activates the death receptor pathway of apoptosis mediated by the Fas/Fas ligand system.

3.7. *FTS does not inhibit cellular proliferation*

No proliferation is observed in livers of untreated control animals (not shown). Expression levels of Ki67 are low in DEN administered animals (Fig. 9A). Surprisingly, Ki67 expression is even slightly higher in DEN + FTS-treated animals (Fig. 9B) with Ki67 positive hepatoctes being principally located outside GSTp positive areas on serial sections (Fig. 9B and C). Analysis of cyclin D expression in liver nuclear extracts does also not show any significant difference between DEN alone and DEN + FTS-treated rats (Fig. 9D) confirming that FTS does not interfere with cellular proliferation.

4. Discussion

The molecular mechanisms of hepatocellular carcinogenesis and means for effective prevention and treatment of HCC still remain poorly defined. Recent data emphasise the potential role of Ras in the development of HCC in humans[18,28] making it a potential target for drug design. We have previously shown that Ras blockage through high dose FTS inhibits synchronous liver cell proliferation after partial hepatectomy and also blocks proliferation of the hepatic tumour cell line HepG2 *in vitro*.[19] We now demonstrate that Ras blockage through FTS also prevents the development, *in vivo*, of preneoplastic foci of altered hepatocytes (FAH) known to evolve to neoplastic nodules[25] in the diethylnitrosamine (DEN) model of liver carcinogenesis previously described by Schiffer et al.[22] Treatment of DEN-induced rats with repeated low doses of FTS, leads to important modifications: (1) FTS elicits a dramatic reduction in number and size of FAH as well as to a strongly reduced expression of GSTp, a marker of neoplastic transformation in hepatocytes[26] and (2) FTS blocks induction of Ras membrane activity. The effect of FTS in rat livers is consistent with observations in Ha-Ras transformed cells *in vitro* and in SCID mice grafted with non-hepatic tumour cell lines where FTS significantly reduces tumour development

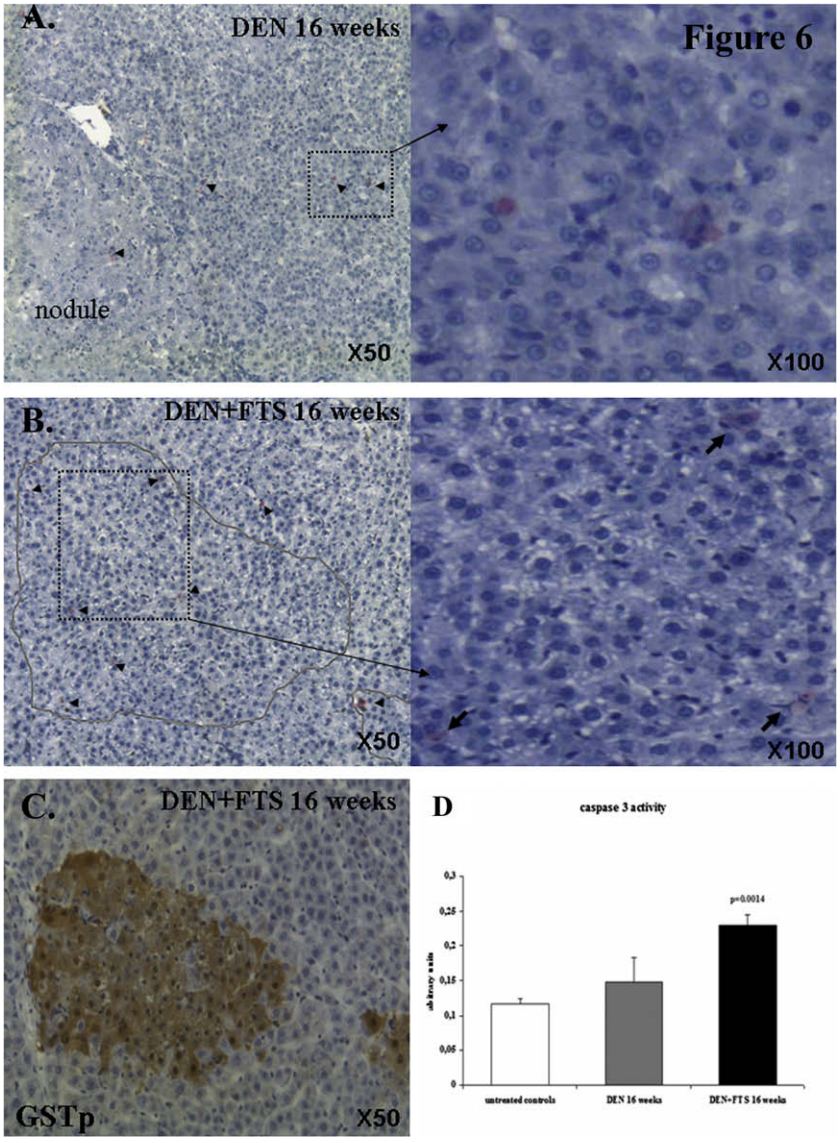

Fig. 6 – Assessment of apoptosis after 16 weeks of DEN or DEN + FTS. Representative Tunel staining (A, B) showing an increased number of dark red apoptotic bodies and cells (arrows) in DEN + FTS-treated animals; (B) principally located in GSTp positive areas (grey line) on corresponding serial sections; (C) liver caspase 3 activity; and (D) confirming increased apoptosis in DEN + FTS-treated animals.

and growth.[8–12] FAH induction by DEN is closely associated with increased expression and activity of Ras in membrane fractions. Administration of FTS to DEN treated rats in our study prevents Ras cell membrane anchorage thereby blocking Ras membrane activity that is consistent with the previously described mechanism of action of this compound.[5] Recent data confirm that FTS displays a high affinity for Ras acting in a specific manner on the active, GTP-bound forms of Ras proteins. FTS principally competes with Ras-GTP for binding to specific binding sites in the plasma membrane preventing active Ras from activating intracellular downstream signalling pathways.[8] Consequently, the physiological impact of potential interactions of FTS with Ras independent targets is likely to be minor. Therefore, it is plausible that Ras inhibition through FTS is responsible for the FAH preventive effect with remarkably low systemic toxicity. Moreover, the tumour preventive effect cannot be attributed to interference of FTS with DEN uptake or DEN metabolism thus lowering DEN toxicity for several reasons: DEN is cleared from both the blood and the liver within 24 h whereas plasma clearance of FTS is even more rapid (8 h after ip injection).[27,29] It is thus impossible that injections that are separated by 24 h or more interact with each other. In addition, concomitant ip injections of both FTS and DEN produce similar early histological and biochemical damage compared to DEN injections alone making it unlikely that FTS interferes with DEN uptake even if administered concomitantly. Finally, FTS is neither an inducer nor an inhibitor of CYP enzymes and is primarily

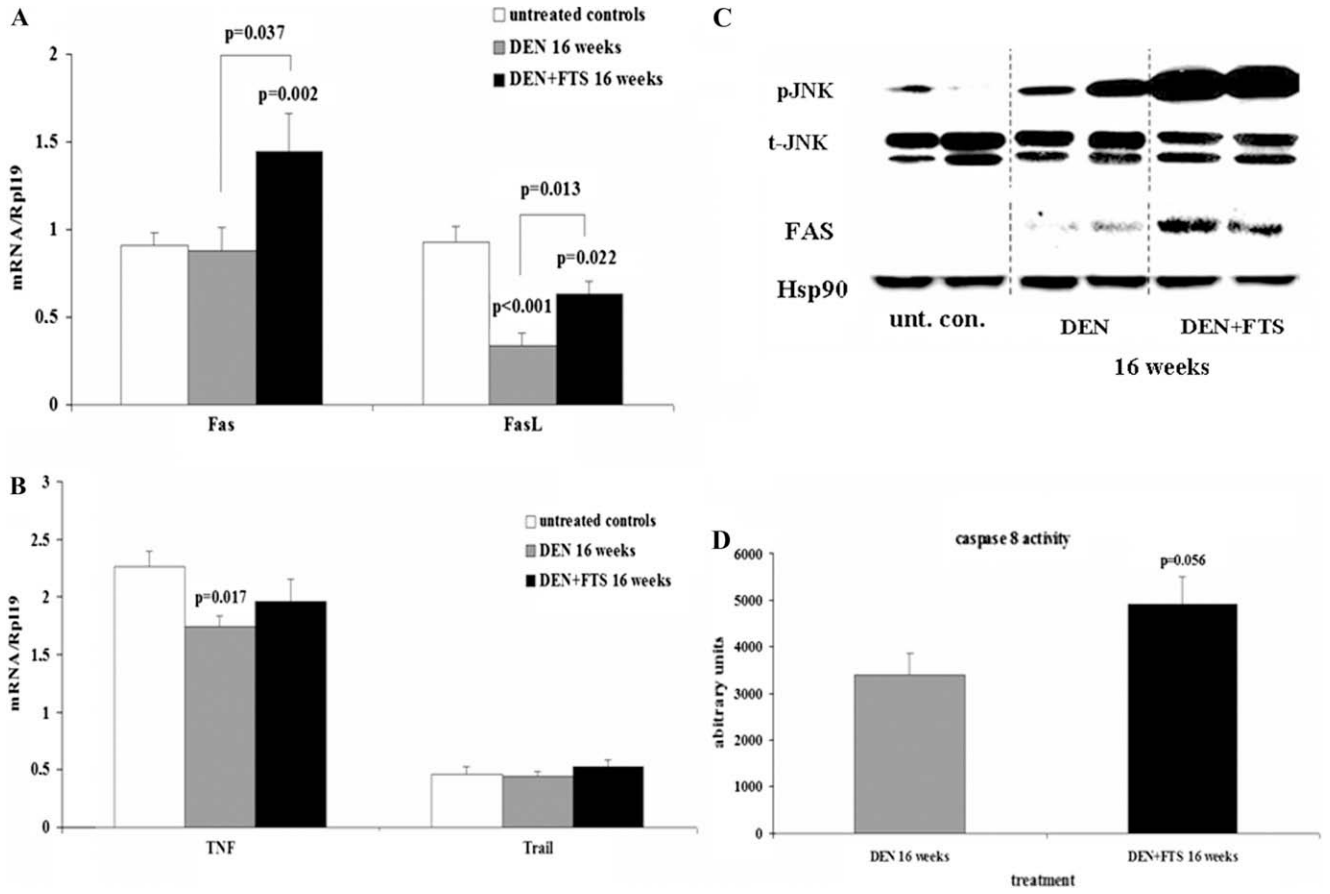

Fig. 7 – Activation of the death receptor pathway of apoptosis after 16 weeks of DEN or DEN + FTS. Relative expression of Fas, FasL (A) and TNF, Trail mRNA (B), representative Western blot of liver Fas and phospho-JNK protein expression (C) and liver caspase 8 activity (D) demonstrating increased caspase 8 activity associated with Fas up-regulation, and overexpression of phospho-JNK in DEN + FTS-treated animals.

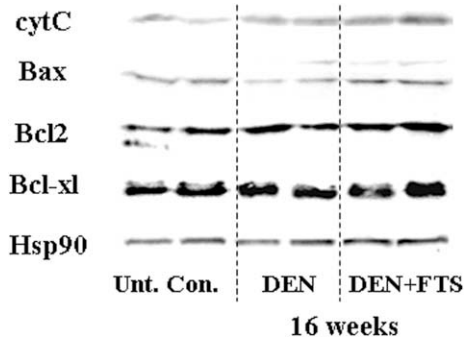

Fig. 8 – Expression mitochondrial markers of apoptosis after 16 weeks of DEN or DEN + FTS. Representative Western blots of cytochrome C release, Bax, Bcl2 and Bcl-xl expression showing no significant differences in expression of any of these markers in DEN + FTS-treated animals compared to their DEN-alone counterparts.

metabolised by the CYP450 2C subfamily (Concordia Pharmaceuticals Inc., personal communication). Therefore, even repeat injections of FTS are very much unlikely to interfere with DEN activation through CYP450 2E1.

The occurrence of cancers may be due to loss of control of normal apoptosis disturbing the balance between cell apoptosis and cell proliferation.[30] Long term treatment with repeated low dose FTS does not seem to interfere with non-synchronised cellular proliferation generally seen in cancer development in contrast to what has been observed with short term repeated high dose FTS in synchronised proliferation after partial hepatectomy.[19] By contrast, caspase 3 activity is significantly elevated in the livers of FTS-treated animals and Tunel positive cells are mainly seen in areas of transformed, GSTp positive hepatocytes. Moreover, FTS treatment is associated with activation of the Fas/Fas ligand system that is generally believed to promote apoptosis.[31] Ras overexpression has been reported to inhibit Fas gene expression and renders tumour cells resistant to Fas-induced cells death.[32] Furthermore, evidence suggests that GSTp positive hepatocytes from DEN-treated rats are less sensitive to Fas-mediated apoptosis[33] and that inhibition of Ras restores sensitivity to apoptotic cell death.[32] Our data is in keeping with these observations suggesting that FTS induced Ras blockage elicits a pro-apoptotic effect that is primarily related to activation of the extrinsic, Fas-mediated pathway of apoptosis in transformed cells. The increase of caspase 8 activity is also consistent with this scenario. In parallel to Fas/Fas ligand

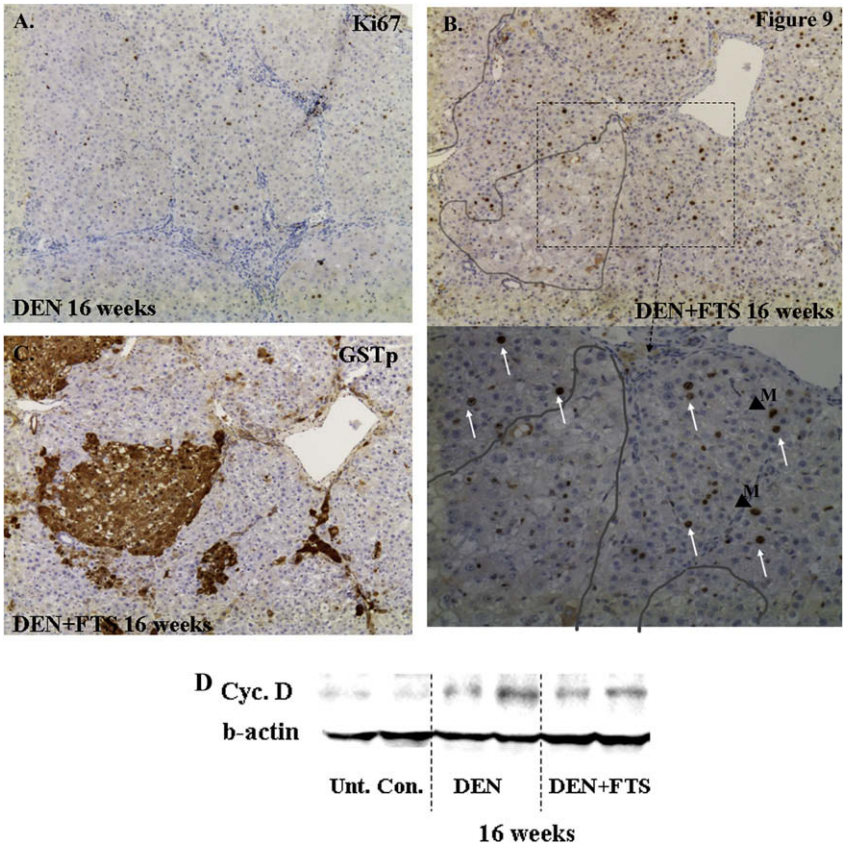

Fig. 9 – Assessment of proliferation after 16 weeks of DEN or DEN + FTS. Representative immunohistochemistry of Ki67 expression (A, B) showing unaffected hepatocytes proliferation (arrows) by FTS; (B) outside GSTp positive areas (B, C: grey lines). M = mitosis. (D) Representative Western blot of nuclear cyclin D expression confirming similar proliferation in DEN and DEN + FTS-treated rats.

up-regulation, we observe a strong activation of JNK in FTS-treated livers suggesting a potential link between JNK and apoptosis. Prolonged overactivation of the JNK signalling pathway, as seen in FTS-treated animals, has been proposed as a central inducer of hepatocyte death. The apoptosis promoting effect of JNK seems to be located above the level of mitochondrial involvement,[34] which is consistent with our data showing no impact of FTS treatment on the intrinsic, mitochondrial pathway of apoptosis. It remains to be determined whether JNK directly regulates apoptosis in our experimental setting or whether a pro-apoptotic effect occurs via crosstalk with the Fas pathway as reported in the literature.[35,36]

In conclusion, our findings indicate that FTS does affect pathological processes involved in hepatocarcinogenesis ultimately reducing formation of FAH. This effect is associated with inhibition of Ras membrane translocation and activity. In addition, the preventive effect of FTS on FAH formation is likely related to induction of apoptosis in transformed cells. The pro-apoptotic effect is associated with activation of the extrinsic, Fas-mediated pathway of apoptosis together with prolonged overactivation of JNK. Whether FTS may also inhibit the progression or induce regression of full blown HCCs remains to be proven. Given the lack of FTS toxicity *in vivo* in our and other studies,[12,13] FTS may thus represent a potential tool in HCC prevention for clinical use.

Conflict of interest statement

None declared.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2009.04.014.

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