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# Hexachlorobenzene induces cell proliferation, and aryl hydrocarbon receptor expression (AhR) in rat liver preneoplastic foci, and in the human hepatoma cell line HepG2. AhR is a mediator of ERK1/2 signaling, and cell cycle regulation in HCB-treated HepG2 cells

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ABSTRACT

Hexachlorobenzene (HCB) is a widespread environmental pollutant, and a liver tumor promoter in rodents. Depending on the particular cell lines studied, exposure to these compounds may lead to cell proliferation, terminal differentiation, or apoptosis. The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that is involved in drug and xenobiotic metabolism. AhR can also modulate a variety of cellular and physiological processes that can affect cell proliferation and cell fate determination. The mechanisms by which AhR ligands, both exogenous and endogenous, affect these processes involve multiple interactions between AhR and other signaling pathways. In the present study, we examined the effect of HCB on cell proliferation and AhR expression, using an initiation-promotion hepatocarcinogenesis protocol in rat liver and in the human-derived hepatoma cell line, HepG2. Female Wistar rats were initiated with a single dose of 100 mg/kg of diethylnitrosamine (DEN) at the start of the experiment. Two weeks later, daily dosing of 100 mg/kg HCB was maintained for 10 weeks. Partial hepatectomy was performed 3 weeks after initiation. The number and area of glutathione *S*-transferase-P (GST-P)-positive foci, in the rat liver were used as biomarkers of liver precancerous lesions.

Immunohistochemical staining showed an increase in proliferating cell nuclear antigen (PCNA)-positive cells, along with enhanced AhR protein expression in hepatocytes within GST-P-positive foci of (DEN HCB) group, when compared to DEN. In a similar manner, Western blot analysis demonstrated that HCB induced PCNA and AhR protein expression in HepG2 cells. Flow cytometry assay indicated that the cells were accumulated at S and G2/M phases of the cell cycle. HCB increased cyclin D1 protein levels and ERK1/2 phosphorylation in a dose-dependent manner. Treatment of cells with a selective MEK1 inhibitor, prevented HCB-stimulatory effect on PCNA and cyclinD1, indicating that these effects are mediated by ERK1/2. Pretreatment with an AhR antagonist, prevented HCB-induced PCNA protein levels, ERK1/2 phosphorylation and alterations in cell cycle distribution. These results demonstrate that HCB-induced HepG2 proliferation and cell cycle progression depend on ERK1/2 phosphorylation which is mediated by the AhR.

Our results provide a clue to the molecular events involved in the mechanism of action of HCB-induced hepatocarcinogenesis.



## 1. Introduction

Hexachlorobenzene (HCB) is a widespread environmental pollutant. It was employed as a fungicide until the 1970s, when such use was prohibited. However, considerable amounts are still generated as waste by-products of industrial processes and emitted into the environment. Because of the relative persistence of this chemical, significant human exposure to HCB has occurred (Carey et al., 1986).

Nowadays there is a concern over the possible involvement of xenobiotics in carcinogenesis and this has led to a solid research stream in both humans and rodents. In experimental animals, long-term HCB feeding induced mainly hepatomas and hemangioendotheliomas in the liver, and adenomas of thyroid, bile ducts

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and renal cortex (ATSDR, 2002). Genotoxicity evaluations of HCB are generally negative and it is considered to be a nongenotoxic carcinogen (Mally and Chipman, 2002).

The aryl hydrocarbon receptor (AhR) is a cytosolic ligand-activated transcription factor that is involved in drug and xenobiotic metabolism. The AhR is highly expressed in multiple organs and tissues, and plays an important role in cellular homeostasis and disease (Safe et al., 2013). The mechanisms by which AhR ligands, both exogenous and endogenous, affect these processes are poorly understood but appear to involve multiple interactions between AhR and other signaling pathways. AhR ligands either enhance or inhibit tumorigenesis, and these effects are highly tumor specific, demonstrating that selective AhR modulators exhibit agonist or antagonist activities (Puga et al., 2002). While it has been demonstrated that AhR acts as a positive regulator of cell proliferation, deregulation of apoptosis may also be important. In this respect, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-treatment, enhanced hepatocellular proliferation and reduced levels of apoptosis in a tumor promotion model in rat liver (Stinchcombe et al., 1995).

Several reports describe a role for the AhR in cell cycle control; most of these reports identified $G_1$ phase as the cell cycle period influenced by AhR activity (Ma and Whitlock, 1996; Puga et al., 2002). Progression through the cell cycle is controlled intrinsically by cyclins and cyclin dependent kinases (CDKs). Cyclin D1 is most closely linked to cell-cycle progression through G1 phase and the commitment of cells to enter S phase.

Other nongenomic effects of the AhR have been proposed in regulating mitogen-activated protein kinase (MAPK) signaling pathways (Tan et al., 2002).

MAPK families play an important role in complex cellular programs like proliferation, differentiation, development, transformation, and apoptosis. Among the MAPK family, extracellular signal-regulated protein kinase (ERK) is the most extensively studied.

Many observations have led to the proposal that sustained ERK activation is an obligatory event for growth factor-induced cell cycle progression, (reviewed by Roovers and Assoian, 2000).

In the present study, we examined the effect of HCB on parameters of cell proliferation, and AhR expression in preneoplastic foci, in an initiation-promotion model in rat liver, and in the human hepatoma cell line HepG2. We also evaluated the potential role of AhR in ERK1/2 signaling pathway, cell cycle progression and cyclin D1 protein expression in HCB-treated HepG2 cells.

## 2. Materials and methods

### 2.1. Reagents

HCB (>99% purity, commercial grade), protease inhibitors, phenylmethanesulfonyl fluoride (PMSF), aprotinin, leupeptin, pepstatin, and 4, 7-o-phenantroline (4, 7-OP), PD98059, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MMT) were purchased from Sigma–Aldrich Co. (St. Louis, MO). Anti-ERK1/2, anti-phospho-ERK1/2, and anti-AhR primary antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA). Anti-β-actin, and anti-cyclin D1 were from Abcam Inc. (Cambridge, MA). Antibody against anti proliferating cell nuclear antigen (PCNA) was from Dako Cytomation, (Glostrup, Denmark); anti-rabbit GST-P antibody was from Assay Designs Inc. (Ann Arbour, MI); anti-BrdU antibody was from Jackson ImmunoResearch Laboratories (Baltimore, PA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Invitrogen Life Technology (Cambridge, MA). Antibiotics were all purchased from PAA Laboratories GmbH (Pasching, Austria). The enhanced chemiluminescence kit (ECL) was from GE Healthcare Life Sciences (Buckinghamshire, UK). All other reagents used were of analytical grade.

### 2.2. Animals and treatment

Female Wistar rats (100 g at the onset of the experiment) were purchased from the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina. The rats were fed Purine 3 rat chow (Cabeca S.C.A, Argentina) and water *ad libitum*. Environmental conditions consisted of a 12 h light-dark cycle, 20–24 °C, and 45–75% humidity. Following a 7-day acclimation period, rats were randomly divided into 4 groups containing 4 animals per group and treated according to Ou et al., (2001). At week 0, animals received a single i.p. injection of DEN (100 mg/kg) dissolved in 0.9% saline. Concurrent controls were administered 0.9% saline without DEN. After 2 weeks, the rats received daily gavage administration of HCB (100 mg/kg/day), or vehicle by the same route, through the remainder of the 10-week study. HCB was administered as a suspension in water, containing Tween 20 (0.5 ml/100 ml). At week 3 (day 21), a partial hepatectomy, which comprised approximately 70% of the total liver weight, was performed on all animals. At the end of the tenth week, the animals were killed by decapitation. The HCB dose used in our *in vivo* studies is consistent with the dose given in medium-term bioassays for carcinogenicity of HCB (Gustafson et al., 2000). No mortalities caused by HCB treatment were encountered, and no clinical changes were apparent. Whole livers were removed, and parts of the left and right lateral lobes were fixed overnight in freshly prepared 4% paraformaldehyde and embedded in paraffin for light microscopy. All animal procedures were undertaken in accordance with the Institutional Guidelines for Animal Care and Research.

### 2.3. Cell culture and treatment

HepG2 human hepatocarcinoma cell line was supplied by the American Type Culture Collection (ATCC). It has been shown that this cell line maintains many of the functions of a normal liver cell, which is to be a good model for *in vitro* studies. HepG2 cells were cultured in DMEM complete growth medium (DMEM, supplemented with 10% BFS (v/v), 50 μg/ml gentamicin and 50 μg/ml penicillin). Cells were maintained under standard conditions at 37 °C in 5% $CO_2$.

### 2.4. Cells treatment for time-course and dose-response studies

HepG2 cells were grown in DMEM complete growth medium followed by 24 h incubation to allow cells to attach. Afterwards, the medium was withdrawn and replaced with fresh serum and HCB dissolved in absolute-ethanol (ETOH), according to the assay. Final ETOH concentration in each treatment was 0.5% and had no influence on the analyzed parameters. For time-course studies, cells were treated with 5 μM HCB or vehicle (ETOH) for 2, 4, 6, 24 and 48 h. For dose–response studies, cells were exposed for 24 h to HCB (0.005, 0.05, 0.5 and 5 μM) in DMEM complete growth medium. Selected doses were in the same range of concentration as that found in serum from humans from a highly contaminated population (To-Figueras et al., 1997). After HCB exposure, cells were washed twice with ice-cold phosphate-buffer saline (PBS), and harvested in 5 vol of lysis buffer containing 20 mM (HEPES-KOH), 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 0.1 mM PMSF, pH 7.5, 20 μg/ml aprotinin, 120 μM leupeptin, and 12 μM pepstatin.

### 2.5. Immunohistochemistry

Cell proliferation was evaluated by immunohistochemical staining. Briefly, the liver slices were fixed in 4% paraformaldehyde in PBS for 24 h. Endogenous peroxidase was quenched in 3% hydrogen peroxide for 30 min. The slices were then embedded in

paraffin for subsequent immunohistochemical examination of PCNA, AhR and GST-P. After being deparaffinized with xylene, quenched with hydrogen peroxide and blocked with normal serum, the liver tissue sections were treated sequentially with normal goat serum, anti-rabbit GST-P antibody (1:200), and biotin-labeled goat anti-rabbit IgG (1:400) conjugated with fluorophores (Texas Red or Alexa Fluor). Finally, the sections were dehydrated with alcohol and mounted. The number of GST-P positive foci/cm$^2$, and area of GST-P-positive foci, were assessed by using a fluorescence confocal Nikon C1 (Plan Apo 40×/0.95)

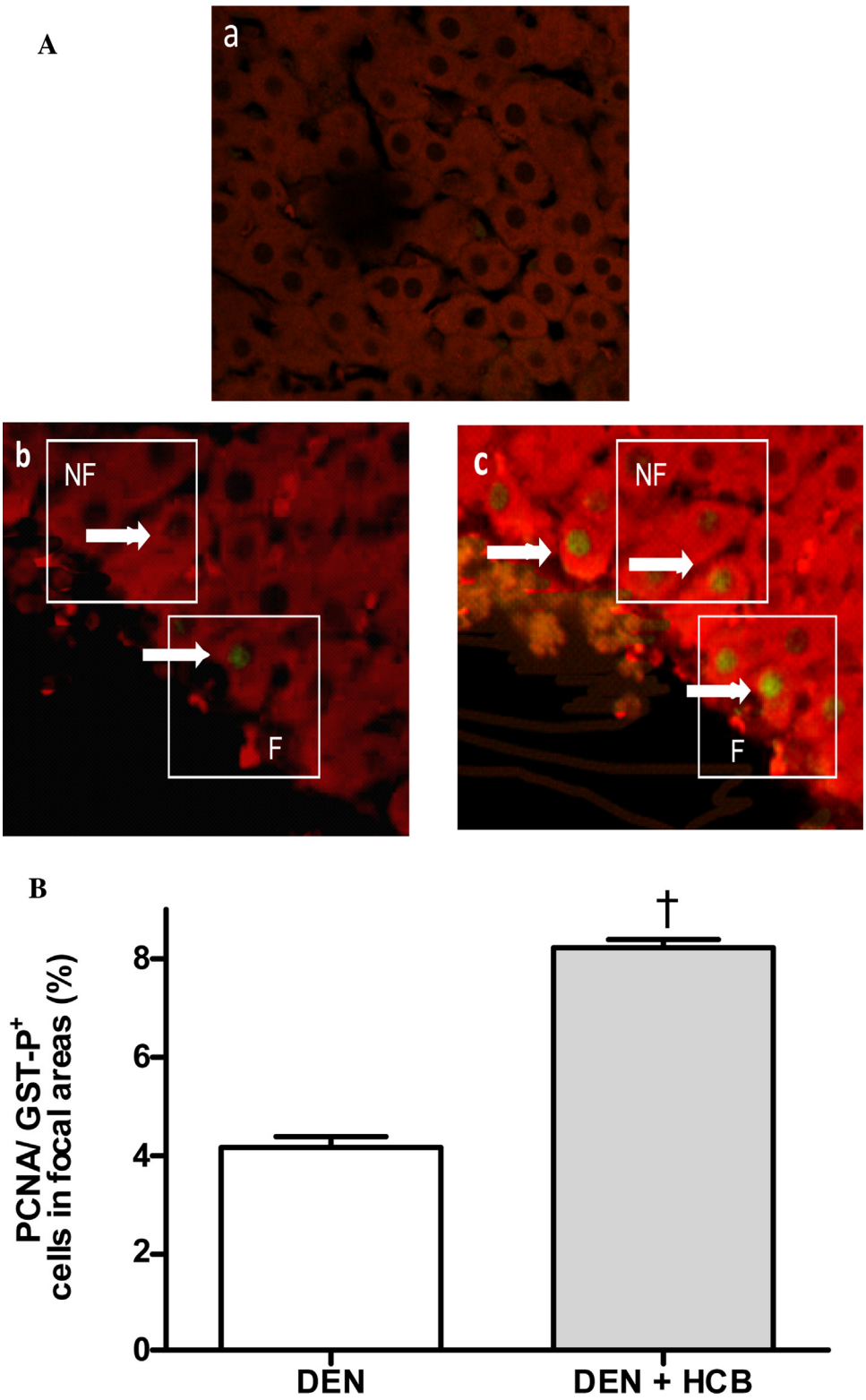


**Fig. 1.** HCB induces cell proliferation in focal areas. (A) PCNA immunoreactivity in (a) control, (b) DEN, and (c) (DEN + HCB). Arrows point to PCNA in GST-P-positive cells. Magnification: 400×. (B) Quantification of percentage of PCNA/GST-P-positive cells in focal areas of DEN + HCB *vs.* DEN group. Values are means ± SEM of three independent experiments. Significantly different from DEN group (†$p < 0.01$). GST-P-positive (GST-P$^+$).

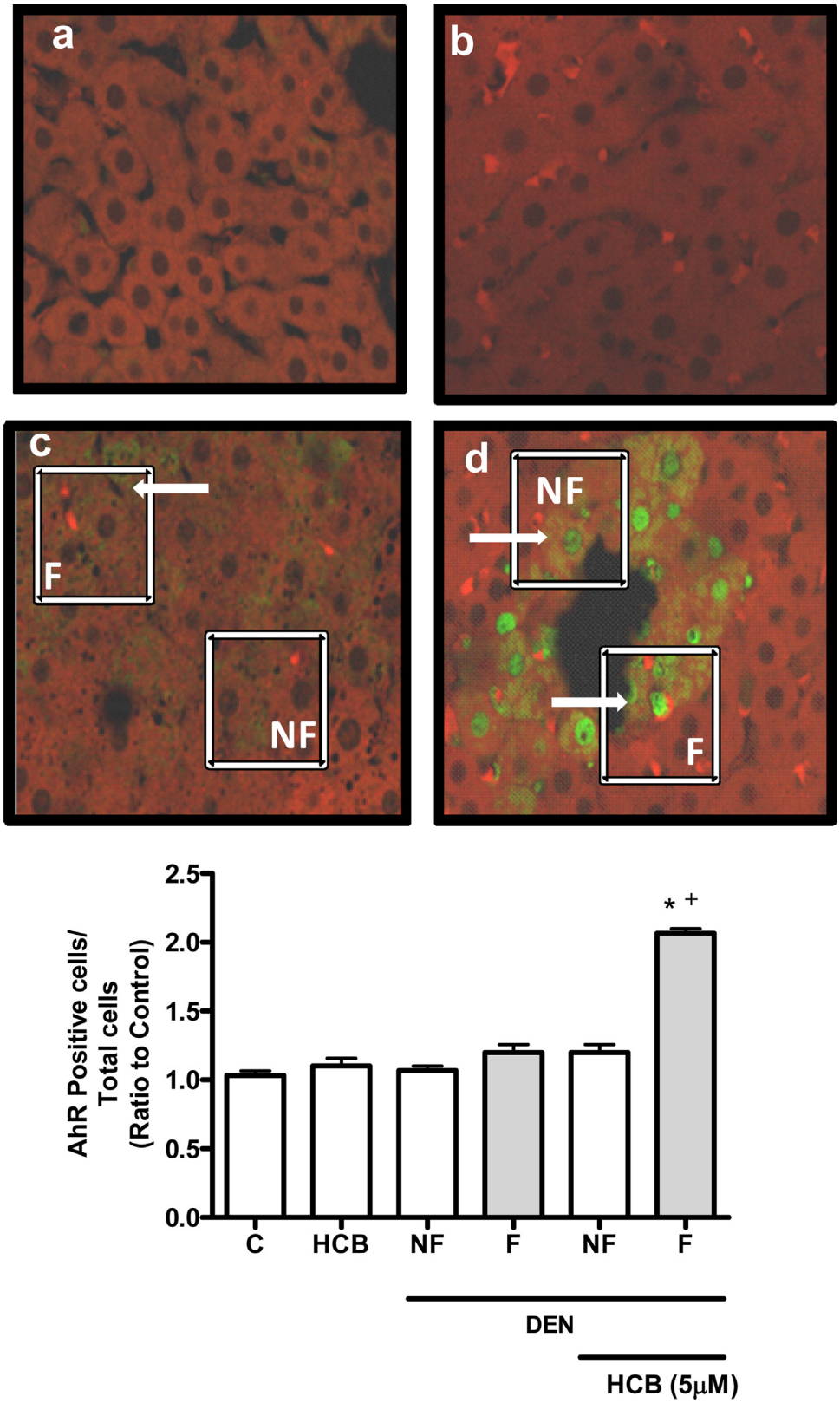


**Fig. 2.** HCB increased AhR expression in focal areas. Immunolocalization of AhR in 1000 cells from focal and non-focal areas of histological sections of rat liver. Representative images are shown in the upper panel. (a) Control, (b) HCB, (c) DEN, and (d) DEN + HCB. Magnification: 400×. Quantitative analysis of AhR per 1000 cells from different groups are shown in the lower panel. Arrows point to AhR-positive cells. Red staining corresponds to GST-P-positive cells. The values are means ± SEM of three independent experiments. *and + significantly different ($p < 0.05$) compared to non-focal area of (DEN + HCB) and focal area of DEN group, respectively. NF: non-focal area, F: focal area.

microscope. The digital images were transferred to Adobe PhotoShop 5.5 software for color channel analysis and figure assembly and analyzed by the Image Pro-Plus image system. The immunohistochemical staining was analyzed according to the staining intensity. Three or more than 3 positive cells, was considered a positive focus.

Quantitation of PCNA-positive nuclei in the immunostained sections was performed in the following manner: a square graticule with 25 equal subdivisions was used for counting labeled cells at 200 × magnification, providing a square counting area that usually contained between 50 and 100 hepatocytes. All PCNA-labeled and unlabeled nuclei in this graticule area were counted. Counts were obtained from 10 graticule areas, resulting in the counting of about 1000 nuclei per animal, and the labeling index was expressed as the percent of PCNA/GST-P labeled hepatocytes.

### 2.6. Western blotting

Total HepG2 cellular protein lysates, were electrophoresed in 10–12% SDS-polyacrylamide gel (SDS-PAGE), prior to transfer to polivinylidene difluoride membranes (PVDF) (Millipore, Bedford, MA), in a semidry transfer cell at 18 V for 1.5 h. Membranes were blocked overnight at 4 °C with 5% nonfat dry milk- 2.5 % BSA in TBST buffer (10 mM Tris–HCl, pH 8.0, 0.5% Tween 20, 150 mM NaCl).

Membranes were incubated with rabbit polyclonal anti-phospho ERK1/2 (Thr-202/Tyr 204) (1:500), anti-ERK1/2 (1:1000), anti-PCNA and anti-β-actin at (1:500) dilution, and incubated overnight at 4 °C. After incubation, membranes were washed five times with TBST, and the suitable peroxidase-conjugated anti-species-specific antibodies were used for protein detection. After washing, blots were reacted using an enzyme-linked enhanced chemoluminescence (ECL) detection kit (Amersham Biosciences, Inc., UK) and quantified by scanning laser densitometry in a Fotodyne (Foto/Analyst), Gel-Pro Analyzer 3.1.

### 2.7. Cell viability

To determine cell viability the colorimetric MTT metabolic activity assay was used. This process requires active mitochondria, and even freshly dead cells do not cleave significant amounts of MTT. The cell viability and cell number are proportional to the value of absorbance measured by spectrophotometry at 570 nm. Briefly, $6 \times 10^3$ HepG2 cells were seeded in 96-well plates and maintained in DMEM complete medium for 24 h. The next day medium was removed, and serum-free DMEM medium was added. Finally, cells were treated with HCB (0.05, 0.5, and 5 μM) or ETOH in complete DMEM medium for 24 h, and MTT (0.5 mg/ml) solution dissolved in DMEM without phenol-red was added to each well and incubated for 1 h at 37 °C. Formazan crystals were dissolved in 100 μl dimethyl sulfoxide, and the absorbance of the solution was measured at 570 nm using the microplate reader Synergy HT (Biotek Instruments, Inc.).

### 2.8. Cell cycle analysis

Cells were cultured for 24 h in complete medium. Synchronized cells were then treated with 5 μM HCB or vehicle, for up to 24 h. Then cells were collected by trypsinization, fixed with ice cold ETOH 70%, centrifuged and resuspended in 0.5 ml of propidium iodide (PI) staining solution (50 mg/ml PI in PBS containing 0.2 mg/ml of DNase-free RNase A). After incubation, samples were evaluated by flow cytometry (Becton Dickinson, USA). Cell cycle distribution was analyzed using Cylchred 1.0.2 software (Cardiff University, UK).

### 2.9. Proliferation assay with BrdU incorporation

To asses proliferative activity of HepG2 cells, we used 5-bromo-2′-deoxyuridine (BrdU) labeling. The incorporated BrdU is detected by an immunoassay, using a primary antibody that specifically recognizes BrdU bound to denaturated DNA. Briefly, to asses proliferation, cells were then incubated with BrdU (0.4 μM) for 1 h. Thereafter, cells were fixed and then incubated for 2 h with anti-BrdU monoclonal antibody, conjugated with a fluorophor (Texas Red). Nuclei were stained with Hoechst (1/1000) for 15 min. The number of BrdU positive cells/1000 cells, were assessed by using a fluorescence confocal Nikon C1 (Plan Apo 40×/0.95) microscope.

### 2.10. Statistical analysis

Data are expressed as mean ± SEM. Differences between treated and control groups were analyzed by one-way ANOVA, at a 95% confidence interval, followed by Tukey post hoc test to identify significant differences between the means of samples and controls, after testing homogeneity of variance using Barlett's procedure. Differences between control and treated animals were considered significant when $p$ values were $<0.05$.

## 3. Results

### 3.1. HCB promotes preneoplastic foci formation in rat liver

The use of GST-P expression as a marker for preneoplastic lesions in liver is a well-established method, and has been used extensively in medium-term bioassays to identify potential hepatocarcinogens (Ogiso et al., 1990).

We focused on the effect of HCB on development of GST-P positive foci initiated by DEN in rat liver. As shown in Table 1, significantly increased (165% and 95%) number and areas, respectively, of GST-P-positive foci, were observed in the (DEN + HCB) treated group compared to DEN-initiated animals.

### 3.2. HCB induces cell proliferation in focal areas

Because cell proliferation in hepatic foci, is useful in identifying hepatic carcinogens, measures of PCNA protein levels within GST-P- positive hepatocytes, permits an estimate of both S-phase hepatocytes within foci and the proportion of cells that are in active cell division. As shown in Fig. 1, the proportion of PCNA-positive hepatocytes in GST-P-positive foci was significantly increased by 102%, in (DEN + HCB) compared with DEN group.

### 3.3. HCB increased AhR expression in focal areas

Because there is convincing evidence for a critical role of AhR in proliferation control in tumor cells (Marlowe and Puga, 2005), we addressed the question of whether AhR was overexpressed in foci compared to non-focal areas, in response to HCB-treatment. Immunohistochemical analysis showed that AhR was not present in control and HCB-treated rats (Fig. 2a, b). AhR expression was the same in focal and non-focal areas of DEN group (Fig. 2c). On the other hand, AhR staining was significantly higher in focal areas of (DEN + HCB) group, when compared to non-focal areas of the same group as well as focal areas of DEN group (Fig. 2d).

### 3.4. Effect of HCB on viable cell number

In order to investigate the effect of HCB on cell proliferation, *in vitro* studies were performed in HepG2 human hepatocellular cells.

MTT assay is based on measurement of a marker activity associated with viable cell number. Our results show an increase in

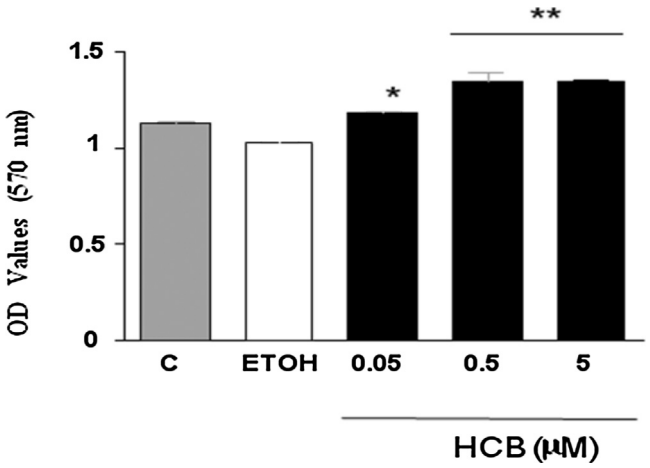


**Fig. 3.** Effect of HCB on HepG2 viable cells number. The viable cells number was evaluated using the MTT assay. The absorbance was measured at 570 nm, and the results were expressed as percentage of ETOH-treated cells. Cells were treated with HCB (0.05, 0.5 and 5 μM) or ETOH for 24 h. The values are means ± SEM of three independent experiments. Significantly different (*$p < 0.05$, **$p < 0.01$) compared to the ETOH group.

OD (570 nm) values in cells treated with HCB (0.05, 0.5 and 5 μM) for 24 h when compared to ETOH (Fig. 3).

### 3.5. HCB effect on parameters of cell proliferation in HepG2 cells

Because we have demonstrated that HCB induced PCNA-positive cells in focal areas of rat liver, we hypothesized that HCB could increase cell proliferation in HepG2 cells. Western blots analysis, showed a dose-dependent increase (51, 120 and 210%) in PCNA protein levels, in HCB (0.05, 0.5 and 5 μM)-treated cells, respectively, compared to ETOH (Fig. 4A).

To evaluate time-response effects of HCB on PCNA expression, HepG2 cells were treated with 5 μM HCB for 2, 4, 6, 24 and 48 h. As shown in Fig. 4B, PCNA levels were increased (59%, 77% and 58%) compared to ETOH, after 6, 24 and 48 h, respectively.

To further confirm the potential of HCB as a promoter of cell proliferation, BrdU cell proliferation assay was performed. Immunocytochemical detection showed that BrdU labeling in nuclei of DNA synthesizing cells, was significantly increased (38%), by 5 μM HCB-treatment, when compared to ETOH-treated cells (Fig. 4C).

It should be remarked that antibody against BrdU is confined to S phase cells, whereas anti-PCNA antibody is against a more broad assessment of proliferation, and does not label only S phase cells (Foley et al., 1993). This difference may explain that dose-response effects of HCB, show different patterns in PCNA and BrdU assays. Altogether, these results demonstrate that HCB induces cell proliferation in a dose-and time-dependent manner.

### 3.6. Effect of HCB on cell cycle progression

The ability of a substance to affect specific phases of the cell cycle may provide clues to its mechanism(s) of action. To determine the effects of HCB on the cell cycle, HepG2 cells forced to quiescence were incubated with HCB (5 μM) or ETOH for 24 h. Cells were stained with propydium iodide (PI) and the DNA content analyzed by flow cytometry. Fig. 5A shows that untreated HepG2 cells showed a normal cell cycle distribution, with 59% in G0/G1 phase, 12% in S phase and 29% in G2/M phase. HCB-treated cells exhibited a significant increase in the S phase, from 9% to 14%, and from 32 to 36% in the G2/M phases, compared to ETOH-treated cells.

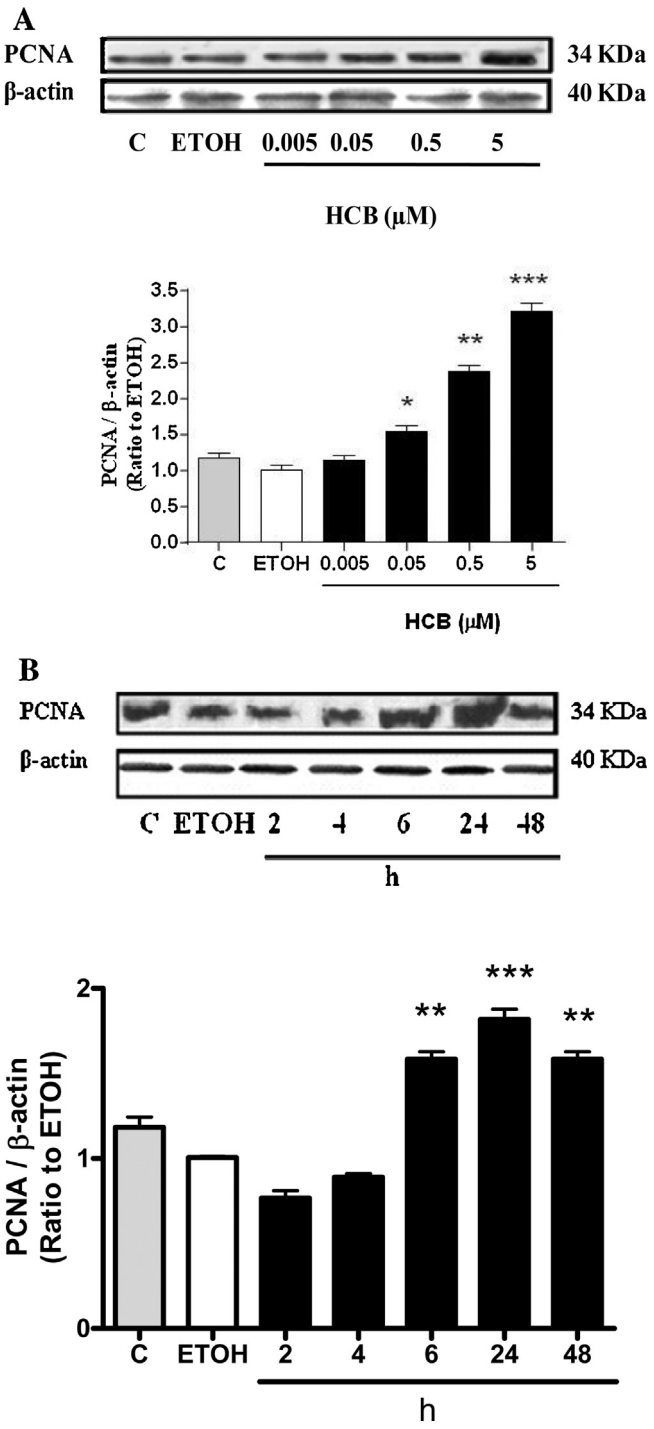


**Fig. 4.** HCB effect on HepG2 cells proliferation. (A) Western blot analysis of PCNA in total cell lysate of HepG2 cells treated with HCB (0.005, 0.05, 0.5 and 5 μM) during 24 h. A Western blot from one representative experiment is shown in the upper panel. Quantification of the PCNA protein levels by densitometric scanning of the immunoblots is shown in the lower panel. (B) Time-course of HCB effect on PCNA protein levels. HepG2 cells were treated with 5 μM HCB during 2, 4, 6, 24 and 48 h, and PCNA protein levels were analyzed by Western blot. Quantification of the PCNA/β-actin ratio to ETOH is shown in the lower panel. (C) Immunohistochemical detection of BrdU labeled cells/1000 cells (LI). Quantification of LI ratio to ETOH is shown in the lower panel. Values are means ± SEM of three independent experiments. Significantly different (*$p < 0.05$, **$p < 0.005$, *** $p < 0.001$) compared to ETOH group.

Cell cycle progression from G1 to S-phase is primarily controlled by the D-type cyclins in association with CDK4/6. Cycin D1 protein levels were analyzed by immunoblotting, in

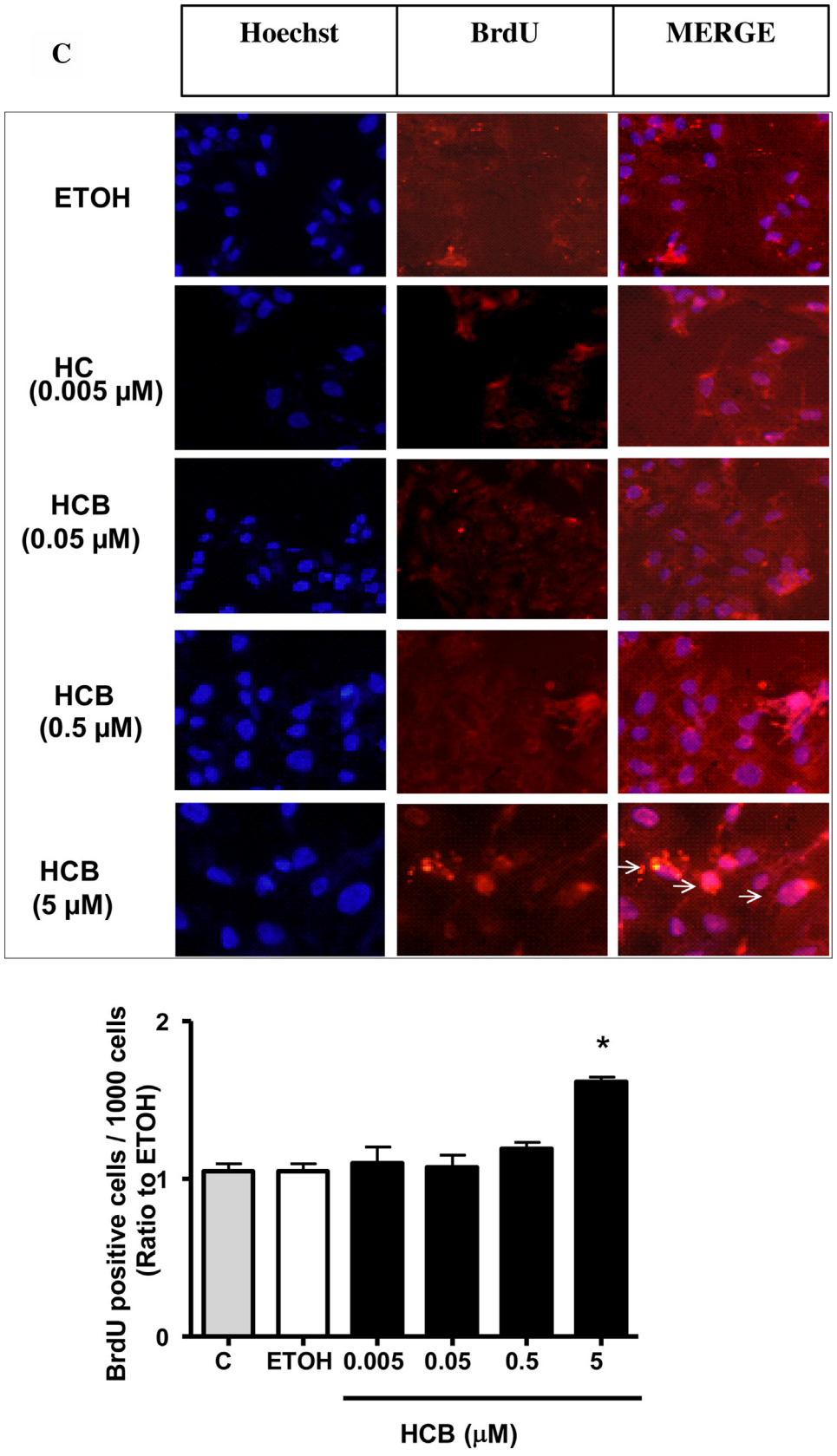


**Fig. 4.** (Continued)

HepG2 cells incubated with HCB (0.005, 0.05, 0.5 and 5 µM), or ETOH, for 24 h. As seen in Fig. 5B, a dose-dependent increase (20, 12, 47 and 39%) of cyclin D1/β-actin ratio to ETOH was observed in total cell lysates after HCB-treatment.

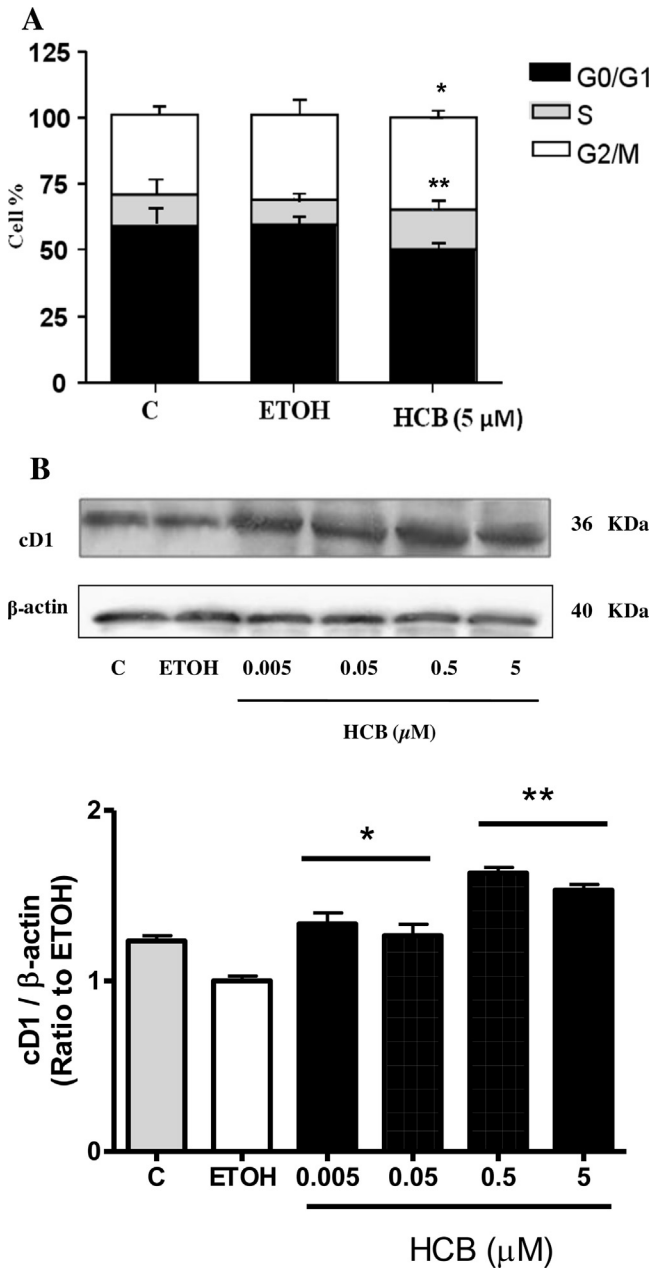


**Fig. 5.** HCB effect on cell cycle progression. (A) HCB increases HepG2 cells accumulation in S and G2/M phase. Cells were synchronized in the medium for 24 h, and then released into complete medium containing 5 μM HCB or ETOH. Percentage of cells in each phase was plotted. (B) Dose-response effect of HCB on cD1 protein levels. HepG2 cells were treated with HCB (0.005, 0.05, 0.5 and 5 μM) during 24 h, and cD1 protein levels were analyzed by Western blot. Quantification of the cD1/β-actin ratio to ETOH is shown in the lower panel. Values are means ± SEM of three independent experiments. Significantly different ($^{*}p < 0.05$, $^{**}p < 0.005$) compared to ETOH group. Cyclin D1 (cD1).

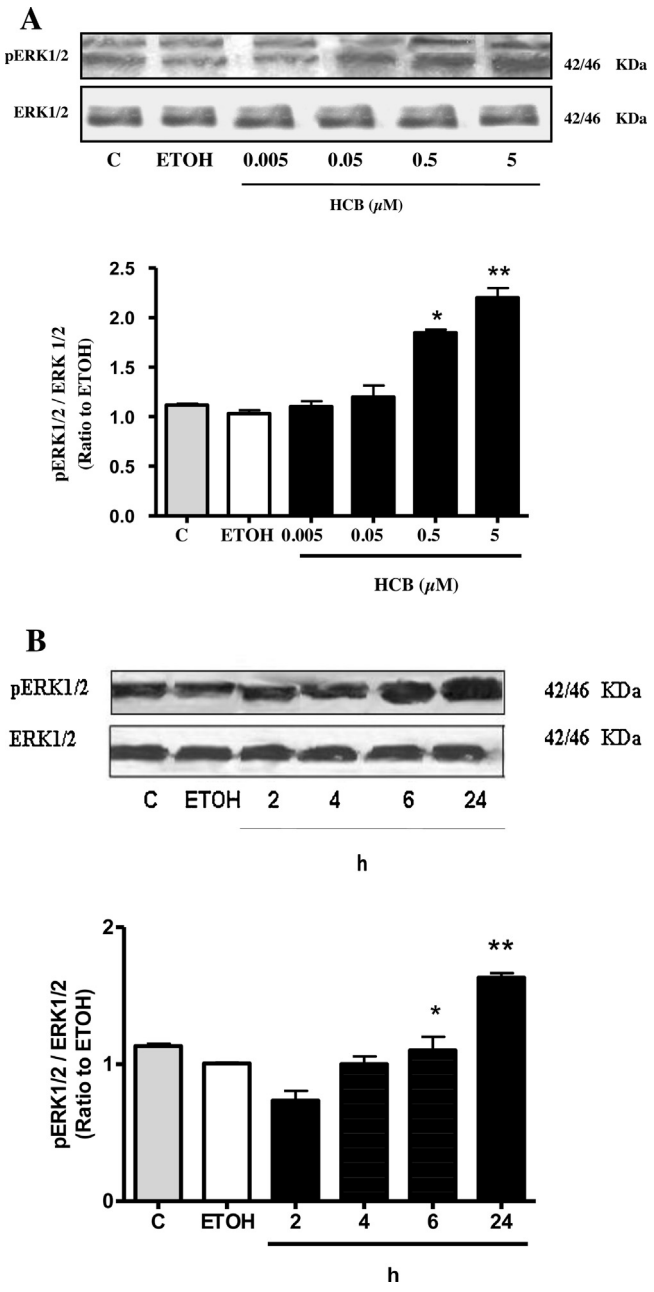


**Fig. 6.** HCB induced ERK 1/2 phosphorylation. (A) Dose-response effect of HCB on ERK1/2 phosphorylation; (B) Time-course of 5 μM HCB treatment. Quantifications of pERK1/2/ERK1/2 ratio to ETOH are shown in the lower panels. Values are means ± SEM of three independent experiments. Significantly different ($^{*}p < 0.05$; $^{**}p < 0.005$) compared to ETOH group.

### 3.7. HCB activates ERK1/2

Because ERK1/2 signaling is typically associated with cell proliferative events, phosphorylation of ERK1/2 was assayed by Western blot in total cell lysates. To evaluate dose–response effects, HepG2 cells were treated with HCB (0.005, 0.05, 0.5 and 5 μM) for 24 h. Our results showed that HCB (0.5 and 5 μM) significantly enhanced ERK1/2 phosphorylation (83 and 116%) respectively, compared to ETOH (Fig. 6A).

Time-dependent studies were performed incubating HepG2 cells with 5 μM HCB for 2, 4, 6 and 24 h. As shown in Fig. 6B, 0.5 and 5 μM HCB significantly increased ERK1/2 phosphorylation, at 24 h of treatment. Altogether, these results indicate that HCB induced a dose- and time- dependent increase in ERK1/2 signal transduction pathway in HepG2 cells.

### 3.8. Role of ERK1/2 on HCB-induced PCNA and cyclin D1expression

We further investigated whether the activation of ERK1/2 was involved in HCB-induced cells proliferation. HepG2 cells were pretreated with the specific MEK1 inhibitor (PD98059) (5 and 10 μM) for 2 h, and further treated with 5 μM HCB during 24 h. Our results showed that HCB-induced PCNA expression was significantly reversed by PD98059 at both assayed concentrations

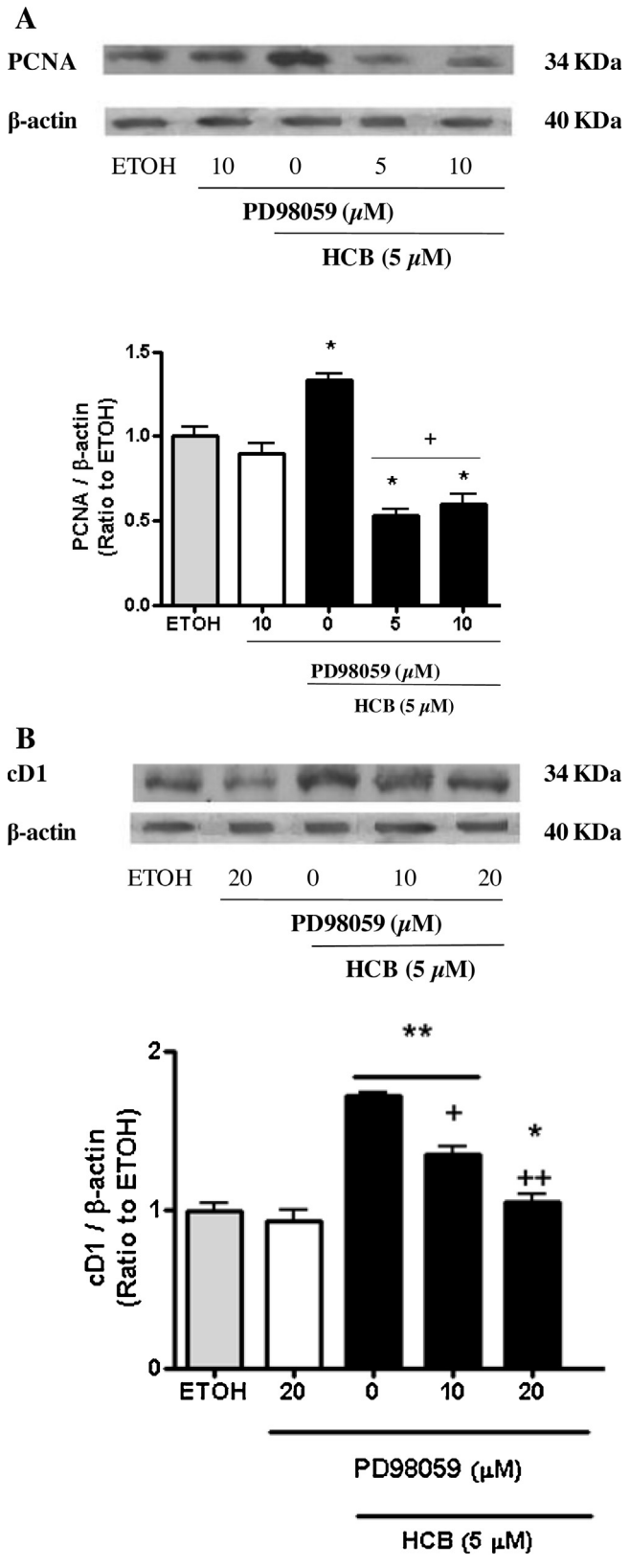


**Fig. 7.** Role of ERK1/2 in HCB induced PCNA and cyclin D1 expression. HepG2 cells were treated with 5 μM HCB for 24 h in the presence or absence of MEK1 inhibitor. (A) Western blot of PCNA in HepG2 cells, pretreated with PD98059 (5 and 10 μM) or DMSO and further treated with HCB or ETOH. Quantification of PCNA/β-actin ratio to ETOH is shown in the lower panel. (B) Western blot of cyclin D1 protein levels in HepG2 cells pretreated with PD98059 (10 and 20 μM) or DMSO and further treated with HCB or ETOH. Quantification of cyclinD1/β-actin ratio to ETOH by densitometric scanning of the immunoblots is shown in the lower panels. Values are means ± SEM of three independent experiments. Significantly different compared to ETOH-treated cells ($^{*}p < 0.05$; $^{**}p < 0.005$), and significantly different compared to 5 μM HCB-treated cells ($^{+}p < 0.01$; $^{++}p < 0.001$).

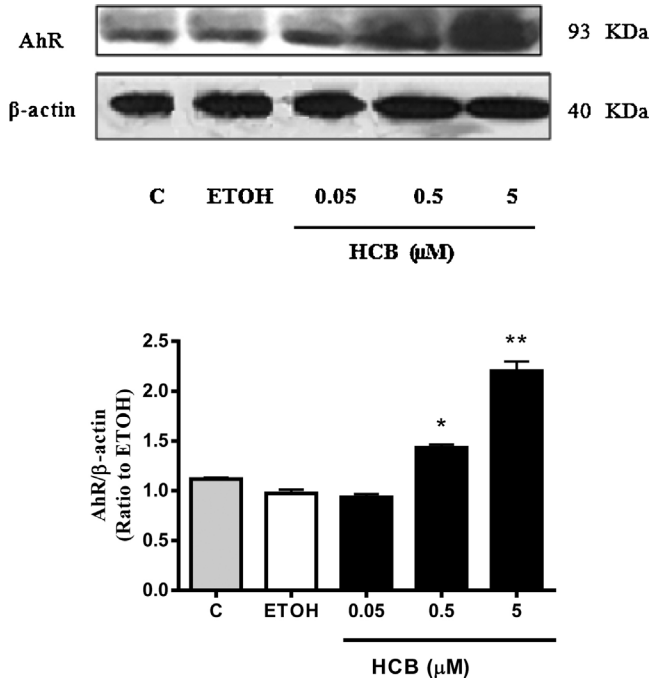


**Fig. 8.** HCB induces AhR expression. HepG2 cells were treated with HCB (0.05, 0.05 and 5 μM) or ETOH, for 24 h. Western blot from one representative experiment is shown in the upper panel. Quantification of AhR/β-actin ratio to ETOH, by densitometric scanning of the immunoblots is shown in the lower panel. Values are means ± SEM of three independent experiments. Significantly different ($^{*}p < 0.05$; $^{**}p < 0.005$) compared to ETOH.

(Fig. 7A). These data demonstrate that HCB-induced cell proliferation is mediated by ERK1/2 signaling pathway.

We next sought to determine whether HCB-induced cyclin D1 protein expression, was dependent on ERK1/2 activation. HepG2 cells were pretreated with (10 and 20 μM) PD98059, for 2 h, followed by 24 h exposure to 5 μM HCB. As shown in Fig. 7B, 20 μM MEK1 inhibitor, reversed HCB-induced cyclin D1 protein levels.

### 3.9. HCB induces AhR expression in HepG2 cells

Because it has been demonstrated that AhR expression is increased in progression of hepatocellular carcinoma (Liu et al., 2013), we examined the effect of HCB (0.05, 0.5 and 5 μM) treatment, on AhR protein expression in HepG2 cells. Fig. 8 shows that HCB significantly increased AhR protein levels in a dose-dependent manner, in total cell lysates, compared to ETOH treatment.

### 3.10. AhR is a mediator of HCB-induced PCNA and pERK1/2 protein expression, and cell cycle progression

It has been reported that cellular AhR protein content is relevant to induce proliferation (Shimba et al., 2002). To determine whether HCB induced PCNA expression and ERK1/2 activation are mediated by AhR, HepG2 cells were pre-incubated for 1 h in the presence or absence of an AhR antagonist, 4,7-OP (5, 10, 20 and 50 μM), and then exposed to 5 μM HCB for 24 h. As shown in Fig. 9A, a significant dose-dependent decrease of PCNA protein levels was observed with increasing concentrations of 4,7-OP,

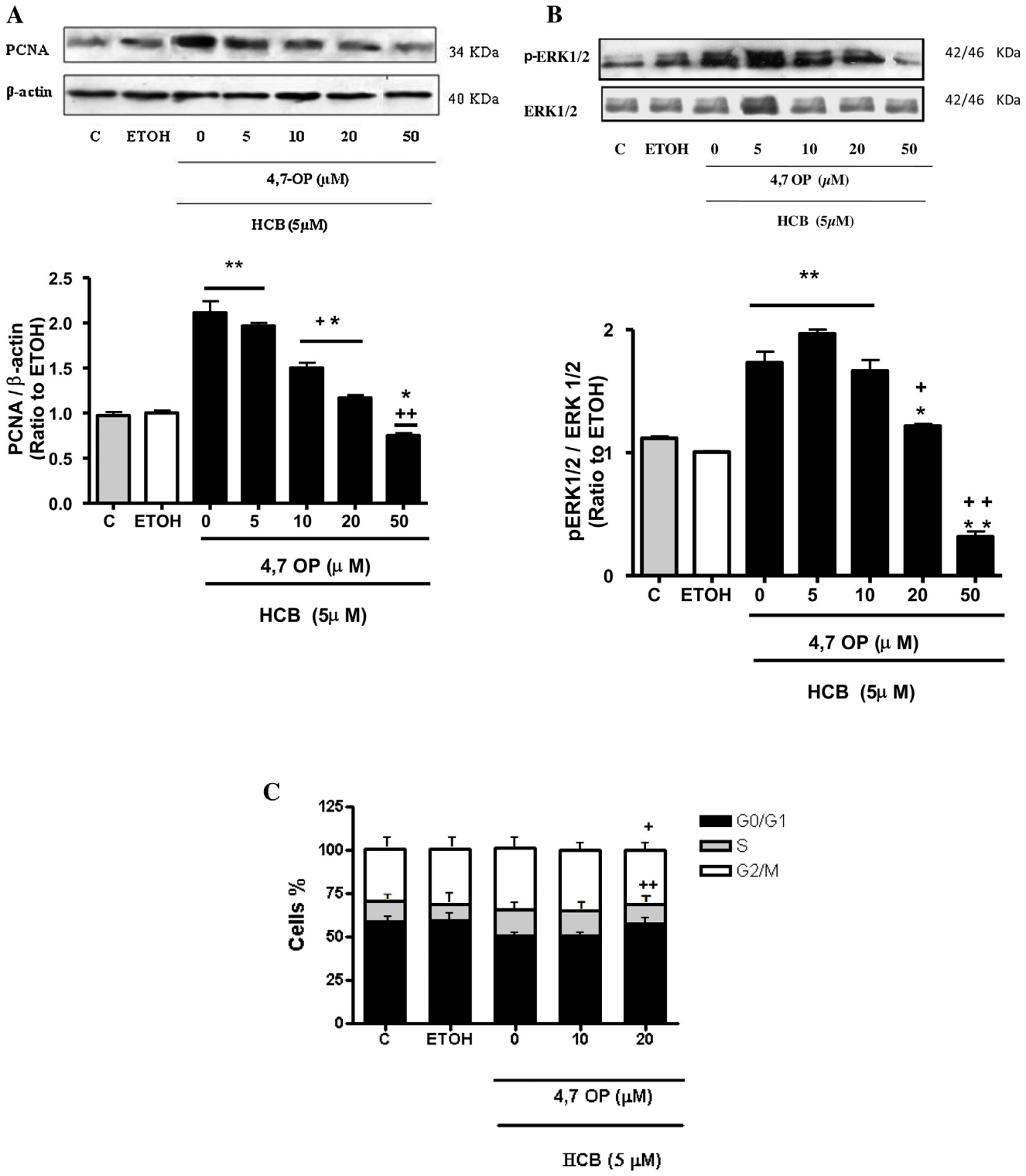


**Fig. 9.** Role of AhR in PCNA and pERK1/2 protein expression, and cell cycle progression. HepG2 cells were pretreated with 4, 7-OP or DMSO and further treated with 5 μM HCB or ETOH for 24 h in the presence of the inhibitor. Western blots of total cell lysate from one representative experiment are shown in the upper panels. Densitometric scanning of the immunoblots is shown in the lower panels. (A) Effect of 4, 7-OP, on PCNA protein expression. (B) Effect of 4, 7-OP, on pERK1/2 and ERK1/2 protein levels. (C) Effect of 4, 7-OP on cell cycle distribution. Cells were pretreated with 4, 7-OP for 2 h, and further treated with 5 μM HCB or ETOH. Values are means ± SEM of three independent experiments. Significantly different compared to ETOH-treated cells. ($^{*}p < 0.05$; $^{**}p < 0.01$), and significantly different compared to 5 μM HCB-treated cells ($^{+}p < 0.01$; $^{++}p < 0.001$).

when compared to 5 μM HCB-treated cells, in the absence of the inhibitor.

Similarly, we sought to determine whether HCB-induced ERK1/2 activation was dependent on AhR signaling pathway. A significant decrease (25% and 58%), of ERK1/2 phosphorylation, was observed in the presence of 20 and 50 μM 4,7-OP, respectively, compared to HCB-treated cells in the absence of the inhibitor (Fig. 9B). Altogether, these findings indicate that HCB-induced

PCNA expression and ERK1/2 phosphorylation were dependent on AhR-mediated signaling pathway.

It has been demonstrated that AhR regulates cell cycle progression (Barhoover et al., 2010). We evaluated the role of AhR on cell cycle distribution of 5 μM HCB-treated HepG2 cells, preincubated in the presence or absence of 4,7-OP (10 and 20 μM), and then exposed to HCB for 24 h. Flow cytometry analysis showed that 20 μM (4,7-OP)-treatment resulted in a significant decrease in the percentage of cells both in S and G2/M phase, from 16 to 10%, and from 34 to 30%, respectively, when compared to HepG2 cells incubated with HCB, in the absence of AhR antagonist (Fig. 9C).

## 4. Discussion

Previous studies from our laboratory have demonstrated that HCB induces dysregulation of cellular growth in rat liver (Giribaldi et al., 2011). The present study demonstrates that HCB increased the development of rat liver preneoplastic lesions in a medium-term initiation/promotion model. These results are consistent with those observed previously by Gustafson et al., (2000). Administration of HCB without DEN initiation resulted in the lack of preneoplastic foci, reinforcing the idea that this compound has no capability for initiating liver carcinogenesis, which suggests that increased promotion of preneoplastic foci probably occurs through non-genotoxic mechanisms. Similar results were also observed with other so-called non-genotoxic carcinogens such as phenobarbital and p,p'-dichlorodiphenyltrichloroethane (DDT) (Tsuda et al., 2003).

Our study shows that PCNA positive cells were increased in GST-P-positive liver foci, which strongly suggests that HCB contributed to the development of preneoplastic foci by enhancing the proliferation of initiated cells. These results are relevant because cell proliferation may increase the risk of mutations, and may be important in selective clonal expansion of initiated cells to form preneoplastic foci and eventually tumors (Ou et al., 2001, 2003). In a similar manner HCB treatment induced PCNA expression and BrdU incorporation into DNA, in HepG2 cells. Based on these data, it is clear that enhanced MTT assay, observed in our study, results from an increase in the number of cells. Our results remark the apparent concordance of HCB effect on cell proliferation in preneoplastic liver foci and in the human-derived hepatoma cell line. Comparative studies have shown that PCNA provides a strong correlation with flow cytometric assessment of S phase proliferating cells (Weisgerber et al., 1993). In agreement, our results show that HCB- induced PCNA expression may be associated with an increase in the number of cells that accumulate in the S phase of the cell cycle.

AhR is expressed in multiple tissue types, in cancer cell lines, and in tumors from animal models. There is considerable evidence to support the critical role played by AhR and AhR-regulated gene batteries in murine hepatocarcinogenesis (Celius and Matthews, 2010). To date the effect of HCB, a weak agonist of the AhR (Hahn et al., 1989), on the expression of AhR in preneoplastic foci has not yet been investigated. Herein, we have demonstrated that HCB induced the expression of AhR in both rat liver preneoplastic foci, and in HepG2 cells, suggesting that this receptor might play a role in HCB-induced liver tumor promotion. Interestingly, mouse models overexpressing a constitutively active form of the AhR, demonstrated the role of this receptor as a proliferation promoter (Moennikes et al., 2004).

Several reports describe a role for the AhR in cell cycle control; most of these reports identified $G_1$ phase as the cell cycle period influenced by AhR activity (Ma and Whitlock, 1996; Levine-Fridman et al., 2004). In the present study, we have demonstrated that HCB increased number of cells transiting into S- and G2/M phases of the cell cycle, in HepG2 cells. On the other hand it has been reported that TCDD inhibits cell proliferation and induces cell cycle arrest in G1 phase, in a rat hepatoma cell line (Huang and Elferink, 2005). These results indicate that the effect of AhR ligands could be associated with their capacity to alter signal transduction pathways controlling cell proliferation and apoptosis, with the ultimate response and responsible mechanism(s) being dependent upon the specific phenotype of the cell.

Within the cell cycle, progression from G (1) to S phase is controlled by sequential phosphorylation of the retinoblastoma protein (RB1) by cyclin D-cyclin-dependent kinase (CDK) 4/6 complexes. Amplification of the cyclin D1 gene and its overexpression has been associated with aggressive forms of human hepatocellular carcinoma (Deane et al., 2001). Our data, demonstrating that HCB induced cyclin D1 protein expression in HepG2 cells, is consistent with a corresponding increase in cells entry into S-phase. Conversely, we have recently demonstrated that HCB induced a decrease in nuclear cyclin D1 that was consistent with a corresponding inhibition in FRTL-5 cells entry into S-phase (Chiappini et al., 2014). These results indicate that HCB effect on cell cycle progression depends on tissue context, but also vary with species. It is worthy to note that elevating the level of cyclin D1 is not sufficient to induce cell cycle entry, because repression of CDK inhibitors (CDKIs) is also a pre-requisite for G1/S phase transition entry. Further experiments are required to investigate the effect of HCB on CDK4 protein levels and CDKIs. Cell cycle progression is also regulated by various mitogenic signal transduction pathways, including the MEK/ERK cascade (Chang et al., 2003). ERK activation plays a fundamental role for G1/S transition since the phosphorylated form is required for the induction of the cyclin D1 protein and E2F family of genes which results in the expression of target genes, such as PCNA, required for S phase entry (Chambard et al., 2007).

Herein, we have reported that HCB induces ERK1/2 phosphorylation in HepG2 cells. In a similar manner we have previously demonstrated that HCB induced ERK1/2 activation, in rat liver (Giribaldi et al., 2011). The role of ERK1/2 as a mediator of HCB-induced PCNA and cyclin D1 expression was demonstrated in the present study. Similarly, heptachlor, an organochlorine compound used as an insecticide, triggers proliferation in rat hepatocytes both by the induction of ERK1/2 phosphorylation and the inhibition of apoptosis (Okoumassoun et al., 2003).

Herein, we demonstrate for the first time, that AhR acts as a mediator of HCB-induced ERK1/2 activation, in HepG2 cell line. However, we have previously demonstrated that HCB could stimulate ERK1/2 activation in MDA-MB-231 cells, through AhR-dependent and independent mechanisms according to HCB concentration (Pontillo et al., 2011).

In the present study, we have also shown that HCB-induced alterations in cell cycle progression were AhR dependent. Similarly, other studies found that RNAi and knockdown of the AhR in human hepatoma HepG2 cells results in growth inhibition, accompanied by downregulation of several cell cycle-related genes including cyclin D1 (Abdelrahim et al., 2003).

In summary, we demonstrate for the first time, that HCB induces cell proliferation and AhR expression in rat liver preneoplastic foci and in HepG2 cells. HCB-induced cell proliferation and regulation of cell cycle progression depend on ERK1/2 phosphorylation which is mediated by the AhR in the human hepatoma cell line. Our results provide a clue to the molecular events involved in the mechanism of action of HCB-induced hepatocarcinogenesis.

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## Conflict of interest statement

The authors declare that there are not conflicts of interest.

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