EUROPEAN JOURNAL OF CANCER 43 (2007) 1755–1763

available at www.sciencedirect.com

journal homepage: www.ejconline.com

The PPARgamma agonist pioglitazone inhibits early neoplastic occurrence in the rat liver

I. Borbath[a,*], *I. Leclercq*[a], *P. Moulin*[b], *C. Sempoux*[b], *Y. Horsmans*[a]

[a]*Gastroenterology Laboratory, Cliniques Universitaires St-Luc, Université catholique de Louvain, 10, avenue Hippocrate, 1200 Brussels, Belgium*

[b]*Pathology Department, Cliniques Universitaires St-Luc, Université catholique de Louvain, 10, avenue Hippocrate, 1200 Brussels, Belgium*

ARTICLE INFO

Article history:
Received 12 January 2007
Received in revised form 25 April 2007
Accepted 4 May 2007
Available online 19 June 2007

Keywords:
Hepatocellular carcinoma
Prevention
Peroxisome proliferator-activated receptor
Proliferation

ABSTRACT

Hepatocellular carcinoma (HCC) is increasing worldwide and is the fifth main cause of cancer-related death. HCC develops on a preneoplastic organ, the cirrhotic liver. Therefore, chemoprevention could play a role in the therapy of HCC.

We evaluated the preventive effects of pioglitazone, a peroxisome proliferator-activated receptor gamma agonist, on the induction of early carcinogenic events. We monitored pre-neoplastic foci induced by a two-stage initiation/promotion model of hepatocarcinogenesis in rats, using diethylnitrosamine and acetylaminofluorene. Pioglitazone treatment was initiated the day after the first diethylnitrosamine injection. By quantitative morphometry and Western blot, we showed that pioglitazone significantly decreases the size of pre-neoplastic foci. Analysis of proliferation and apoptosis, assessed by immunohistochemistry, demonstrated decreased proliferation but no effect on cell death in rats treated with pioglitazone. These events were associated with an increased expression of the cyclin-dependent kinase inhibitor $p27^{kip1}$, compared to the non treated group.

In conclusion, pioglitazone inhibits early carcinogenic transformation in a two-step rat model. As pioglitazone has a low toxicity profile, we believe it would be interesting to evaluate its effect in chemoprevention of HCC in humans in a clinical setting.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth main cause of cancer-related death. Its incidence is in progression, with 2–5 new cases per 100,000 inhabitants/year in western countries and more than 20 per 100,000 inhabitants/year in Asia.[1,2] Curative treatment can be proposed in only 10–20% of patients.

The particularity of HCC is that it develops in an organ considered to be pre-cancerous, namely the cirrhotic liver. After surgical resection, tumoural relapse is observed with an incidence of 20% per year. Therefore, chemoprevention could play an important role in the therapeutic strategy of this disease.

In experimental carcinogenesis, pre-neoplastic foci of altered hepatocytes emerge weeks or months before the appearance of hepatocellular adenomas and HCC.[3] Similar progression has been described in human hepatocarcinogenesis.[4] This fact has led to the development of a number of *in vivo* systems to study early neoplasia in rat liver.[5] The initiation-promotion or two-stage model of cancer development used in this study mimics the early events of the latent period of human carcinogenesis. The initiation stage of cancer development can be produced in rat liver by the administration of diethylnitrosamine (DEN),[6] a carcinogen that causes DNA ethylation and mutagenesis.[7] Necrogenic doses of DEN cause massive necrosis followed by regeneration, associated with

* *Corresponding author*: Tel.: +32 2 764 28 20; fax: +32 2 764 89 27.
E-mail address: ivan.borbath@clin.ucl.ac.be (I. Borbath).
0959-8049/$ - see front matter
doi:10.1016/j.ejca.2007.05.005

genomic mutations. Administration of promoting agents, such as 2-acetylaminofluorene (AAF), induces selective enhancement of proliferation of the initiated cell populations over non-initiated cells in the target tissue.[8] This model has proven to give rise to HCC several months after initiation.[9]

Peroxisome proliferator-activated receptors (PPAR) are members of the superfamily of steroid nuclear receptors. Three isotypes have been identified: α, β and γ. The PPARγ functions as a transcription factor activated upon ligand binding. It heterodimerises with the Retinoid X receptor (RXR) and binds to specific response elements called peroxisome proliferating response elements,[10] thereby regulating the transcription of target genes. PPARγ is involved in the metabolism of lipids, the insulin sensitisation of peripheral cells, it has anti-inflammatory action, can induce differentiation and inhibit proliferation of cancer cells.[11,12] Thiazolidinediones (TZDs) are synthetic ligands for PPARγ that act as agonists. Owing to their insulin sensitising effect, they are used in the treatment of type II diabetes mellitus. The effect of these drugs on growth of tumoural cell lines has been extensively studied. They have been shown to inhibit proliferation and enhance apoptosis.[13] This effect is assumed to be dependent on the level of PPARγ expression. Among the TZDs, troglitazone has been shown to inhibit cell proliferation through interfering with cyclins and cyclin-dependent kinases (CdK) inhibitors such as $p21^{waf1}$ and $p27^{kip1}$; thereby inducing cell cycle arrest and terminal differentiation.[14–19] Troglitazone also enhanced apoptosis of liver cancer cell lines *in vitro* through activation of caspase 3.[19–21] Pioglitazone has also been shown to reduce survival and proliferation of liver preneoplastic cells *in vitro*[14] as well as in animal models *in vivo*.[22,23] Furthermore, pioglitazone prevented collagen synthesis and hepatic stellate cell activation in animal hepatic fibrosis models, suggesting additional benefit of PPARγ agonists in the context of hepatic cirrhosis.[24,25] This could have an indirect effect on primary liver cancer prevention.

In this study, we focused on the evaluation of the effects of pioglitazone on the early carcinogenic events induced by a two-stage initiation/promotion model of hepatocarcinogenesis in rats. We studied its effects after initiation by DEN and after initiation/promotion induced by administration of DEN and AAF, on the formation of pre-neoplastic hepatic foci, both in terms of number and size. These foci are best identified by the antibody raised against the placental form of rat glutathione S-transferase (GSTp). This iso-enzyme has been described as the most effective single marker of hepatic preneoplasia in the rat.[26]

We evaluated the effect of pioglitazone on proliferation and apoptosis in the pre-neoplastic foci and the surrounding liver, to gain insight into the mechanisms of the drug effects. Finally, we analysed the expression of cell cycle regulators $p21^{waf1}$ and $p27^{kip1}$, which are known to inhibit cyclin-dependent kinases and block the cell cycle in the G1 phase in liver cancer cell models *in vitro*.[15,16,18]

2. Materials and methods

2.1. Animals and treatment

Male Wistar rats (180 ± 20 g) were purchased from Charles River Laboratories, Brussels, Belgium. They were maintained on an automatic 12 h light/dark cycle. All animals received humane care in compliance with the regulations of the Université catholique de Louvain (UCL), Belgium.

All animals were subjected to a two-phase carcinogenic model, as described elsewhere.[27] Briefly, initiation was induced by DEN (Sigma Chemical Co., St. Louis, MO), injected intraperitoneally at a dose of 150 mg/kg body weight at weeks 0 and 2. Then, the promotion phase was performed using AAF (Sigma Chemical Co.), dissolved in methyl sulfoxide and then suspended in tricaprylin (Sigma Chemical Co.) to a final concentration of 8 mg/mL. The rats received 20 mg/kg body weight of AAF/tricaprylin suspension by gavage for 4 consecutive days per week for 3 weeks or 6 weeks. The rats were randomly assigned to one of the two experimental groups after the first DEN injection and received either pioglitazone ($n = 15$, kind gift of Takeda chemicals, Osaka, Japan) mixed with food at a concentration of 0.01% *wt/wt*, either a standard rat chow ($n = 30$, AO4, Usine d'Alimentation Rationnelle, Epinay-sur-Orge, France). Food and water were accessible ad libitum. A scheme of the experimental set-up is shown in Fig. 1. Ten rats from the induced group and five rats from the pioglitazone group were sacrificed at the end of the initiation period (DEN), after 3 weeks promotion course (DEN + 3 weeks AAF) and after 6 weeks promotion course (DEN + 6 weeks AAF). They were anaesthetised by ether inhalation, livers were excised and samples of anterior and posterior lobes were immersed in 4% buffered formaldehyde for histological and immunohistochemical analyses. The remaining liver tissue was snap-frozen in liquid nitrogen and stored at –80 °C for Western blotting.

2.2. Immunohistochemistry (IHC)

Identification of pre-neoplastic foci: Immunohistochemical staining was performed with an antibody raised against the placental form of rat glutathione S-transferase (GSTp; 1:100 dilution, MBL, Nagoya, Japan) revealed by En-Vision system (Dako, Denmark). Sections were counterstained with haematoxylin. For each animal, several low magnification fields (2.75 μm/pixel) that cover 1–1.5 cm^2 of the liver slides were taken at random in a representative area and digitised through a Zeiss microscope using a KS-400 system (Zeiss, Munich, Germany) coupled with a DAGE-MTI CCD72 camera (Michigan City, IN). The images were segmented by the operator in order to define GSTp-labelled foci. The area of each focus and the ratio of stained area to total area were then measured. The number of pre-neoplastic foci per liver were derived from the digitised images using the Foci 3D estimation software (kind gift of Dr. Xu and Dr. Pitot, McArdle Laboratory for Cancer Research, Madison, USA).[28]

2.2.1. Determination of proliferative index

Liver sections (5 μm thick) from each animal were stained using an anti-Ki67 antibody (1:50 dilution DAKO, Denmark) and counterstained with haematoxylin. Using a 10× magnification field, at least 1000 hepatocytes were assessed for Ki67 labelling. Proliferative index was expressed as positive cells scored per 100 hepatocytes. Proliferative index was also determined using an antibody against the phospho-Histone H3 (Ser10) (1:100 dilution, Cell Signaling Technologies, Beverly,

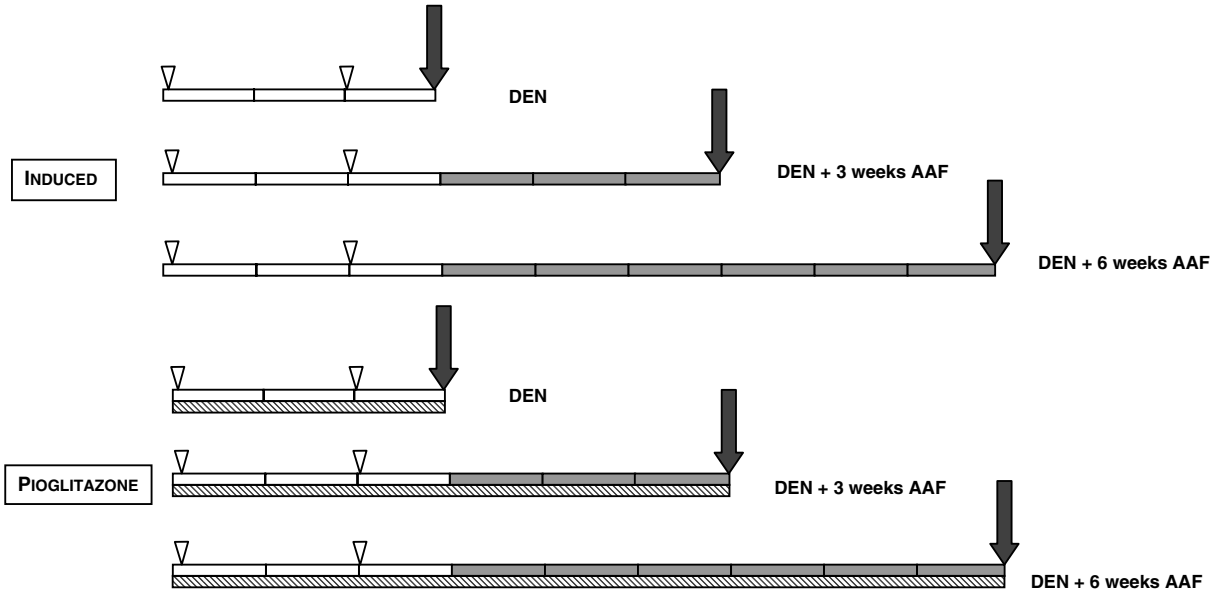

Fig. 1 – Scheme of the two-stage carcinogenic model. Rats were allocated to no treatment (induced, *n* = 30) or treatment with pioglitazone (pioglitazone, *n* = 15, dashed line). Each group was submitted to DEN I.P. injections at week 0 and week 2 (DEN, open arrowheads), followed by one week rest. Then AAF was given by gavage four times a week for 3 weeks (DEN + 3 weeks AAF) or 6 weeks (DEN + 6 weeks AAF) course (grey bars). Ten rats of the induced group and five rats from the pioglitazone group were sacrificed (vertical gray arrow) at end of initiation (DEN), end of initiation followed by 3 weeks promotion (DEN + 3 weeks AAF) and end of initiation followed by 6 weeks promotion (DEN + 6 weeks AAF). Pioglitazone was given mixed with food at 0.01% *wt/wt*, starting just after the first DEN injection (dashed line).

USA). To assess the proliferation activity in GSTp positive foci compared to surrounding hepatocytes between the experimental groups, liver sections were examined after a double immunohistochemical staining with anti–GSTp revealed in red with Fast Red (Sigma, Chemical Co., St. Louis, MO) and anti-Ki67 antibody revealed in brown. The number of proliferating cells within GSTp–positive foci and surrounding liver tissues was determined by examining at least 1000 and 5000 hepatocytes, respectively.

2.2.2. *Determination of apoptotic index*

The apoptotic index was determined by double immunostaining using an antibody against anti-cleaved caspase 3 (1:100 dilution, Cell Signaling Technologies, Beverly, USA) revealed in brown with diaminobenzidine and anti-GSTp revealed in red with Fast Red (Sigma, Chemical Co., St. Louis, MO).[29] At least 1000 and 5000 cells were counted in pre-neoplastic foci and surrounding tissues respectively. Apoptotic index was expressed as the number of apoptotic cells per 100 hepatocytes.

2.3. *Western blot*

Liver homogenates (50 to 100 μg of proteins) were resolved on a SDS–PAGE gel and transferred to a PVDF membrane (Hybond P, Amersham Pharmacia, UK). The following antibodies and incubation conditions were used: rabbit anti-GST polyclonal antibody (MBL, Nagoya, Japan) 1/100 for 2 h at room temperature; mouse polyclonal anti-cyclin D1 antibody (clone M-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1/2000 for 2 h at room temperature, rabbit polyclonal anti- $p21^{waf1}$ (C-17, Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1/1000 overnight at 4 °C, rabbit polyclonal anti- $p27^{kip1}$ (C-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1/1000 overnight at 4 °C. All incubations were carried out in TBS containing 1% skimmed milk. The antigen–antibody reaction was visualised using the Enhanced Chemiluminescence detection system (Amersham, UK) or the Renaissance Enhanced Luminol Reagent (NEN Life Science) and exposure of the membranes to X-Omat Blue XB-1 films (Kodak Scientific Imaging, Rochester, NY, USA). All membranes were stripped and reprobed with beta-actin antibody to assess equivalence of protein loading. The amount of immunoreactive protein was quantified using a Gel Doc 2000 scanning device and software (Bio Rad, Nazareth, Belgium) and normalised to beta-actin signal.

2.4. *Statistical analysis*

All results are expressed as mean ± SE. Data were compared using the Mann–Whitney U test, with a p value less than 5% being considered as significant.

3. Results

There was no mortality during the experimental period. Administration of pioglitazone did not significantly affect rat body weight compared to the induced group. However, mean liver weight was significantly less in pioglitazone treated rats after DEN + 6 weeks AAF: 18.4 ± 3.3 versus 13.3 ± 1.3 g ($p < 0.01$). Also liver to body weight ratio was decreased significantly: 5 ± 0.3% versus 3.6 ± 0.12% in rats receiving the induction scheme only ($p < 0.01$).

3.1. *Effect of pioglitazone on pre-neoplastic foci extension*

In the induced group, pre-neoplastic foci represented 2% of the liver section after DEN, it increased to 21% and 63.5% of the liver section surface after 3 and 6 weeks of promotion, respectively (Fig. 2). Conversely, because of the confluence of the foci, the number of pre-neoplastic foci per liver significantly decreased, from 23×10^4 after DEN to 7.7×10^4 after DEN + 6 weeks AAF (data not shown).

Pioglitazone treatment did not affect the pre-neoplastic foci surface after DEN. After DEN + AAF, pioglitazone treatment noticeably reduced pre-neoplastic foci surface compared to that observed in rats receiving the induction scheme only: from 21 ± 25% in the induced group to 4.9 ± 3.6% in the pioglitazone-treated group after DEN + 3 weeks AAF ($p = 0.1$) and from 63.5 ± 14% in the induced group to 47 ± 21% in pioglitazone-treated rats after DEN + 6 weeks AAF ($p = 0.05$). This decrease in GSTp expression in pioglitaz-

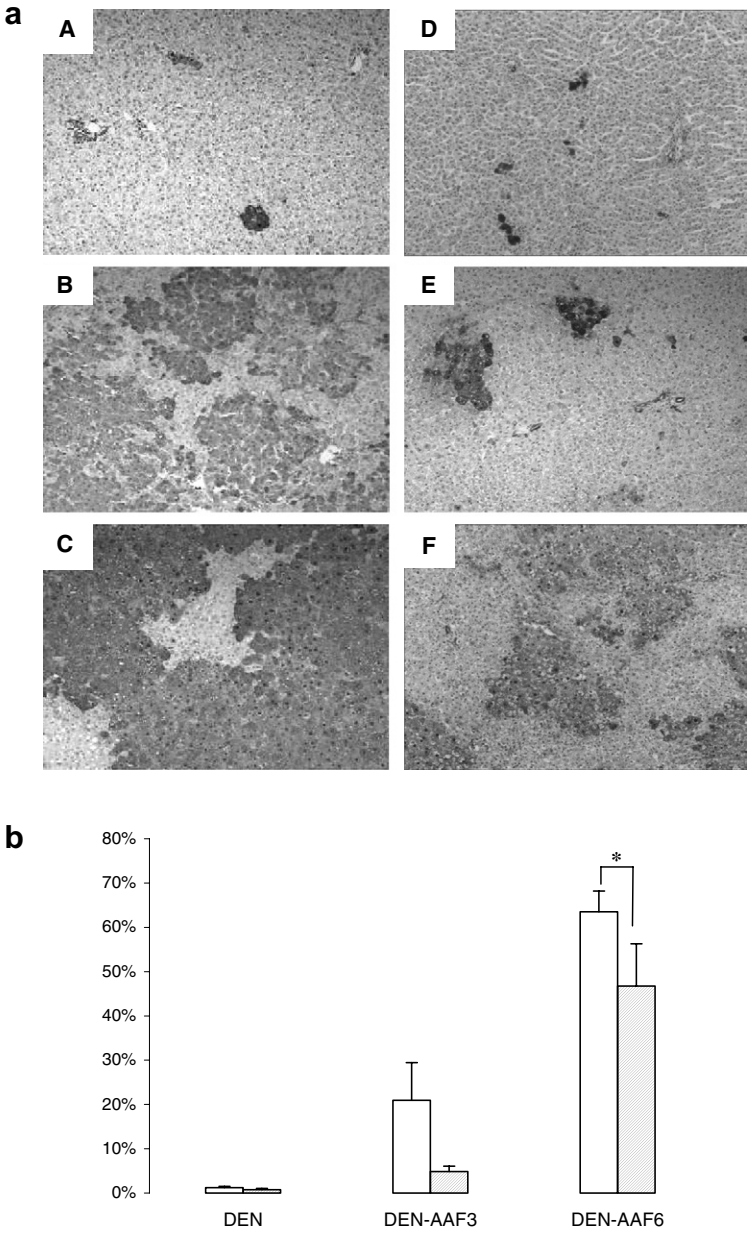

Fig. 2 – (a) Representative photomicrographs of immunohistological determination of GSTp positive area in liver sections of induced rats, expressed at the end of initiation (DEN), end of initiation followed by 3 weeks promotion (B) and end of initiation followed by 6 weeks promotion (C). D, E, F: **Photomicrographs of liver sections of pioglitazone-treated rats after initiation, end of initiation followed by 3 weeks promotion and end of initiation followed by 6 weeks promotion respectively. (b) Graphs representing the surface of GSTp positively stained foci as % of liver sections from rats after initiation (DEN), short (DEN-AAF3) and long (DEN-AAF6) promotion period, in non treated rats (white bars) and rats treated with pioglitazone (dashed bars). Each bar is mean + SE of 4–5 rats ($^*p < 0.05$).**

one-treated animals was confirmed by Western blot (Fig. 3A, B), which showed a 60–70% inhibition of GSTp expression in the whole liver ($p < 0.05$).

A significant increase in the number of pre-neoplastic foci per liver was observed after DEN + 3 weeks AAF in the pioglitazone-treated group compared to the induced (2.4×10^5 versus 1.4×10^5 respectively, $p < 0.05$). After DEN + 6 weeks AAF, the number of pre-neoplastic foci per liver was similar in both groups (7.7×10^4 in induced versus 7.4×10^4 in pioglitazone, NS). This was mainly due to the fact that the pre-neoplastic foci were better individualised in pioglitazone treated livers due to their smaller size. Thus treatment with pioglitazone reduced the size of transformed hepatocyte foci rather than it influenced their number.

3.2. *Proliferation index analysis*

Results of proliferation index determined by Ki67 are shown in Fig. 4. Induced rat livers showed significant proliferation, with a Ki67 expression in 13%, 14% and 18% of hepatocyte nuclei in DEN, DEN + 3 or 6 weeks AAF, respectively. Compared to the induced group, pioglitazone-treated rat livers showed similar high proliferative indices after DEN, but the proliferation decreased significantly to 6% and 5% after DEN + 3 or 6 weeks AAF, respectively ($p < 0.05$). Proliferation assessed by phospho-histone H3 showed similar results, with a significant

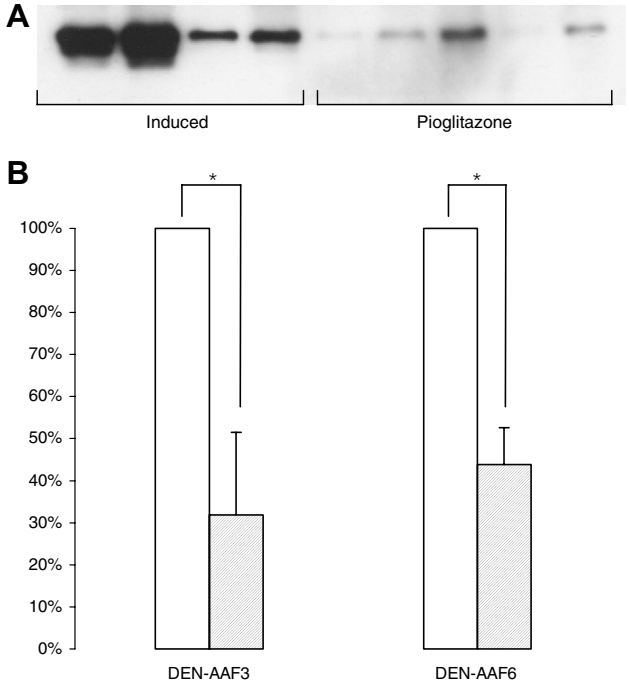

Fig. 3 – (A) Histogram of a Western blot showing the decreased expression of GSTp in the pioglitazone group compared to the induced group, after DEN and 3 weeks AAF. (B) Expression of GSTp in induced (white bars) and pioglitazone (dashed bars) treated rats, after initiation + 3 weeks promotion (DEN-AAF3) and after initiation + 6 weeks promotion (DEN-AAF6), assessed by Western blot. Data are expressed as mean + SE, in %. Values of the induced group are set to 100%. *$p < 0.05$.

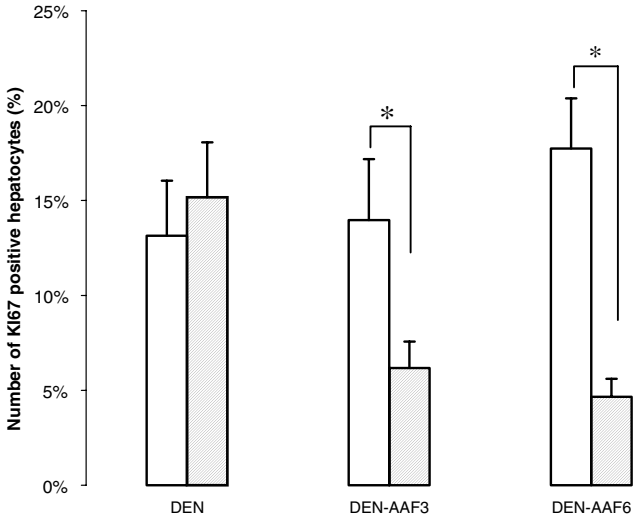

Fig. 4 – Percentage of hepatocyte nuclei labelled with Ki67 in induced (white bars) and pioglitazone treated (dashed bars) rats after initiation (DEN), initiation + 3 weeks promotion (DEN-AAF3) and after initiation + 6 weeks promotion (DEN-AAF6). Data are expressed as mean + SE, in %. *$p < 0.05$.

decrease in the pioglitazone treated group compared to the animals receiving the induction scheme: 1.6 ± 0.8% versus 3.5 ± 1.5% after DEN + 3 weeks AAF and 1.9 ± 0.8% versus 3.8 ± 1.9% after DEN + 6 weeks AAF ($p < 0.05$). In response to DEN, proliferative cells were found at random in the liver in both induced and pioglitazone groups (Fig. 5A and B) with no clustering. On the contrary, after promotion by AAF, in the induced group, clusters of positive cells were found throughout the liver slices, with no predisposition for a specific area, suggesting the preferential proliferation of some hepatocytes clones. This clustering was also found in the pioglitazone group, but the clusters were much smaller, with only groups of two to three cells found (Fig. 5D–F).

We performed a double immunostaining detecting together Ki67 and GSTp to see if the clustering of proliferative cells co-localised with area of transformed hepatocytes. Indeed we observed that in the induced group after DEN + 3 weeks AAF, significantly more hepatocytes expressed Ki67 in pre-neoplastic foci compared to the surrounding liver (Table 1). An enhanced proliferation was also found in the pre-neoplastic foci of the induced group after DEN + 6 weeks AAF, but this difference was no longer significant.

Pioglitazone-treated animals showed significantly decreased proliferation in pre-neoplastic foci after DEN + 3 weeks AAF compared to the induced group; this decrease was not significant after the longer promotion period (in DEN + 6 weeks AAF). By contrast with the induced group, in the pioglitazone-treated group there was virtually no difference between pre-neoplastic foci and surrounding parenchyma, either after DEN + 3 weeks or 6 weeks AAF.

3.3. *Effect of pioglitazone on $p21^{waf1}$ and $p27^{kip1}$ expression*

Because TZDs have shown to inhibit expression of CdK inhibitors *in vitro*, we analysed protein expression of both CdK

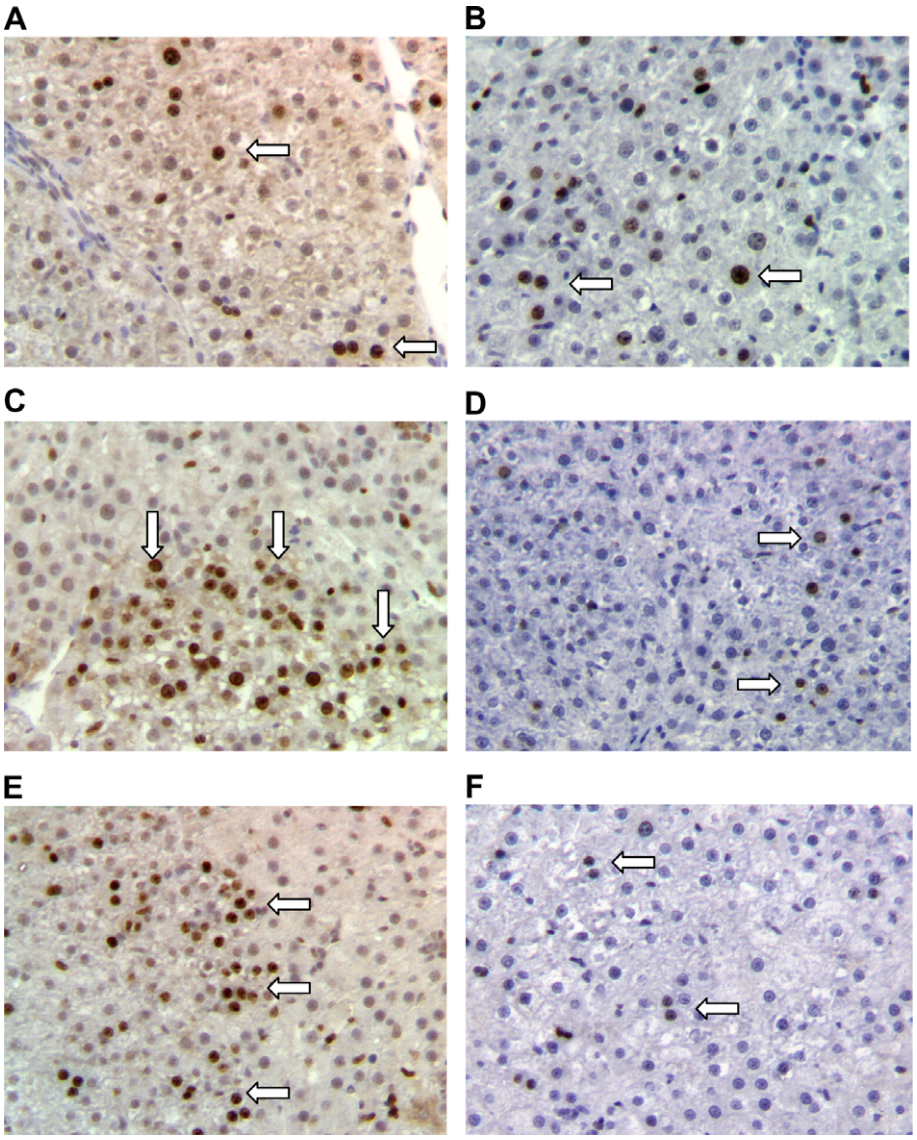

Fig. 5 – Representative photomicrographs of Ki67 staining of induced rat liver sections after initiation DEN (A) initiation + 3 weeks promotion (DEN-AAF3) (C) and after initiation + 6 weeks promotion (DEN-AAF6) (E). B, D, F: liver sections of pioglitazone-treated rat livers at same time. Magnification 10×, Ki67 antibody 1:100 dilution. Positive hepatocyte nuclei are brown, showed by white arrows. Note the cluster pattern of staining in C and E.

Table 1 – Proliferation index in pre-neoplastic foci and surrounding liver

		DEN-AAF3	DEN-AAF6
Induced	Pre-neoplastic foci	25.5 ± 1.9	19.7 ± 3.9
	Surrounding liver	13.3 ± 3*	11.5 ± 4.5
Pioglitazone	Pre-neoplastic foci	11.8 ± 3.5**	12.1 ± 0.9
	Surrounding liver	10.8 ± 2.3	12.4 ± 2

Percentage of nuclei labelled with Ki67 in pre-neoplastic foci and surrounding liver after initiation followed by 3 weeks promotion (DEN-AAF3) and initiation followed by 6 weeks promotion (DEN-AAF6). All values are mean ± SE.

* $p < 0.05$ for preneoplastic foci versus surrounding liver in the induced group.

** $p < 0.05$ for preneoplastic foci in the induced group versus pioglitazone group.

inhibitors $p21^{waf1}$ and $p27^{kip1}$, by Western blot (Fig. 6). $p21^{waf1}$ was downregulated in both groups after DEN. Promotion by AAF resulted in progressive overexpression of $p21^{waf1}$ whether in the induced or in the pioglitazone group with, after DEN + 6 weeks AAF, a two-fold increase compared to normal liver taken as control. $p27^{kip1}$ was significantly decreased in the pioglitazone treated group compared to induced after DEN. However, after DEN + 6 weeks AAF, $p27^{kip1}$ was highly and significantly overexpressed in the pioglitazone treated group compared to CTL and even more so to the induced group (Fig. 6).

3.4. *Apoptotic index analysis*

In induced rats, we noted an increased apoptosis in the surrounding liver versus pre-neoplastic foci after DEN + 3 weeks AAF. This finding was confirmed after a longer promotion

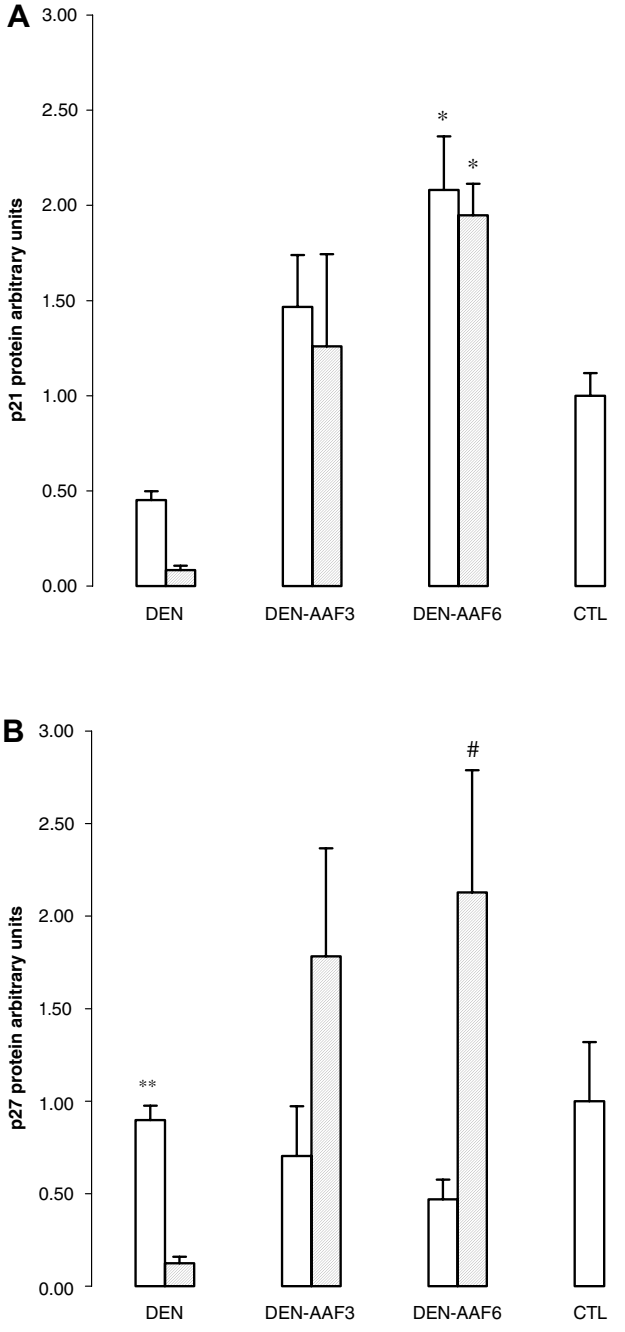

Fig. 6 – (A) $p21^{waf1}$ and (B) $p27^{kip1}$ protein expression assessed by Western blot in whole liver, in the induced group (white bars) and pioglitazone treated group (dashed bars) after initiation by DEN (DEN), initiation followed by 3 weeks promotion with AAF (DEN-AAF3) or 6 weeks promotion with AAF (DEN-AAF6). CTL represents normal rat liver taken as control. Each bar is mean + SE of five rats. $^{*}p < 0.05$, DEN-AAF6 versus CTL, $^{\#}p < 0.05$, induced versus pioglitazone; $^{**}p < 0.005$ induced versus pioglitazone.

period (DEN + 6 weeks AAF), with a three-fold increase in the number of cleaved caspase 3 positive cells in the surrounding liver compared to pre-neoplastic foci ($p < 0.05$). This difference was not found in the pioglitazone treated group (Table 2).

Table 2 – Apoptotic index in pre-neoplastic foci and surrounding liver

		DEN-AAF3	DEN-AAF6
Induced	Pre-neoplastic foci	0.54 ± 0.21	0.22 ± 0.04
	Surrounding liver	0.81 ± 0.37	0.65 ± 0.06*
Pioglitazone	Pre-neoplastic foci	0.54 ± 0.15	0.35 ± 0.08
	Surrounding liver	0.47 ± 0.04	0.51 ± 0.08

Percentage of nuclei labelled with cleaved caspase-3 in pre-neoplastic foci and surrounding liver after initiation followed by 3 weeks promotion (DEN-AAF3) and initiation followed by 6 weeks promotion (DEN-AAF6). All values are mean ± SE.
* $p < 0.005$ for pre-neoplastic foci versus surrounding liver in the induced group.

4. Discussion

There is a solid rationale to justify a chemopreventive approach to treat hepatocellular carcinoma. This cancer arises on a well-known pre-neoplastic field, i.e. the cirrhotic liver. The disease is gaining incidence, the etiologic factors are known (mainly alcohol, hepatitis B and C viruses), and the surveillance program of cirrhotic patients is getting more common. Though no established drug has been proved unequivocally to be efficient in the chemoprevention of HCC, interferon-α showed some effect in hepatitis C cirrhotic patients in primary[30] and secondary[31–33] prevention, but only in Japanese patients. With less evidence, acyclic retinoids also showed effect in secondary prevention.[34]

In this study, we analysed the inhibitory effect of pioglitazone on the appearance of early hepatic pre-neoplastic events, using a two-stage carcinogenic model combining DEN and AAF.

In this model, initiation by DEN followed by promotion with AAF induces the appearance of phenotypically modified cell foci called altered hepatic foci.[35] The number of these pre-neoplastic foci reflects the amount of initiated cells capable of undergoing clonal expansion, whereas the surface percentage reflects the growth rate and total cellular population of the pre-neoplastic foci.[36] In the model we used, we observed the formation of pre-neoplastic foci, revealed by GSTp positive staining, already after the injection of DEN. Their size increased in parallel with the duration of AAF administration. Due to the confluence of the foci, the number of pre-neoplastic foci decreased along the promotion phase. Our results demonstrate that pioglitazone significantly decreases the size of these pre-neoplastic foci. This decrease was observed after the initiation period as well as after the long promotion period in animals receiving AAF for 6 weeks. This was shown by quantitative analysis of the GSTp positive surface of the liver sections and confirmed by quantification of GSTp protein by Western blot on whole liver homogenates.

As a result of the diminished size of pre-neoplastic foci under pioglitazone treatment after DEN + 3 weeks AAF, the foci were less confluent and therefore better individualised. This is a likely explanation for the significant increase in the number of foci in the pioglitazone-treated animals compared to those induced after DEN + 3 weeks AAF. Taken together, our

results suggest that pioglitazone does not influence the appearance of modified hepatocytes, but may rather affect the growth of existing foci.

We could show that at all time points, the carcinogenic model induced high levels of proliferation. As the pictures and the analyses of proliferative index in pre-neoplastic foci demonstrate, this proliferation rate was mainly seen in pre-neoplastic foci of the induced population, compared to surrounding liver, as already described when AAF is used as the promoting agent, in contrast with other agents such as phenobarbital.[37] Pioglitazone did not decrease the proliferation rate after the DEN injections, suggesting again that it is not active on the induction of transformed hepatocytes. More interestingly, we could show that pioglitazone significantly and specifically inhibited proliferation in pre-neoplastic foci after promotion with AAF, with identical proliferative indices in pre-neoplastic foci and surrounding liver. Such an effect of pioglitazone on proliferation has been described *in vitro* in liver cancer cell models,[14] although under certain metabolic conditions it can act synergistically with insulin to have a growth-promoting effect on hepatoma cells.[38]

Several *in vitro* studies have supported that PPARγ agonists may control liver cancer cell proliferation by inducing the expression of both $p21^{waf1}$ and $p27^{kip1}$, which are known to inhibit cyclin-dependent kinases and block the cell cycle in the G1 phase.[15,16] Furthermore, an *in vivo* study showed that $p21^{waf1}$ was involved in the inhibition of cell proliferation in pre-neoplastic foci by interferon-alpha.[39] In our study, there was no difference in $p21^{waf1}$ protein expression, which was high in both groups compared to normal liver. Therefore it seems difficult to speculate on the role of $p21^{waf1}$ in our model. On the contrary, we could show that the $p27^{kip1}$ protein expression was clearly upregulated in the pioglitazone group. Therefore, our data favours the hypothesis that $p27^{kip1}$ is a factor responsible for the inhibitory effect of pioglitazone on proliferation in this model.

Cell death by apoptosis is another mechanism that could explain the reduced proportion of transformed hepatocytes in our model. Apoptosis was enhanced in surrounding liver of induced animals compared to pre-neoplastic foci, which is in contradiction with previous reports,[27] but favours the hypothesis that AAF confers a survival advantage to initiated cells.[8,37] This is also supported by the fact that pre-neoplastic foci express less p53 than surrounding liver.[40] Discrepancies between our study and previous reports may be explained by the methodology used. Indeed, the TUNEL method used by Alvarez and colleagues[27] has been criticised for its lack of sensitivity and specificity.[41] The longer duration of promotion in our study may also explain these differences. Pioglitazone did not affect apoptosis in our *in vivo* model. *In vitro* data report conflicting effects of PPARγ agonists pioglitazone or troglitazone on apoptosis of cancer cells.[19,21]

In conclusion, pioglitazone showed activity in inhibiting the development of pre-neoplastic foci in a carcinogenic animal liver model using DEN and AAF. This resulted from a reduction in the size of the pre-neoplastic foci rather than in their number, suggesting that pioglitazone controls the growth of pre-neoplastic foci, as shown by the decreased proliferation in transformed hepatocytes. Our data suggest that this effect on proliferation, which has never been shown *in vivo*, may be mediated through an increased expression of the CdK inhibitor $p27^{kip1}$, which occurred during the promotion phase of this two-stage model. However, we believe that although pioglitazone shows promising activity in this model and is known to have a very good toxicity profile, its use as a chemopreventive agent in HCC would need to be combined with an agent able to prevent hepatocyte transformation.

Conflict of interest statement

None declared.

Acknowledgement

The authors wish to acknowledge Mrs. C. Desaeger, Mrs. V. Lebrun and Mr. J Abarca-Quinones for expert technical assistance.

REFERENCES

1. Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. *Int J Cancer* 2001;**94**(2):153–6.
2. El-Serag H, Mason A. Rising incidence of hepatocellular carcinoma in the United States. *New England Journal of Medicine* 1999;**340**:745–50.
3. Williams GM. The significance of chemically-induced hepatocellular altered foci in rat liver and application to carcinogen detection. *Toxicol Pathol* 1989;**17**(4 Pt 1):663–72. discussion 673-4.
4. Altmann HW. Hepatic neoformations. *Pathol Res Pract* 1994;**190**(6):513–77.
5. Goldsworthy TL, Hanigan MH, Pitot HC. Models of hepatocarcinogenesis in the rat–contrasts and comparisons. *Crit Rev Toxicol* 1986;**17**(1):61–89.
6. Solt D, Farber E. New principle for analysis of chemical carcinogenesis. *Nature* 1976;**263**:701–3.
7. Becker RA, Shank RC. Kinetics of formation and persistence of ethylguanines in DNA of rats and hamsters treated with diethylnitrosamine. *Cancer Res* 1985;**45**(5):2076–84.
8. Dragan YP, Hully J, Crow R, Mass M, Pitot HC. Incorporation of bromodeoxyuridine in glutathione S-transferase-positive hepatocytes during rat multistage hepatocarcinogenesis. *Carcinogenesis* 1994;**15**(9):1939–47.
9. Ogiso T, Tatematsu M, Tamano S, Tsuda H, Ito N. Comparative effects of carcinogens on the induction of placental glutathione S-transferase-positive liver nodules in a short-term assay and of hepatocellular carcinomas in a long-term assay. *Toxicol Pathol* 1985;**13**(4):257–65.
10. Mangelsdorf DJ, Evans RM. The RXR heterodimers and orphan receptors. *Cell* 1995;**83**(6):841–50.
11. Suh N, Wang Y, Williams CR, et al. A new ligand for the peroxisome proliferator-activated receptor-gamma (PPAR-gamma), GW7845, inhibits rat mammary carcinogenesis. *Cancer Res* 1999;**59**(22):5671–3.
12. Wang Y, Porter WW, Suh N, et al. A synthetic triterpenoid, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), is a ligand for the peroxisome proliferator-activated receptor gamma. *Mol Endocrinol* 2000;**14**(10):1550–6.
13. Panigrahy D, Shen LQ, Kieran MW, Kaipainen A. Therapeutic potential of thiazolidinediones as anticancer agents. *Expert Opin Investig Drugs* 2003;**12**(12):1925–37.

14. Rumi MA et al. Peroxisome proliferator-activated receptor gamma ligand-induced growth inhibition of human hepatocellular carcinoma. *Br J Cancer* 2001;**15**(84):1640–7.
15. Koga H, Harada M, Ohtsubo M, et al. Troglitazone induces p27Kip1-associated cell-cycle arrest through down-regulating Skp2 in human hepatoma cells. *Hepatology* 2003;**37**(5):1086–96.
16. Koga H et al. Involvement of p21WAF1/Cip1, p27Kip1, and p18INK4c in troglitazone-induced cell-cycle arrest in human hepatoma cell lines. *Hepatology* 2001;**33**:1087–97.
17. Yoshizawa K, Cioca DP, Kawa S, Tanaka E, Kiyosawa K. Peroxisome proliferator-activated receptor gamma ligand troglitazone induces cell cycle arrest and apoptosis of hepatocellular carcinoma cell lines. *Cancer* 2002;**95**(10):2243–51.
18. Motomura W, Takahashi N, Nagamine M, et al. Growth arrest by troglitazone is mediated by p27Kip1 accumulation, which results from dual inhibition of proteasome activity and Skp2 expression in human hepatocellular carcinoma cells. *Int J Cancer* 2004;**108**(1):41–6.
19. Toyoda M, Takagi H, Horiguchi N, et al. A ligand for peroxisome proliferator activated receptor gamma inhibits cell growth and induces apoptosis in human liver cancer cells. *Gut* 2002;**50**(4):563–7.
20. Li MY, Deng H, Zhao JM, Dai D, Tan XY. PPARgamma pathway activation results in apoptosis and COX-2 inhibition in HepG2 cells. *World J Gastroenterol* 2003;**9**(6):1220–6.
21. Yamamoto Y, Nakajima M, Yamazaki H, Yokoi T. Cytotoxicity and apoptosis produced by troglitazone in human hepatoma cells. *Life Sci* 2001;**70**(4):471–82.
22. Kawaguchi K, Sakaida I, Tsuchiya M, Omori K, Takami T, Okita K. Pioglitazone prevents hepatic steatosis, fibrosis, and enzyme-altered lesions in rat liver cirrhosis induced by a choline-deficient L-amino acid-defined diet. *Biochem Biophys Res Commun* 2004;**315**(1):187–95.
23. Guo YT, Leng XS, Li T, Zhao JM, Lin XH. Peroxisome proliferator-activated receptor gamma ligands suppress liver carcinogenesis induced by diethylnitrosamine in rats. *World J Gastroenterol* 2004;**10**(23):3419–23.
24. Leclercq IA, Sempoux C, Starkel P, Horsmans Y. Limited therapeutic efficacy of pioglitazone on progression of hepatic fibrosis in rats. *Gut* 2006;**55**(7):1020–9.
25. Galli A, Crabb DW, Ceni E, et al. Antidiabetic thiazolidinediones inhibit collagen synthesis and hepatic stellate cell activation in vivo and in vitro. *Gastroenterology* 2002;**122**(7):1924–40.
26. Imai T, Masui T, Ichinose M, et al. Reduction of glutathione S-transferase P-form mRNA expression in remodeling nodules in rat liver revealed by in situ hybridization. *Carcinogenesis* 1997;**18**(3):545–51.
27. Alvarez M et al. The in vivo apoptotic effect of interferon alfa-2b on rat preneoplastic liver involves Bax protein. *Hepatology* 2002;**35**:824–33.
28. Xu YH, Pitot HC. An improved stereologic method for three-dimensional estimation of particle size distribution from observations in two dimensions and its application. *Comput Methods Programs Biomed* 2003;**72**(1):1–20.
29. Duan WR, Garner DS, Williams SD, Funckes-Shippy CL, Spath EA, Blomme EA. Comparison of immunohistochemistry for activated caspase-3 and cleaved cytokeratin 18 with the TUNEL method for quantification of apoptosis in histological sections of PC-3 subcutaneous xenografts. *J Pathol* 2003;**199**(2):221–8.
30. Camma C, Giunta M, Andreone P, Craxi A. Interferon and prevention of hepatocellular carcinoma in viral cirrhosis: an evidence-based approach. *J Hepatol* 2001;**34**(4):593–602.
31. Ikeda K, Arase Y, Saitoh S, et al. Interferon beta prevents recurrence of hepatocellular carcinoma after complete resection or ablation of the primary tumorA prospective randomized study of hepatitis C-related liver cancer. *Hepatology* 2000;**32**:228–32.
32. Kubo S et al. Randomized clinical trial of long-term outcome after resection of hepatitis C virus-related hepatocellular carcinoma by postoperative interferon therapy. *Br J Surg* 2002;**89**(4):418–22.
33. Suou T, Mitsuda A, Koda M, et al. Interferon alpha inhibits intrahepatic recurrence in hepatocellular carcinoma with chronic hepatitis C: A pilot study. *Hepatol Res* 2001;**20**:301–11.
34. Muto Y, Moriwaki H, Saito A. Prevention of second primary tumors by an acyclic retinoid in patients with hepatocellular carcinoma. *N Engl J Med* 1999;**340**(13):1046–7.
35. Pitot HC. Altered hepatic foci: their role in murine hepatocarcinogenesis. *Annu Rev Pharmacol Toxicol* 1990;**30**:465–500.
36. Dragan YP, Campbell HA, Baker K, Vaughan J, Mass M, Pitot HC. Focal and non-focal hepatic expression of placental glutathione S-transferase in carcinogen-treated rats. *Carcinogenesis* 1994;**15**(11):2587–91.
37. Tatematsu M, Aoki T, Kagawa M, Mera Y, Ito N. Reciprocal relationship between development of glutathione S-transferase positive liver foci and proliferation of surrounding hepatocytes in rats. *Carcinogenesis* 1988;**9**(2):221–5.
38. Weinstock RS, Murray FT, Diani A, Sangani GA, Wachowski JL, Messina JL. Pioglitazone: in vitro effects on rat hepatoma cells and in vivo liver hypertrophy in KKAy mice. *Pharmacology* 1997;**54**(4):169–78.
39. Nakaji M, Yano Y, Ninomiya T, et al. IFN-alpha prevents the growth of pre-neoplastic lesions and inhibits the development of hepatocellular carcinoma in the rat. *Carcinogenesis* 2004;**25**(3):389–97.
40. Finnberg N, Silins I, Stenius U, Hogberg J. Characterizing the role of MDM2 in diethylnitrosamine induced acute liver damage and development of pre-neoplastic lesions. *Carcinogenesis* 2004;**25**(1):113–22.
41. Labat-Moleur F, Guillermet C, Lorimier P, et al. TUNEL apoptotic cell detection in tissue sections: critical evaluation and improvement. *J Histochem Cytochem* 1998;**46**(3):327–34.