Chemoprevention of hepatocellular carcinoma. Proof of concept in animal models

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Abstract

In the present work, we have evaluated the possibility of preventing liver carcinogenesis in rats at two stages of development. In the first series of experiments, we induced *foci of altered hepatocytes*, (FAH) which represent the first events in rodent liver carcinogenesis, using the chemical mutagens diethylnitrosamine (DEN) and acetylaminofluorene (AAF). In the second part of the work, we used repeated weekly injections of DEN only that gave rise to significant fibrosis at 11 weeks and the development of malignant tumours at 16 weeks. We chose to assess the chemopreventive effect of three different drugs : pioglitazone, lanreotide and S-trans-trans-farnesylthiosalicylic acid (FTS). Pioglitazone (PGZ) is an agonist of peroxisome proliferator-activated receptor gamma (PPARg), itself a member of the nuclear receptor superfamily, responsible for the modulation of a number of metabolic pathways, including cell differentiation, metabolism of lipids and inflammation. Lanreotide (LAN) is a somatostatin analogue that has an inhibitory effect on the release of several hormones, such as growth hormone and serotonine. FTS is a specific antagonist of the protooncogene Ras, tested here based on the rationale that Ras is activated in many hepatocellular carcinomas (HCC).

We showed that both PGZ and LAN were efficient in the first, pre-neoplastic model, by reducing the size of FAH, decreasing proliferation specifically in FAH by interacting with proteins of the cell cycle. We could also demonstrate that LAN increased apoptosis. In the second model, LAN was able to diminish the number of established HCC by decreasing proliferation, in parallel with an anti-fibrotic action. Furthermore, enhanced apoptosis and anti-angiogenic effects were observed when LAN was given from the start of the carcinogenic induction by DEN. The cellular mechanisms leading to its effects warrant further investigations. FTS also strongly inhibited the appearance of FAH and HCC in the second model, through a complete inhibition of Ras activation and the induction of pro-apoptotic pathways. On the contrary, PGZ did not prevent the appearance of neoplastic lesions. For these reasons, we did not analyse further its mechanism of action in the second model. Altogether, the results we obtained demonstrate an activity of both LAN and FTS, at the early onset of liver carcinogenesis, and later on when advanced fibrosis, cirrhosis and HCC are induced. These anti-tumoural effects could be complementary and will be tested in combination in the future. (Acta gastroenterol. belg., **2011**, 74, **34-44**).

Key words : hepatocellular carcinoma, chemoprevention, animal models, lanreotide, FTS.

Introduction

Hepatocellular carcinoma : Epidemiology and risk factors

Hepatocellular carcinoma (HCC) is the fifth cancer in the world and the third most common cause of cancer mortality (1). Its distribution is highly heterogeneous around the globe. This important variation reflects the many different causal factors that can lead to HCC. The major risk factor for HCC is liver cirrhosis, itself a consequence of chronic liver injury, mainly due to hepatitis B and C viruses, alcohol intake, but also to non-alcoholic steatohepatitis (NASH) in the context of diabetes mellitus and obesity (2).

The cirrhotic liver as a pre-neoplastic organ

Numerous epidemiologic evidences support the concept of cirrhosis being a condition associated with carcinogenesis, which develops after decades of chronic liver disease (3). First, it has been shown that HCC incidence increases in parallel to the fibrosis stage (4), with a risk that remains low the during chronic hepatitis stage, but rises exponentially at the cirrhotic stage. Second, when recurrence of HCC occurs within 2 years after a complete resection, it is considered as arising from occult intra-hepatic metastasis. By contrast, if HCC recurs later, it is considered as *de novo* HCC. Risk factors and prognosis are different in these 2 categories of recurrence (5,6). Recently, Hoshida *et al*. demonstrated convincingly that late recurrence of HCC was closely linked to the gene expression profile from the cirrhotic background rather than to the gene profile of the first tumour (7).

Current status on chemoprevention of HCC

Several levels of prevention can be considered (Fig. 1) : first, primary prevention represents treating or preventing the diseases leading to cirrhosis. Anti-viral treatments or vaccination are the main ways to achieve such prevention. Secondary prevention is the prevention of the first occurrence of HCC once cirrhosis is established. Finally, tertiary prevention refers to treatments that aim at preventing a second occurrence of HCC after the curative treatment of a first tumour. The national HBV vaccination campaign in Taiwan (8) reflects the most compelling proof of concept that primary prevention is indeed efficient. Ten years after having started the

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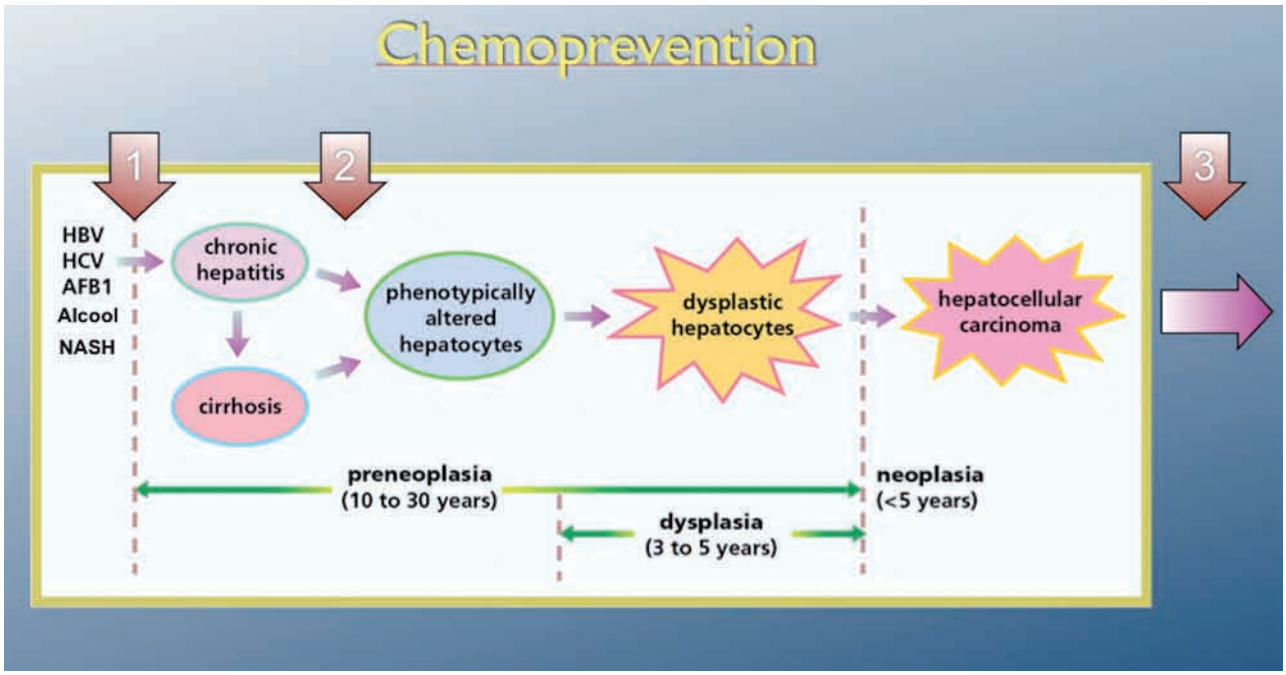

Fig. 1. — Liver carcinogenesis. Adapted from Thorgeirsson *et al*., 2002 (3). Arrows 1, 2, and 3 represent targets for primary, secondary and tertiary prevention respectively.

vaccination of newborns, a significant decline in mortality due to HCC was observed in vaccinated children over 6 years. For patients with HCV-related HCC, a meta-analysis showed a slight reduction in the occurrence of HCC in sustained responders after interferon (IFN) therapy (9). However, Lok *et al*. failed to show a beneficial effect of the pegylated form of IFN in preventing HCC occurrence in a large cohort of patients who did not have a response to anti-viral treatment (10). Other drugs have been tested for tertiary prevention of HCC. Polyprenoic acid has affinity for nuclear retinoid receptors, thereby inducing cell differentiation (11) and apoptosis (12). Polyprenoic acid prevented recurrence of a second HCC by nearly 50% after ablative treatment in a small cohort of Japanese patients (13). Since then, no study has been published that reproduced these impressive results and therefore the drug has not been implemented so far. More recently, other synergistic combinations using acyclic retinoid with trastuzumab, a monoclonal antibody blocking the *ErbB2* receptor (14), or acyclic retinoid and vitamin K2 (15) demonstrated anti-tumoural effect *in vitro*.

Peroxisome proliferator-activated receptor gamma (PPARg) and agonists

PPARg is a nuclear transcription factor that, once activated by its ligand, binds to specific response elements in the nucleus, called peroxisome proliferating response elements (PPRE), thereby activating target genes involved in proliferation, cell differentiation and angiogenesis. Activation of PPARg, by agonists such as thiazolidinediones (TZDs), has been shown to have an anti-cancer effect *in vitro* and *in vivo* in many cancer types (16,17). Anticancer effects of PPARg agonists on HCC have been observed in the last years both in cell cultures and in animal models suggesting a role for the PPARg pathway in the prevention and treatment of HCC. It is not clear whether these preclinical data can be extrapolated to human disease as we do not know with certainty whether PPARg is expressed in cirrhotic non-tumoural liver, or in HCC. This anticancer activity is due to inhibition of cell proliferation, by interfering with important cell cycle cyclins and cyclin-dependent kinase inhibitors, such as $p27^{kip1}$, but also to a pro-apoptotic action. The link between activation of the PPARg pathway and the anticancer activity of TZDs is, however, not clearly established. Finally, PPARg antagonists have shown anti-tumour activity by different mechanisms, mainly involving loss of cell adherence, migration and invasion. All these data are extensively reviewed in a paper we published recently (18).

Somatostatin analogues

Somatostatins (SMS) are natural hormones, produced by the endocrine cells of the GI tract as well as tumours (19). Their effects are mainly inhibitory on hormone secretion, but also anti-proliferative, both on normal and tumour cells (20). Due to their very short half-life, their use is limited in practice. Hence, synthetic analogues with longer half-lives have been developed : octreotide (OCT) and lanreotide (LAN). SMS and analogues have biological effects through binding with membranous somatostatin receptors (sstr). Five sstr have been identified (sstr1-5), with SMS binding with high affinity to all five, while SMS analogues have high affinity to

sstr2, moderate affinity to sstr3 and sstr5, and low affinity to sstr1 and sstr4 (19,21). In the clinical setting, SMS analogues inhibit Growth Hormone (GH) and Insulin Growth Factor 1 and are used to treat GH producing tumours (22) and digestive neuro-endocrine tumours (DNET). The anti-tumoural activity of analogues resides on the presence of sstr2, generally in great abundance, on the cancer cell surface (23). SMS analogues have direct (i.e mediated through sstr binding) and indirect anti-cancer effects (24). Direct effects, shown in many cancer cell types both *in vitro* and *in vivo* (25-27), comprise inhibiting proliferation, inducing apoptosis or initiating specific signal transduction pathways. Each receptor subtype induces different pathways and has a specific biological activity (28). Indirect anti-tumour effects mainly consist of inhibition of the release of growth factors, anti-angiogenic effects and immunomodulatory effects.

Many tumour vessels express sstr, with high affinity for SMS analogues, independently of sstr expression in the tumour (29). In contrast to normal liver, where no significant expression of sstr is found (30), sstr are heterogeneously expressed in human HCC (31,32). Authors have found that at least one sstr was expressed in 82% of the 56 HCC analyzed (32). The main anti-angiogenic mechanism seems to be related to a decrease in vascular endothelial growth factor (VEGF) synthesis (33), but also to inhibition of both the release and activity of other growth factors, such as platelet-derived growth factor (PDGF), IGF-1 and basic fibroblast growth factor (34). Studies have shown a direct anti-tumour effect *in vitro* in HCC cell lines, using OCT (35) or LAN (36). Furthermore, OCT has shown indirect anticancer activity by inhibiting angiogenesis in a HCC xenograft model (33). Because of the potential activity of SMS analogues on relevant pathways in liver carcinogenesis (i.e. cell cycle arrest, induction of apoptosis, inhibition of angiogenesis), we have used LAN in a preventive setting in animal models, an approach that has not been evaluated by others.

S-trans-trans-farnesylthiosalicylic acid (FTS)

The Ras gene products are 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 to downstream cytoplasmic or nuclear events resulting in various cellular responses, like proliferation, differentiation and/or apoptosis (37). Membrane anchorage is important for the biological activity of Ras proteins and depends on their farnesylcysteine group, being subject to post-translational modifications, such as isoprenylation (e.g. farnesylation) (38). Activation of genes of the Ras family (39) and/or Ras mutations have been reported in human HCC (40,41) or in human hepatoma cell lines (42). Recently, it has been outlined that Ras might be a potential target in human HCC (39,43).

FTS has been shown to act as a functional Ras antagonist in cells. It structurally resembles the farnesylcysteine group common to all Ras proteins. FTS acts predominantly on the active, GTP-bound forms of Ras proteins. It affects docking of active GTP-bound Ras on the cell membrane in a rather specific manner, by competition for putative membrane anchorage sites. As a consequence, Ras proteins are dislodged from their anchorage domains, their intracellular degradation is facilitated, thus reducing cellular Ras contents (44-46). FTS has been found to be a potent growth inhibitor of non-hepatic Ras expressing cancer cell lines in culture (47,48), of non-hepatic tumour cells xeno-grafted to nude mice and of hepatic stellate cells in vivo (49,50). Taking advantage of the properties of FTS and given the frequent involvement of Ras-dependent signalling pathways (e.g. the Ras/Mek/Erk pathway) in liver carcinogenesis, we prompted to evaluate the effect of specific Ras inhibition by FTS on tumour prevention.

Animal models of HCC

HCC arises in an inflammatory environment, driving proliferation through growth factor production and stimulating senescent cells that escape cell cycle checkpoints due to chromosomal instability. Reproducing such events in animal models is difficult, so that no perfect animal model exists. In line with the various pathways involved in the carcinogenesis of HCC, a multitude of models have been evaluated. A thorough review of animal models of HCC has recently been published (51). To evaluate possible similarities between rodent and human carcinogenesis, one interesting approach is the comparison of gene expression patterns in transgenic mouse models and human HCC (52). Using this approach, authors found expression profiles in different mouse models that were similar to expression profiles from HCC patients with good or bad prognosis. Eventually, this comparative approach applying genome wide microarray analysis could help identifying the best-fit mouse models. In order to understand liver carcinogenesis, several chemical agents have been used for induction of pre-neoplastic and neoplastic lesions in animals. Sequential administrations of carcinogens allow the study of very early carcinogenic events, thought to be similar between rodents and humans. It has been proposed, in line with a general assumption, that also in the liver, the first event in carcinogenesis would be an "initiation phase", caused by a mutagen resulting in irreversible mutations. *Per se*, these mutations would not be sufficient to give rise to neoplasms, but in the liver of rodents, would be associated with the appearance of so-called "foci of altered hepatocytes" (FAH). To grow, these foci need to be boosted through a "promotion phase", where most mutated cells undergo apoptosis but a few respond to promoting agents presumably due to a selective advantage and evolve to dysplastic foci, and then to nodules. If the promoting stimulus is removed, these foci will disappear, undergoing massive apoptosis (53). Finally, a third step, called "progression phase" is driven by a mutagen, causing irreversible DNA changes sufficient for neoplasms to appear and to be maintained.

Many chemicals have been used, either in a combined or in a sequential fashion. Their mechanisms of action are pure genotoxic effects (e.g. DEN, AAF), oxidative stress induction (thioacetamide), or other (phenobarbital). Diethylnitrosamine (DEN) is a DNA alkylating agent, producing reactive metabolites that interact with DNA to form adducts leading to genetic alterations (54). In rats, depending on the dose and mode of administration, it can either give rise to cirrhosis without evidence of HCC (55), HCC without cirrhosis (56), or cirrhosis and HCC in 16-18 weeks (57). This latter model is very interesting because of the coexistence of the cirrhotic microenvironment with HCC. An overexpression of the Epidermal Growth Factor Receptor ligand TGF□has been found in these DEN-induced HCCs (58). In a slightly different model using DEN, authors have shown beta-catenin mutations in 45% of HCC (59). When used in mice, DEN induces HCC that express a panel of genes also observed in human HCC with a poor prognosis (52). The administration of DEN initiates the appearance of cells expressing specific proteins that can be highlighted by immunohistochemistry. One protein specifically overexpressed in FAH is the « placental form of Glutathione S-transferase » of the rat, called GSTp or GST 7-7 (60). GSTp positive cells and foci can be quantified both in size and number using morphometric methods (61,62), allowing precise analysis of the effect of various carcinogens, but also of the effect of chemopreventive drugs.

Our work has focused on two different steps in the chronology of liver carcinogenesis. First, we used a sequential two-stage model using DEN and acetylaminofluorene (AAF), to analyse the effect of our drugs during the very early events of carcinogenesis, i.e. cellular initiation and promotion that lead to preneoplasia (Fig. 2a). Because these molecular events have been well characterized in this model and because of similarities of these events with human carcinogenesis, we believe that the model confers to our findings some validity with regard to the clinical setting. Second, we wanted to use a model that provided the inflammatory and fibrotic micro-environment and that could give rise to dysplastic foci, neoplastic nodules and HCC. Therefore the model using repeated injections of DEN only was chosen, because this model gives rise to significant fibrosis in 12 weeks, and coexisting cirrhosis and HCC after 16 weeks (Fig. 2b). Using this model, we aimed at assessing the effect of the medications at a more advanced stage, where cells are irreversibly engaged in carcinogenesis.

Is targeting the PPARg pathway with its agonist pioglitazone (PGZ) an effective chemopreventive approach in liver carcinogenesis ?

Based on the data available in the literature, we believed that targeting the PPARg pathway by using one of its potent synthetic agonist, i.e. PGZ, could be an interesting approach in HCC. As described in the introduction section, PPARg agonists elicit anti-fibrotic effects but also a re-differentiation effect on tumour cells. In the first part of the study, in a model that sequentially initiates hepatocytes using DEN, and then promotes the initiated cells with AAF, giving rise to FAH in a non-fibrotic setting, we showed, using quantitative morphometry and Western blot, that PGZ significantly decreases the size of these foci. Analysis of proliferation and apoptosis, assessed by immunohistochemistry, demonstrated decreased proliferation but no effect on cell death in rats treated with PGZ. These events were associated with an increased expression of the cyclin-dependent kinase inhibitor $p27^{kip1}$, compared to the non-treated group (63). Based on these encouraging results, we tested PGZ in a chemical model inducing sequentially significant fibrosis, then cirrhosis and HCC (57). HCC was induced by repeated injections of DEN, every week for 16 weeks, in male Wistar rats. PGZ was administered to the animals either early (from the 1st week onwards) or later (from the 11th week onwards), when significant fibrosis was present. Rats receiving only DEN formed the control group. The following results were obtained : first, only 4/10 rats survived the experiment when receiving PGZ from week 1 to 16, and 9/10 when receiving PGZ from week 11 to 16. Although the surviving rats in the group given PGZ from week 1 onwards were significantly leaner compared to rats receiving DEN only, the number of HCC found in the livers was not statistically different between groups. The results of this unpublished study show that PGZ does not prevent the appearance of hepatocellular carcinoma induced by DEN in the rat liver.

Parallel to this work, we have analysed the effect of PGZ in other models of liver injury, i.e. fibrosis induced by carbon tetrachloride (CCl_4), bile duct ligation (BDL) and choline deficient diet. We noticed that although PGZ was able to reduce the amount of fibrosis in the CCl_4 and choline deficient diet when introduced early in the fibrotic course, it was ineffective when administered later on, when significant fibrosis was already present. Furthermore, PGZ was not able to influence fibrogenesis in the BDL group (64). We also tested PGZ in a mouse model and in vitro in hepatic stellate cells (HSC). We observed that, although PGZ was biologically active, it did not prevent activation of HSC into myofibroblasts, nor was it effective in inhibiting fibrogenesis in vivo in two different mice strains (65). Based upon these results, we concluded that PGZ, although preventing the appearance of preneoplastic foci in rats in a model of very early carcinogenesis, is finally not effective in the prevention of HCC. This lack of effect could be due to a limited effect on fibrogenesis through the inability to prevent HSC activation, the key event in liver fibrogenesis.

The somatostatin analogue lanreotide is able to inhibitit early preneoplastic events and chemically induced hepatocellular carcinoma in the rat liver

Somatostatine analogues have shown anti-cancer effect, based on their ability to decrease proliferation,

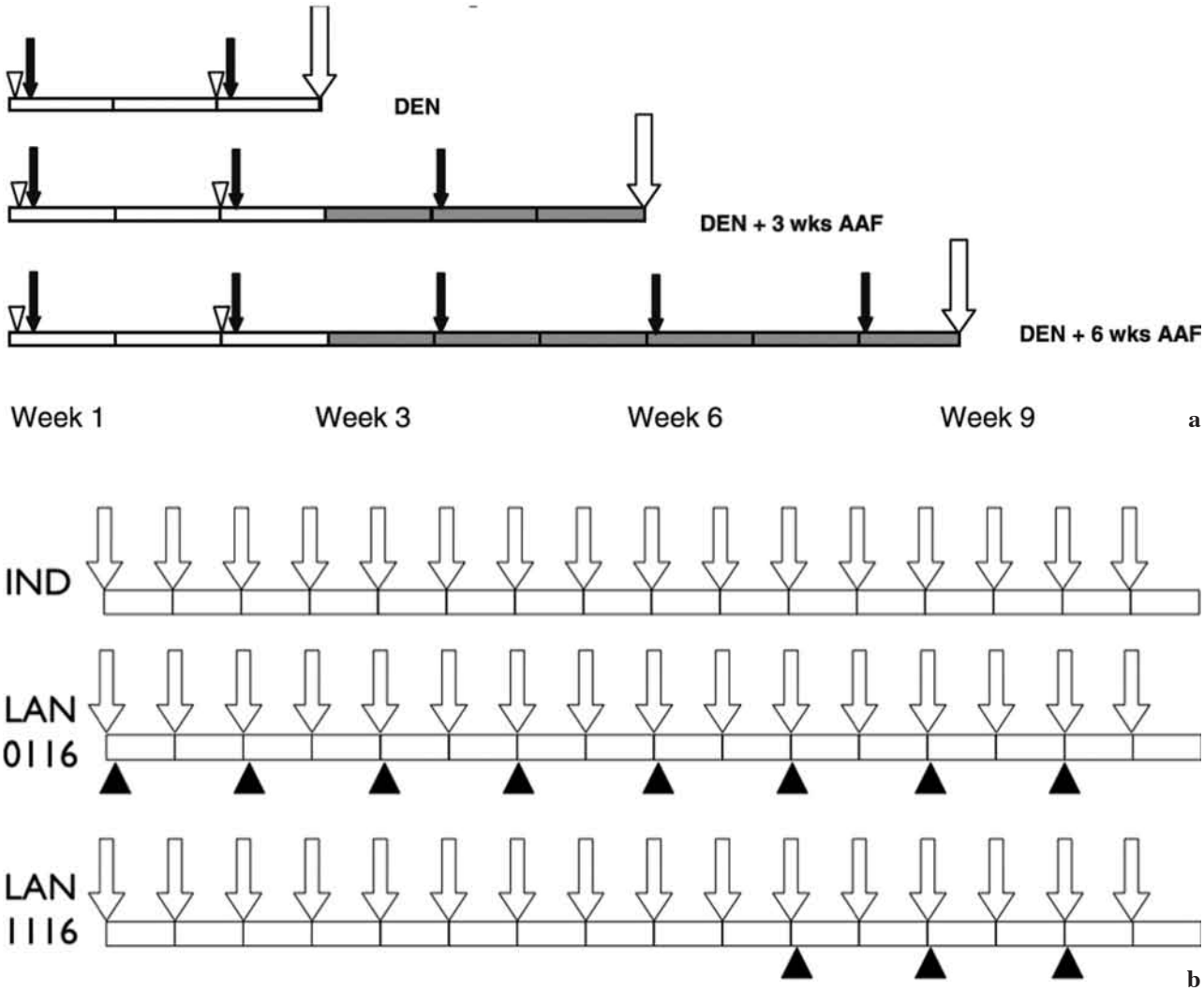

Fig. 2. — a. Scheme of the 2-stage carcinogenic model, using DEN and AAF. Lanreotide was injected I.M. in the thigh, 3 mg/kg diluted in saline, once every 2 weeks, starting the day after the first DEN injection (black arrows). Each group was submitted to DEN I.P. injections at week 0 and week 2 (DEN, open arrowheads), followed by one week rest. Then AAF was given by gavage 4 times a week for a 3 weeks (DEN + 3 wks AAF) or 6 weeks (DEN + 6 wks AAF) course (grey bars). Ten rats of the induced group and 5 rats from the lanreotide group were sacrificed (vertical white arrows) at end of initiation (DEN), after 3 weeks promotion (DEN + 3 wks AAF) or after 6 weeks promotion (DEN + 6 wks AAF) ; b. Scheme of the experimental model using DEN only. All animals received the same carcinogenic drug, i.e. DEN (white arrows), given intraperitoneally at a dose of 50 mg/kg body weight dissolved in saline. This dose was given every 7 days for 16 weeks. The rats were randomly assigned to one of the 3 experimental groups after the first DEN injection. Lanreotide (black arrowheads) was dissolved in saline at a concentration of 3 mg/kg and injected into the right thigh muscle every 14 days, starting at different times after the first DEN injection : either after 11 DEN injections (LAN 1116 group, n = 10) or directly after the first DEN injection (LAN 0116 group, n = 10). In the last group (IND, n = 10), rats received DEN only. DEN : diethylnitrosamine ; AAF : acetylaminofluorene ; LAN : Lanreotide.

induce apoptosis and inhibit angiogenesis. In liver injury models, they also elicited a modest anti-fibrotic effect. Thus, we wanted to assess capacity of somatostatine analogues to prevent liver preneoplastic and neoplastic lesions. We tested LAN in the two rat models described above (section '*Animal models of HHC*') and also used in the PGZ studies. In the first study, we showed a decrease of FAH induced by DEN and AAF. We demonstrated an anti-proliferative but also a pro-apoptotic effect of LAN on preneoplastic hepatocytes. The mechanisms that are believed to underlie these effects are increased expression levels of both p21 and p27, linked to a profound inhibition of cyclin D1, an important actor of the cell cycle, responsible for the G1-S transition phase (66). In the second study, we first demonstrated the presence of sstr subtypes 2, 3 and 5 in normal and cirrhotic liver of rats. We then showed that LAN was able to diminish the number of HCC (Fig. 3a-b) by decreasing proliferation, in parallel with an anti-fibrotic action, shown in Fig. 4a-d. LAN was either started just after DEN injections, or after 11 weeks, when significant fibrosis was present. It is remarkable to note that even

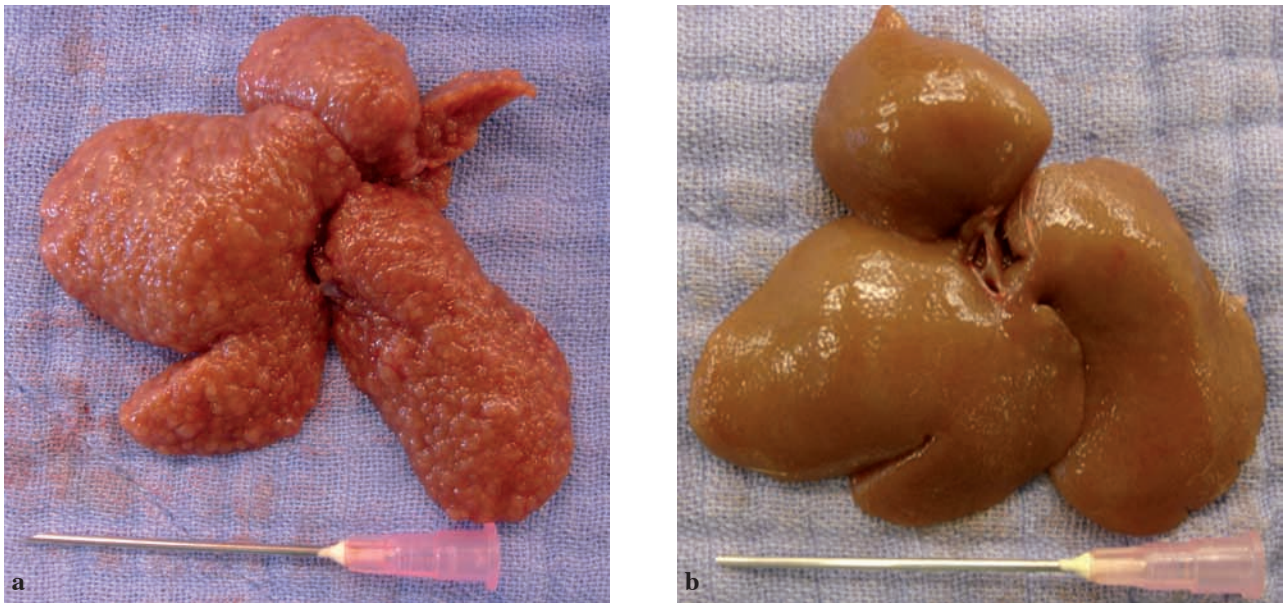

Fig. 3. — a. Macroscopic appearance of the liver in an animal injected with DEN for 16 weeks, showing multiple whitish, dyschromic nodules ; b. Macroscopic appearance of livers in the group treated with LAN from week 1 to 16. Note the smooth aspect of the liver surface, with very few dyschromic nodules at the liver surface. DEN : diethylnitrosamine ; LAN : Lanreotide.

when LAN was administered at that late moment in the carcinogenic process, we observed a significant effect both on fibrosis and on the number of HCCs. However, increased apoptosis and anti-angiogenic effects were only observed when LAN was given from the start of the carcinogenic induction by DEN, suggesting that an additional effect could be obtained when the chemopreventive action of LAN is initiated early. This series of experiments support the concept of chemoprevention with LAN in HCC, acting both on early and late events of liver carcinogenesis. LAN showed an anti-proliferative effect on hepatocytes, mainly through inhibition of key actors of the cell cycle. LAN also stimulated apoptotis of altered hepatocytes and inhibited neo-angiogenesis, which are key elements of liver carcinogenesis (67).

Targeting Ras with its inhibitor farnesylthiosalicyclic acid (FTS) is an effective way to prevent human HCC cell proliferation in vitro and HCC formation in the rat liver

Because activation of Ras dependent signalling pathways such as the MAPK (mitogen-activated protein kinase : Ras/Raf/Mek/Erk) pathway has been shown to be frequently involved in liver carcinogenesis, and because targeting these pathways has shown to be clinically relevant, we wanted to evaluate the effect of specific Ras inhibition on tumour prevention. To achieve Ras inhibition upstream of its intracellular signalling cascades, we used FTS, a molecule that has been shown to act as a functional Ras antagonist in cells. We tested the efficacy of FTS in three different pre-clinical situations : *in vivo*, in a well described model of liver regeneration following partial hepatectomy (PH) in the rat, but also in the model of sequential fibrosis and HCC, using DEN, as for our previous experiments with PGZ and LAN and *in vitro*, in human HCC cell lines. In the first experiments, rats were administered FTS intraperitoneally and killed 12, 24 and 48 h after PH. Cell proliferation, phosphorylation of members of the MAPK pathway and levels and activity of cell cycle effectors were assessed in FTS-treated rats compared with controls. FTS significantly decreased proliferation of hepatocytes after PH. Unlike control rats, cell membrane expression of Ras was decreased in FTS-treated animals after PH, resulting in decreased Raf membrane recruitment and phosphorylation and in reduced phosphorylation of ERK1/2 (extracellular-signal-regulated kinase 1/2). The antiproliferative effect of FTS was linked to a decrease in expression and activity of the cyclin E/Cdk2 complex, without affecting cyclin D and Cdk4. In addition, this anti-proliferative effect of FTS was also observed in the human HCC cell line HepG2 *in vitro (*68). In our second series of experiments, we used the model of chemical hepatocarcinogenesis induced by weekly intraperitoneal injection of DEN. In animals receiving DEN only, we found an increased membranous expression and activation of the Ras protein, which was completely abrogated by the administration of FTS. FTS prevented tumour development (Fig. 5a-c) and dramatically reduced foci expressing the tumour marker GSTp (Fig. 6). We found that FTS induced several apoptotic features : increased Tunel positive cells in transformed hepatocytes, up-regulated caspase 3 and 8 activity, induced Fas, Fas ligand and JNK phosphorylation that occured independently of TNF□and Trail. Cytochrome C release, Bax, Bcl2, Bcl-xl, actors of

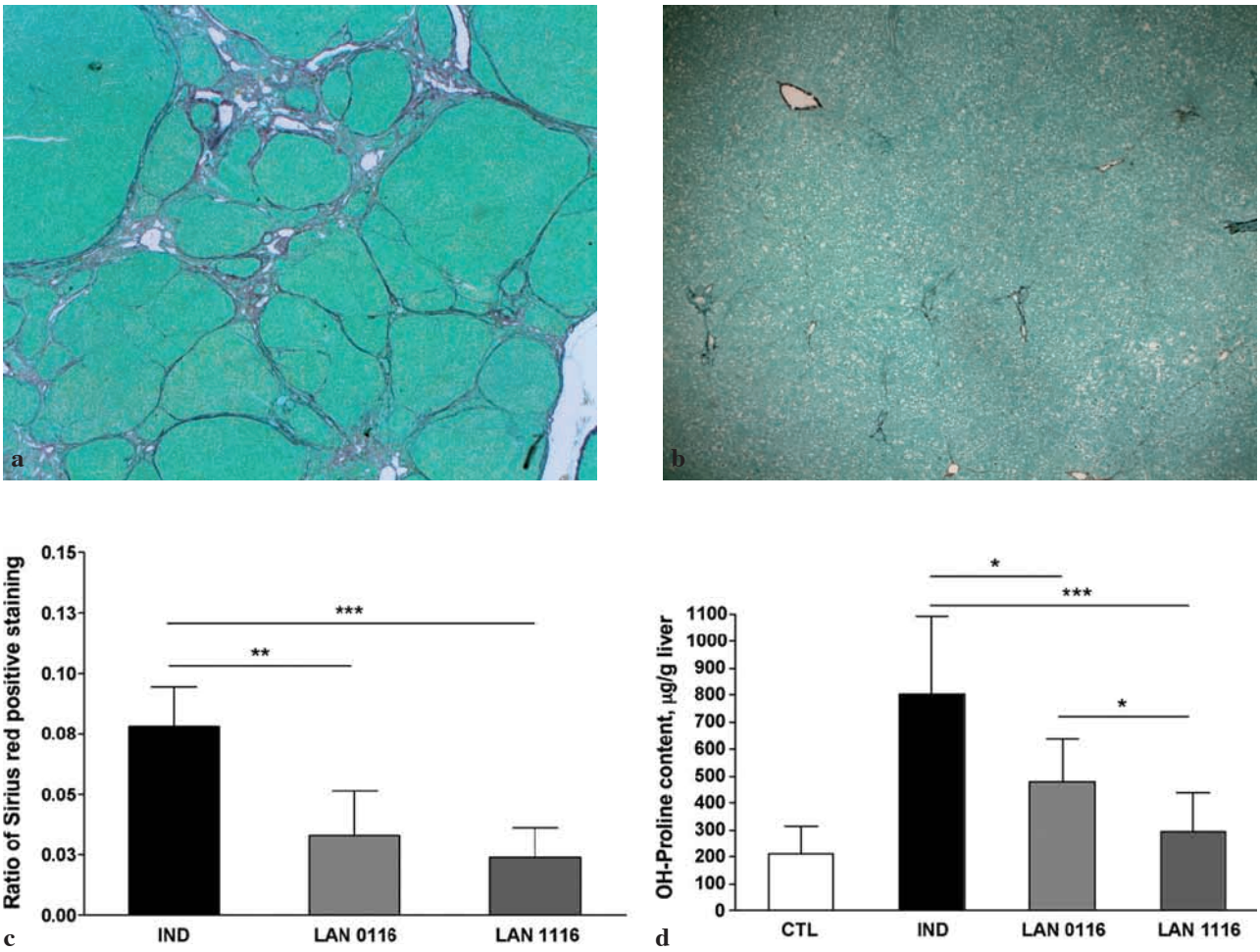

Fig. 4. — a. Sirius red expression, determined by immunohistochemistry in the induced group (IND) ; b. Sirius red expression, determined by immunohistochemistry in animals treated with lanreotide (LAN) for 16 weeks ; c. Morphometric quantification of Sirus red expression in the induced group (IND), in animals treated with LAN for 16 weeks (LAN 1116) and 6 weeks (LAN 0116) ; d. Hydroxyproline content in normal liver (XTL), taken as reference, in animals treated with LAN either for 6 weeks (LAN 1116) or for 16 weeks (LAN 0116), and in the induced group (IND).

mitochondrial-induced apoptosis, were not affected by FTS, nor was proliferation (Ki67 and cyclin D expression) in GSTp negative (non-transformed) areas of the liver. So we concluded that FTS elicited a dual effect of increased apoptosis in DEN-transformed cells, and compensatory proliferation in presumably non-transformed cells (69). These data provide arguments that targeting the Ras pathway with a specific inhibitor results in a significant chemopreventive effect, by inhibition of proliferation of cancer cells and mainly through activation of apoptotic processes in transformed hepatocytes in a model of fibrosis and cancer.

Conclusions and perspectives

The rationale for chemoprevention in hepatocellular carcinoma is obvious. The disease is frequent, the population at risk can be identified and once the tumour is present, the prognosis is poor.

Here, we report the results obtained with three drugs, belonging to very different therapeutic classes. **1**. PGZ, a PPARg agonist, used in clinical practice for type 2 diabetes, shows an excellent safety profile and is able to induce cellular differentiation, an important property in chemoprevention. **2**. LAN, a synthetic analogue of somatostatin, also presents a good toxicity profile in the clinical setting. SMS analogues have shown anti-neoplastic effects in many cancer types (24) and anti-fibrotic effects in rats (70). **3**. FTS, a specific antagonist of the proto-oncogene Ras that has recently been developed as an anti-cancer drug, proved its efficacy in several *in vitro* and *in vivo* tumour models (49). In addition, data from a recently published phase I study show good toxicity profile (71).

The animal models used in the present work address two questions : First, to test their effects in primary prevention, asking the question whether PGZ and LAN can inhibit very early changes in liver carcinogenesis ? To

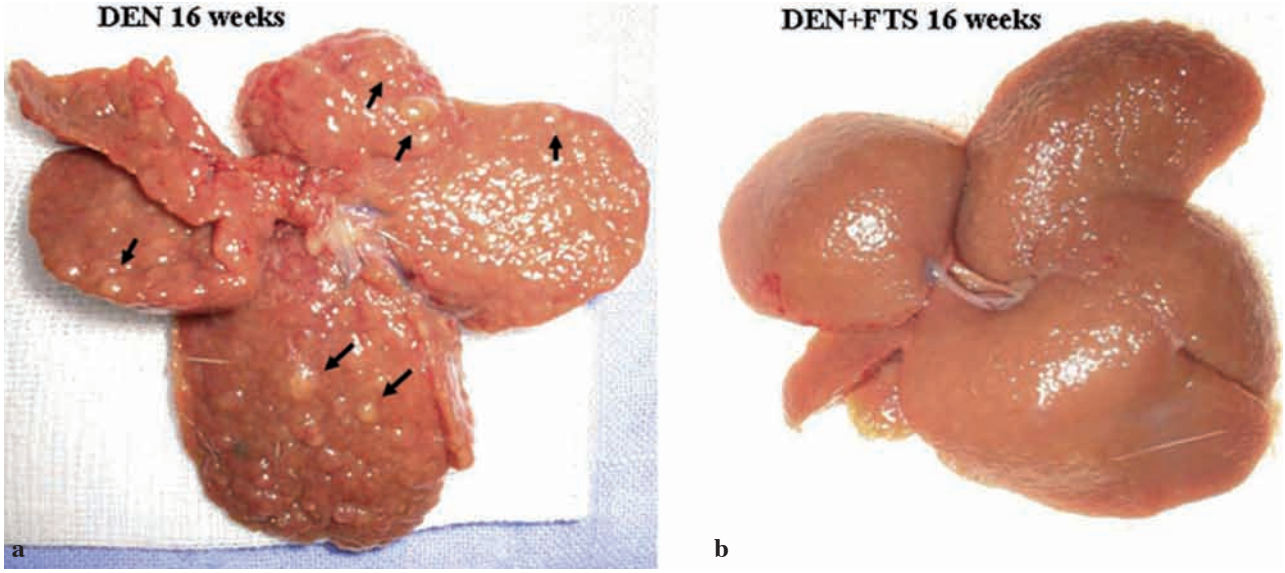

Fig. 5. — Macroscopic nodule formation after 16 weeks of DEN or DEN + FTS. Macroscopic appearance of livers showing multiple whitish, dyschromic nodules (arrows) at the liver surface of DEN-induced animals (a) that is prevented by FTS treatment (b). Overall nodule count in individual animals (c) showing inhibition of surface nodule formation in DEN + FTS treated animals. DEN : diethylnitrosamine ; FTS : farnesylthiosalicylic acid.

answer this question, we induced foci of altered hepatocytes (FAH) in rats in a sequential way, using DEN and AAF as initiator and promoter of preneoplastic hepatocytes. Second, in secondary prevention, can PGZ, LAN or FTS prevent the appearance of HCC in the setting of significant fibrosis ? To address this point, we induced fibrosis and malignant tumours in rats by using DEN.

We demonstrated that PGZ decreases FAH in the model of early carcinogenesis, mainly by inhibiting cell proliferation, through interference with cyclins and cyclin inhibitors. We thus confirmed *in vivo* the results observed by authors using PGZ in *in vitro* models (72,73). However, PGZ was unsuccessful in preventing cirrhosis and the onset of and dysplastic nodules, in the second model used. This lack of efficacy in chemoprevention precluded us from further exploring the effect of PGZ in HCC.

LAN was able to decrease FAH in the early carcinogenesis model, by acting both on proliferation and apoptosis of the altered hepatocytes. Furthermore, it was also effective in a model of more advanced carcinogenesis, with established cirrhosis and HCC. In this context, LAN significantly decreased the number of HCC and, interestingly, also the fibrosis induced by DEN given weekly for 16 weeks in male rats suggesting that LAN could be a potential drug for chemoprevention.

FTS was also very efficient in reducing the number of pre-neoplastic and neoplastic lesions in the model inducing cirrhosis and HCC. FTS specifically and completely inhibited Ras activation in this model, suggesting the causative relation between Ras inhibition and the chemopreventive effect of FTS.

Liver carcinogenesis is a highly complex process, involving many different pathways. Up to now, existing animal models, including the one we used, do only partially explore the complex network of interactions between the different pathways in the carcinogenic process. Although the second model, using DEN, recapitulates the inflammatory and fibrotic environment, similar to that faced in humans, it is still difficult to affirm a parallelism between the animal and human situation, as the molecular pathways involved in both human and chemical-induced carcinogenesis are not completely understood. In addition, the lesions of interest, i.e. the FAH in the first model and the dysplastic and HCC nodules in the second model, were difficult to identify macroscopically. Therefore, the possibility to isolate those areas for mechanistic analyses remains challenging. Nevertheless, our experiments provide a solid basis for future studies exploring the potential of chemopreventive molecules such as LAN and FTS.

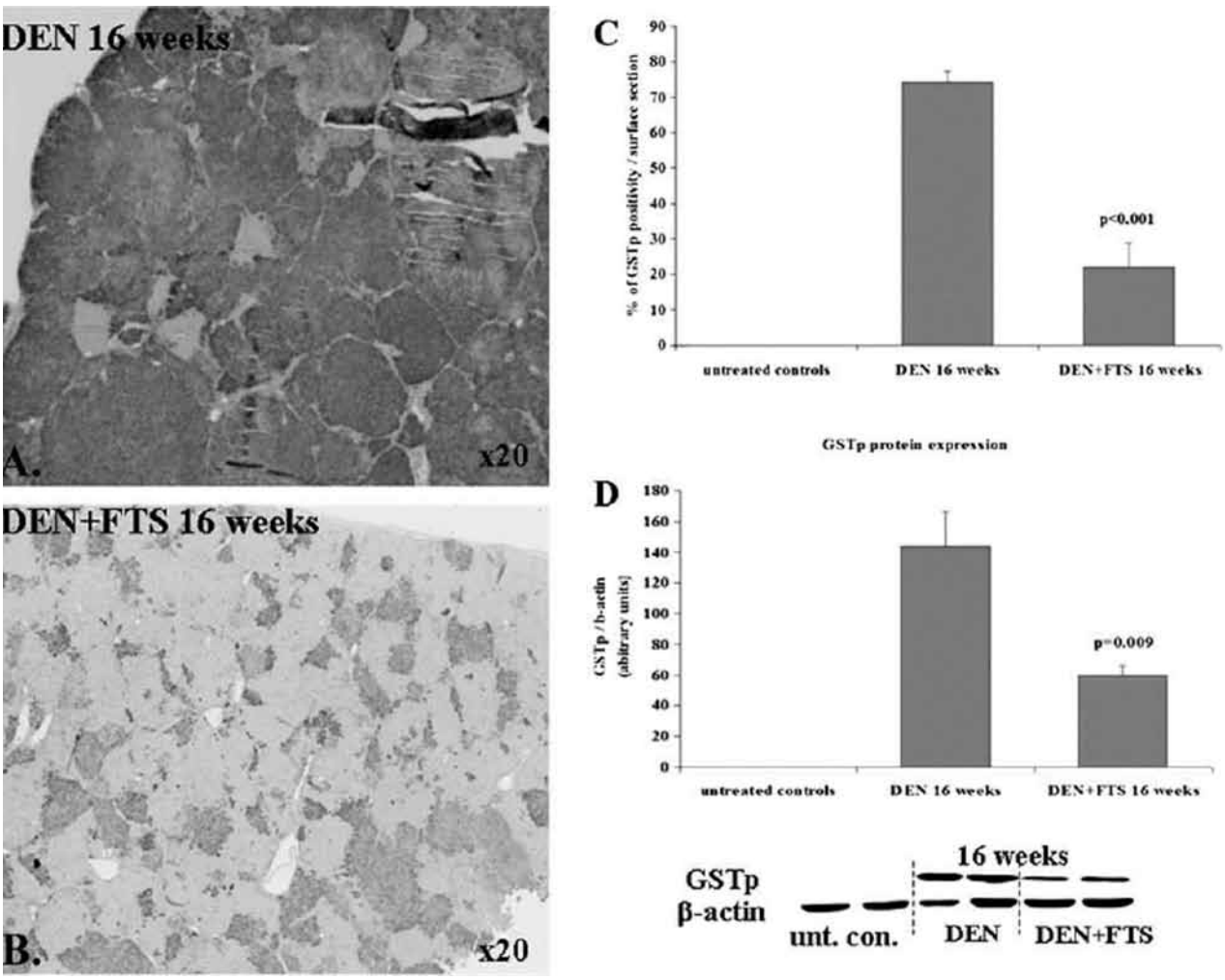

Fig. 6. — GSTp expression after 16 weeks of DEN or DEN + FTS. (A, B) Representative immunohistochemistry of GSTp expression. (C) Quantitative morphometry of GSTp positive areas normalised to the section surface showing strongly reduced GSTp expression in DEN + FTS-treated animals. (D) Representative Western blot and quantification of GSTp expression in liver homogenates confirming the immunohistochemistry findings. DEN : diethylnitrosamine ; FTS : farnesylthiosalicylic acid ; GSTp : placental form of Glutathione S-transferase.

Consequently, the effect of both LAN and FTS will have to be evaluated in cellular models in the near future, to get a better understanding of the subcellular mechanisms and pathways involved in their efficacy. In particular, the effect of LAN on fibrosis induced by DEN deserves further analysis, as well as its anti-angiogenic and anti-proliferative properties. One should try to demonstrate and locate the presence of sstr on isolated hepatocytes, liver endothelial cells, Küpffer cells and stellate cells. The effect of LAN *in vitro* on the main actors in liver fibrogenesis, i.e. hepatic stellate cells should be assessed. To evaluate the anti-angiogenic properties of LAN, the production, by neoplastic cells and endothelial cells, of the main growth factors involved in angiogenesis, including VEGFa (74) but also other VEGF isoforms and receptors should be studied. Finally, to understand the molecular pathways by which LAN decreases proliferation, one would like to explore in more details the actors of the cell cycle, and of known important gate-keepers involved in liver carcinogenesis. These pathways should be studied on neoplastic cells, but also on surrounding cells, as it has been shown that recurrence of HCC after resection is much more dependant on genetic signature from the cirrhotic background tissue than from the initial tumour itself (7). As FTS has been evaluated in the same *in vivo* model, with the same drawbacks, mechanistic pathways downstream of Ras, in particular the MAPK pathway, but also other Ras effectors such as the AKT-mTOR pathway need to be studied thoroughly. Finally, it could be worthwhile to combine LAN and FTS in animal models to analyse their potential synergic effect.

We believe that in the future, LAN and FTS, given their good tolerance profile and their efficacy, deserve to be evaluated in humans, for example after the curative treatment of a first HCC, in tertiary prevention.

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