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Hexachlorobenzene induces TGF-β1 expression, which is a regulator of p27 and cyclin D1 modifications

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HIGHLIGHTS

- Hexachlorobenzene induced cell cycle arrest at G2/M and at G0/G1 phase.
- Cytosolic and nuclear p27 protein levels were increased by hexachlorobenzene.
- Hexachlorobenzene induced a decrease in nuclear cyclin D1 protein levels.
- Hexachlorobenzene-treatment resulted in an increase in TGF-β1 expression.
- TGF-β1 is a regulator of p27 and cyclin D1 expression.

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ABSTRACT

Hexachlorobenzene (HCB) is an organochlorine pesticide widely distributed in the environment. In this study we have demonstrated that HCB induced loss of cell viability and alterations in cell cycle regulation in FRTL-5 rat thyroid cells. Analysis of cell cycle distribution by flow cytometric analysis demonstrated that HCB induced cell cycle arrest at G2/M and at G0/G1 phase, inhibiting cell cycle progression at the G1/S phase, after 24 h and 72 h of treatment.

HCB-treatment resulted in an increase in transforming growth factor-beta (TGF-β1) mRNA levels, a negative regulator of cell growth in thyroid epithelial cells. Time-dependent studies showed that both cytosolic and nuclear p27 protein levels were increased by 5 μM HCB. After 24 h of treatment, total p27 in whole cells lysate was increased. Dose-dependent studies, demonstrated that HCB (0.005, 0.05, 0.5 and 5 μM) increased p27, both in the cytosol and nucleus. HCB (5 μM) induced a concomitant decrease in nuclear cyclin D1 protein levels, in a time-dependent manner. We have also demonstrated that TGF-β1 Smad signaling is involved in HCB-induced alterations of p27 and cyclin D1 protein levels. On the other hand, ERK1/2 activation is not involved in the alteration of cell cycle regulatory proteins.

1. Introduction

Hexachlorobenzene (HCB) is a persistent environmental pollutant, currently formed as a byproduct in several industrial processes. HCB exposure is associated with a wide variety of toxic effects in humans and experimental animals. Serious hepatotoxic, neurotoxic, reproductive, developmental and carcinogenic effects have been reported (ATSDR, 2002). HCB is a dioxin-like compound and a weak ligand of the aryl hydrocarbon receptor (AhR) (van Birgelen, 1998), which is a ligand-dependent transcription factor that modulates processes such as apoptosis, proliferation and migration (Dietrich and Kaina, 2010). Alterations in thyroid metabolism and a risk excess of thyroid cancer have been reported in human populations exposed to organochlorinated compound mixtures with a high content of HCB (Grimalt et al., 1994; Meeker et al., 2007).

Tight regulation of cell number is essential for the maintenance of tissue homeostasis. In many physio-pathological circumstances, growth activating and inhibiting stimuli must be perfectly balanced in order to achieve the right amount of cell proliferation. Loss of cell viability may be due to increased apoptosis, necrosis or cell cycle arrest. In previous works, we have demonstrated that

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HCB induces apoptosis in rat thyroid, and in FRTL-5 cells (Chiappini et al., 2009, 2013).

Cellular proliferation is a tightly regulated sequence of progression from G0/G1 to S to G2/M that requires the activation of specific cyclin dependent kinases (CDKs) and simultaneous inhibition of CDK inhibitors. In mammalian cells, Gl progression is controlled by CDK4 or CDK6 associated with any one of three cyclin D isoforms and CDK2 associated with cyclin E. Two groups, the INK4 family (p15, p16, p18, p19) and the Cip/Kip family (p21, p27, p57), have been described. Physiologically, p27 is believed to act primarily to regulate progression of cells from late G1 into S through its interaction with cyclin E-CDK2 complexes (Polyak et al., 1994).

Iodine and transforming growth factor-β1 (TGF-β1) are the only known negative regulators of thyroid cell number (Knobel and Medeiros-Neto, 2007; Mincione et al., 2011). The thyroid gland expresses TGF-β1 protein, which under physiologic conditions regulates thyroid growth and function. It has been demonstrated that TGF-β1 controls thyroid growth, through the modification of cell cycle regulatory proteins (Carneiro et al., 1998). TGF-β exerts its effects by binding to a type II receptor located at the cell membrane. The TGF-β type II receptor complex then recruits type I receptor, and this new complex stimulates the phosphorylation of Smads 2 and 3, which are subsequently transferred to the nucleus, where they regulate gene transcription (Massagué et al., 2000). TGF-β1 also induces other none-Smad signaling pathways, which include activation of JNK/SPAK, p38 and ERK1/2 pathways (Zhang, 2009). The main anti-proliferative mechanism of TGF-β1 on the thyroid cells has been reported as G1 phase arrest (Carneiro et al., 1998). p27 has been implicated as a mediator of growth arrest due to TGF-β1, cAMP, and other extracellular factors (Kato et al., 1994).

The objective of this study was to determine if alterations in cell cycle regulatory proteins and cell cycle progression contribute to the mechanism of HCB-induced loss of cell viability in FRTL-5 cells. Since TGF-β1 is a negative regulator of thyroid cell number, we assessed the effect of HCB on the expression of TGF-β1, and its involvement in the molecular mechanism of cell cycle progression.

2. Materials and methods

2.1. Chemicals

HCB (>99% purity, commercial grade), Coons' modified F-12 medium, thyroid stimulating hormone (TSH), insulin, hydrocortisone, transferrin, somatostatin, glycyl-L-histidyl-L-lysine, protease inhibitors, phenylmethanesulfonyl fluoride (PMSF), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), aprotinin, leupeptin, and pepstatin, and specific inhibitors SB431542 and PD98059 were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-cyclin D1 and anti-p27 antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA). Anti-β-actin was purchased from Abcam Inc. (Cambridge, MA, USA). Dulbeccós modified Eaglés medium (DMEM) was obtained from Invitrogen Life Technology (Cambridge, MA). DMEM-high glucose, without phenol-red, and antibiotics were all purchased from Sigma Chemical Co. (St. Louis, MO). The enhanced chemiluminescence kit (ECL) was from GE Healthcare Life Sciences (Buckinghamshire, UK). All other reagents used were of analytical grade.

2.2. Cell culture

Fisher rat thyroid cell line (FRTL-5) is derived from Fisher rat thyroids and has an obligate requirement for TSH for growth in cell culture. FRTL-5 cells were routinely cultured in Coons' modified F-12 medium (50%) and DMEM-high glucose (50%), containing a six-hormone (6H) mixture composed of insulin (10 μg/ml), hydrocortisone (10 nM), transferrin (5 μg/ml), glycyl-L-histidyl-L-lysine (10 ng/ml), somatostatin (10 ng/ml), and bovine thyroid stimulating hormone (TSH) (1 mU/ml), and supplemented with penicillin (10,000 IU/ml), streptomycin (10 mg/ml), amphotericin B (25 μg/ml), and 5% fetal bovine serum (FBS), in 5% CO_2 – 95% O_2 at 37 °C in a humidified incubator until confluence. The medium was replaced with fresh medium every 2 and 3 days. Doubling time was 36 h.

2.3. Cell treatment for time–course and dose–response studies

FRTL-5 cells were seeded in 100 mm dishes (1.5×10^6 cells) in DMEM/F12 complete growth medium followed by overnight incubation to allow cells to attach. Afterwards, the medium was withdrawn and replaced with fresh serum and TSH free medium (5H medium without FBS), and 24 h later, cells were exposed to 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 in DMEM/F12 complete growth medium, or vehicle for 2, 4, 6, 8, 24 and 30 h. For dose–response studies, cells were exposed for 6 or 24 h to HCB (0.005, 0.05, 0.5 and 5 μM) in DMEM/F12 complete growth medium. Selected doses were in the same range 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 processed according to the experiment.

2.4. Cell treatment for inhibitor assays

For assays performed in the presence of specific ERK1/2, and TGF-β1 SMAD signaling inhibitors, cells were pre-treated for 1 h with 10 μM of MEK inhibitor, PD-98059 and 0.5 μM of TGF-β1 SMAD signaling specific inhibitor, SB431542, respectively. 5 μM HCB or vehicle was added to the media during the time indicated in the corresponding figures, in the presence or absence of inhibitors and then cells were washed with PBS.

2.5. Subcellular fractionation

FRTL-5 cells were washed three times with ice-cold PBS, and harvested in 5 volume of lysis buffer containing 20 mM

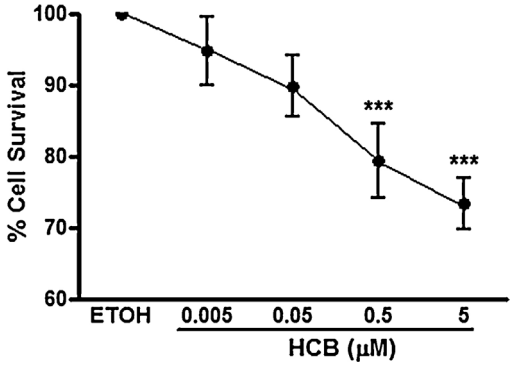

Fig. 1. Effect of HCB on FRTL-5 cell survival. The viability of cells was evaluated using the viability MTT assay. Then the absorbance was measured at 570 nm, and the results were expressed as percentage of ETOH-treated cells. Data are expressed as means ± SEM of three independent experiments. Asterisks indicate significant differences versus ETOH-treated cells. (***$p < 0.001$). Statistical comparisons were made by one-way ANOVA with a 95% confidence interval followed by Tukey post hoc test to identify significant differences between mean values and indicated controls.

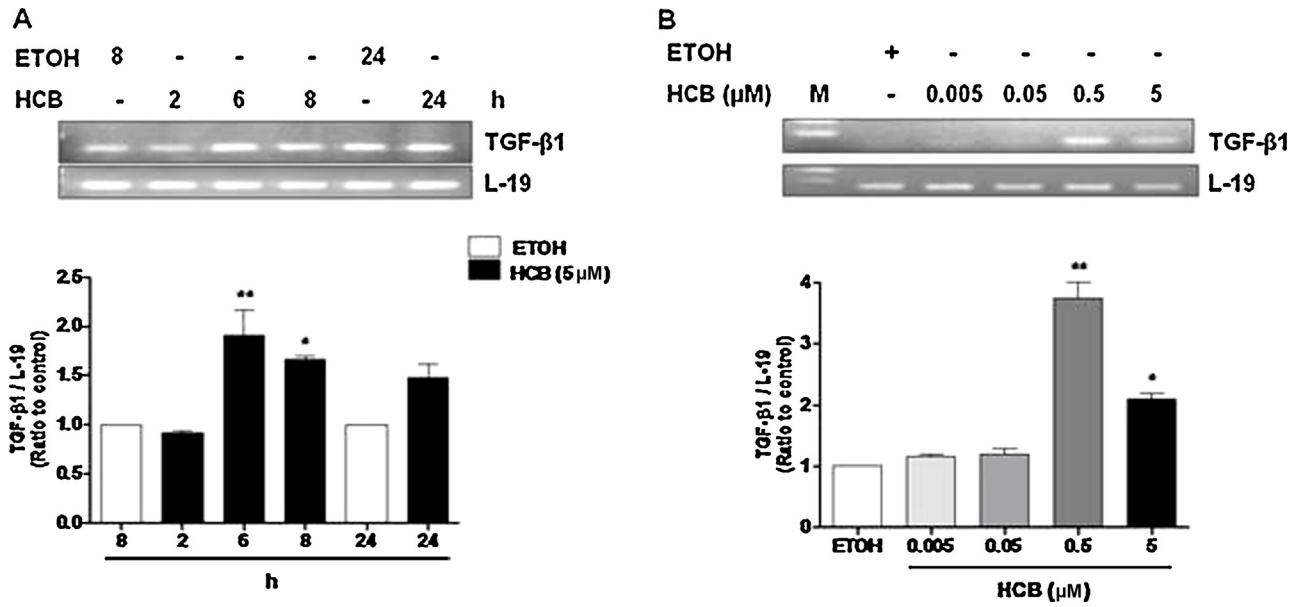

Fig. 2. Analysis of TGF-β1 expression in HCB-treated FRTL-5 cells. (A) Cells were incubated with HCB (5 μM) or ETOH during 2, 6, 8 or 24 h. (B) Cells were incubated with HCB (0.005, 0.05, 0.5 and 5 μM) for 6 h. L-19 was used as a loading control. Representative patterns of RT-PCR amplification of TGF-β1 cDNA from control and HCB-treated cells, synthesized from total RNA are shown in the upper panels. Quantification of TGF-β1 mRNA, after correction with L-19 is shown in the lower panels. Values are means ± SEM of three independent experiments. Asterisks indicate significant differences versus ETOH-treated cells. ($^{*}p < 0.05$, $^{**}p < 0.01$). Statistical comparisons were made by one-way ANOVA with a 95% confidence interval followed by Tukey post hoc test to identify significant differences between mean values and indicated controls.

(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. Nuclei were pelleted by centrifugation at 750 × *g* for 10 min. The supernatant layer was centrifuged for 15 min at 10,000 × *g* to remove mitochondria, and the resulting supernatant was used for cytosolic proteins assays. The nuclear pellet was resuspended in lysis buffer containing 0.5% Triton-X100, 0.15 M NaCl, 20 mM Tris–HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 50 mM NaF, 1 mM PMSF, 2 mM Na_3VO_4, 20 μg/ml aprotinin, 120 μM leupeptin and 12 μM pepstatin, and the resulting suspension was used for the assay of nuclear proteins. Protein concentration was determined according to Bradford (1976); using bovine serum albumin (BSA) as a standard.

2.6. Cell viability

The measurement of cell viability was evaluated by MTT colorimetric assay. 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, 6×10^3 FRTL-5 cells were seeded in 96-well plates, and maintained in DMEM/F-12 complete medium, for 24 h. The next day medium was removed and TSH and serum-free DMEM/F12 medium was added. Finally cells were treated with HCB (0.005, 0.05, 0.5 and 5 μM) or ETOH, in complete DMEM/F12 medium for (2, 6, 8, 24 and 48 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 sulphoxide (DMSO), and the absorbance of the solution was measured at 570 nm using the microplate reader Synergy HT (Biotek Instruments, Inc., USA).

2.7. Cell cycle analyses

Cells were cultured for 24 h without FBS and TSH. Synchronized cells were then treated with 5 μM HCB or vehicle, in 6H media and harvested for up to 24 and 72 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 μg/ml PI in PBS containing 0.2 mg/ml of DNase-free RNase A). After incubation for 30 min at 37 °C, samples were evaluated by flow cytometry (Becton Dickinson, USA). Cell cycle distribution was analyzed using Cylchred 1.0.2 software (Cardiff University, UK).

2.8. Western blotting

Total cellular protein lysates, cytosolic or nuclei proteins 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 overnight at 4 °C with mouse monoclonal antibodies, anti-cyclin D1 (1:1000), anti-p27 (1:500), and anti-β-actin (1:2000). After incubation, membranes were washed five times with TBST, and

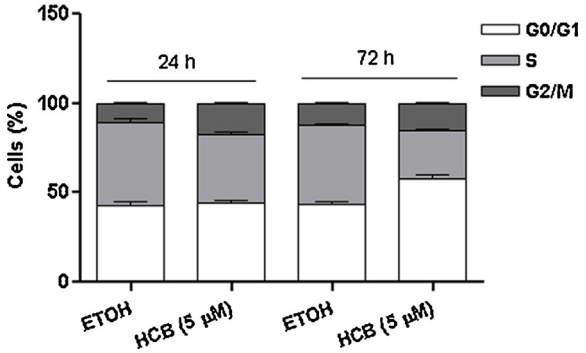

Fig. 3. HCB induces G1/S and G2/M cell cycle arrest in FRTL-5 cells.

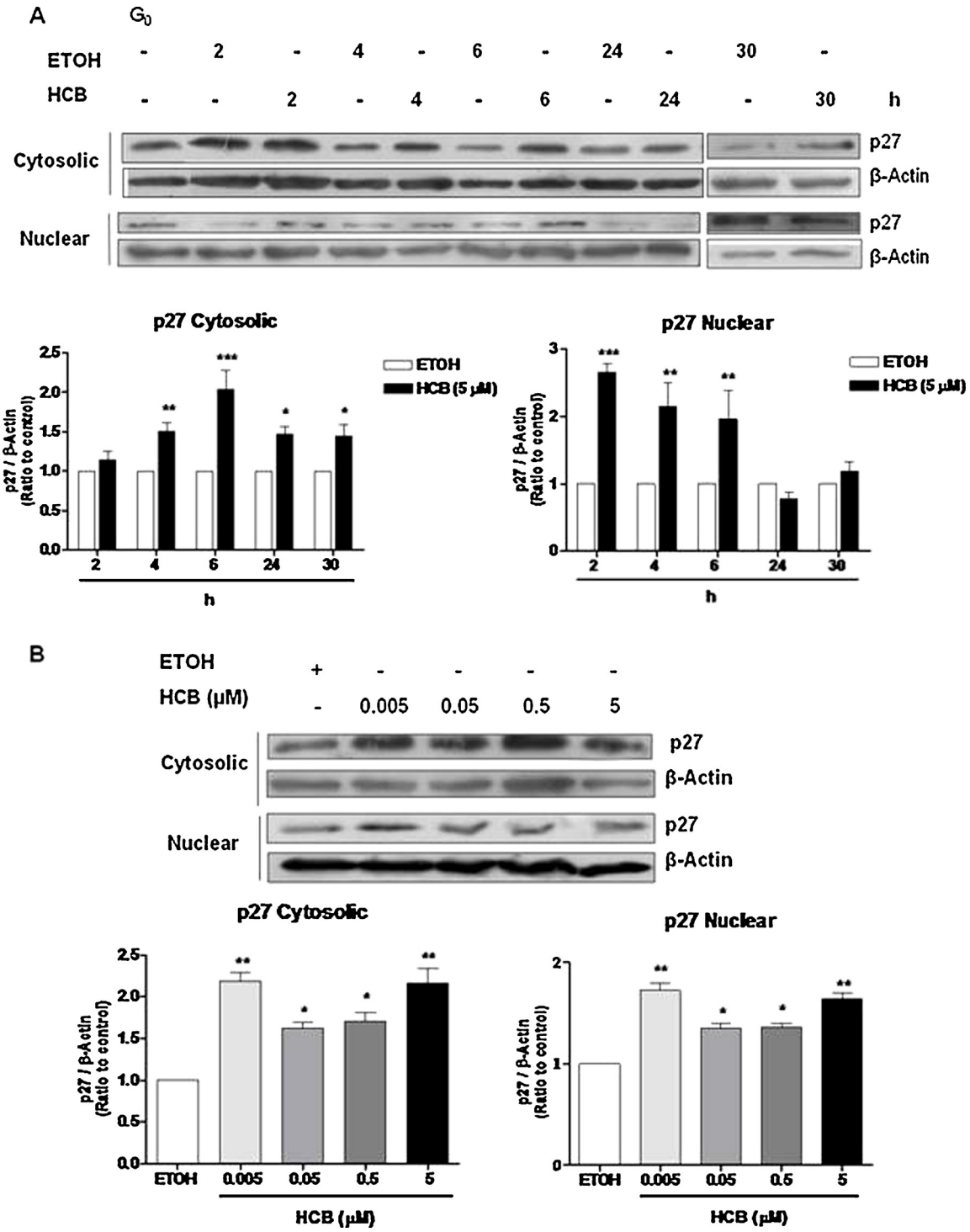

Fig. 4. HCB increases p27 protein levels. FRTL-5 cells were cultured with HCB (5 μM) or ETOH for 2, 4, 6, 24 and 30 h, or HCB (0.005, 0.05, 0.5 and 5 μM) for 6 h. Cytosolic and nuclear fractions were prepared, and proteins were resolved by SDS-PAGE and blotted with the corresponding specific antibodies. Western blots from one representative experiment are shown in the corresponding upper panels. Densitometric scanning of the immunoblots is shown in the lower panels. (A) Time course of HCB effect on cytosolic and nuclear p27 levels. (B) Dose–response effect of HCB-treatment for 6 h, on cytosolic and nuclear p27 protein levels. Quantification of p27/β-actin ratio to control are shown in the lower panels. Data are expressed as means ± SEM of three independent experiments. Asterisks indicate significant differences versus ETOH-treated cells. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Statistical comparisons were made by one-way ANOVA with a 95% confidence interval followed by Tukey post hoc test to identify significant differences between mean values and indicated controls.

the suitable peroxidase-conjugated anti-species-specific antibodies were used for protein detection. After washing, blots were reacted using and ECL detection kit (Amersham Biosciences, Inc., UK) and quantified by scanning laser densitometry in a Fotodyne (Foto/Analyst), Gel-Pro Analyzer 3.1.

2.9. Statistical analysis

Data are expressed as means ± 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 samples and their respective controls, after testing homogeneity of variance using Barlett's procedure. Differences between control and treated cells were considered significant when p values were <0.05. For each experiment, at least three independent assays were performed.

3. Results

3.1. HCB effect on FRTL-5 cell viability

To determine whether HCB affects cell viability, FRTL-5 cells, were incubated with increasing concentrations of HCB ranging from 0.005 to 5 μM, for 24 h. As shown in Fig. 1, HCB decreased cell viability in a dose-dependent manner. Maximal loss of cell viability (25%) was induced by 5 μM HCB, and this concentration was used to treat cells in subsequent experiments.

3.2. HCB increases TGF-β1 mRNA levels

As we have previously demonstrated that TGF-β1 was increased in thyroids of HCB-treated rats (Chiappini et al., 2009), we evaluated TGF-β1 mRNA content in FRTL-5 cells exposed to the pesticide. Time-dependent studies demonstrated that TGF-β1 content significantly increased (95% and 70%) after 6 and 8 h of HCB exposure, respectively, and remained higher than control values after 24 h of treatment (Fig. 2A).

Dose-dependent studies showed that TGF-β1 mRNA content, was increased 285% and 100%, respect to ETOH treated cells, after 0.5 and 5 μM HCB-treatment, respectively (Fig. 2B). We have also demonstrated that 5 μM HCB significantly increases, TGF-β1 protein levels after 6 h of treatment (results not shown). Altogether, the above results demonstrate that HCB increases TGF-β1 gene expression.

3.3. HCB induces G0/G1 and G2/M cell cycle arrest

To determine if exposure to HCB perturbs cell cycle progression, FRTL-5 cells, forced to quiescence by TSH and serum withdrawal, were incubated with 5 μM HCB or ETOH, for 24 and 72 h, in 6H media, and assayed for cell cycle phase distribution by flow cytometry. The pesticide was used at the same concentration as that used in the apoptosis assays reported in our previous study (Chiappini et al., 2013).

Fig. 3 shows that HCB-treated cell exhibited significant G2/M arrest at 24 h (10.4–17.2%), ($p < 0.05$) compared to ETOH-treated cells, respectively. S-phase population decreased from 46.8% to 38% ($p < 0.05$). We next investigated HCB-induced G1 arrest over a longer period of time (up to 72 h). Similar to above, G2/M arrest persisted, but without significantly alterations at 72 h. The percentage of G0/G1 population increased from 43.5 to 58%, ($p < 0.01$) and consequently fewer cells progressed to S-phase (44.1 versus 26.4%) ($p < 0.01$) compared to ETOH-treated cells.

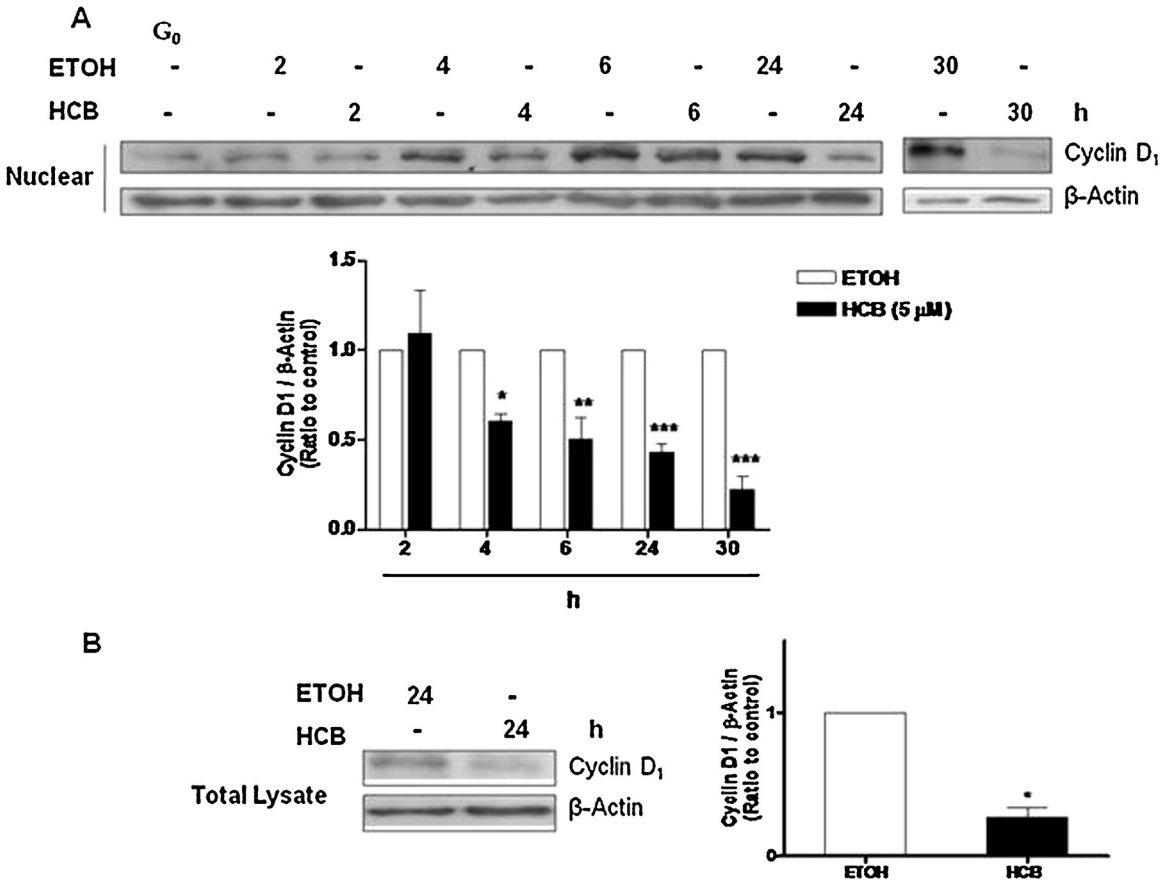

Fig. 5. HCB induces modulation of cyclin D1 expression. (A) Time-course of HCB effect on nuclear cyclin D1 protein levels. Western blot from one representative experiment is shown in the upper panel. Quantification of cyclin D1/β-actin ratio to control is shown in the lower panel. (B) HCB effect on total lysate cyclin D1 protein levels in FRTL-5 cells cultured with 5 μM HCB or ETOH for 24 h. Data are expressed as means ± SEM of three independent experiments. Asterisks indicate significant differences versus ETOH-treated cells. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Statistical comparisons were made by one-way ANOVA with a 95% confidence interval followed by Tukey post hoc test to identify significant differences between mean values and indicated controls.

These results showed that HCB treatment inhibited entry into S-phase, arresting FRTL-5 cells in the G0/G1 phase.

Cell cycle distribution after 0.5 μM HCB-treatment, showed a slight but not significative arrest in G2/M phase after 72 h (results not shown).

Altogether these results showed that HCB arrested cell cycle progression, depending on HCB concentration and time of exposure.

Cells were synchronized in the medium without TSH and FBS for 24 h and then released into complete medium containing 5 μM HCB or ETOH. After incubation for 24 h or 72 h, the cells were harvested, stained with PI, and analyzed for DNA contents as described under Section 2. Percentage of cells in each phase was plotted for 24 and 72 h of incubation. Values are means ± SEM of three independent experiments.

3.4. HCB induces modulation of cell cycle regulators

Considering that the most relevant HCB-induced cell cycle arrest, affects S-phase entry, which is under negative control by p27, we examined the effect of the pesticide on p27 protein levels by western blotting.

Our results showed that HCB (5 μM) treatment significantly increased cytosolic p27 protein (48%) at 4 h and thereafter when compared to their corresponding controls (Fig. 4A). In a similar manner, HCB significantly increased nuclear p27, by 165, 114 and 96%, after 2, 4 and 6 h respectively, compared with ETOH groups, returning to ETOH levels at 24 and 30 h (Fig. 4A).

Dose–response experiments demonstrated that cells-treatment in the range of HCB (0.005, 0.05, 0.5 and 5 μM), significantly increased both cytosolic and nuclear p27 protein levels, showing a U-shaped relationship, with a maximum effect at 0.005 and 5 μM HCB (Fig. 4B).

Cell cycle progression from G1 to S-phase is primarily controlled by the D-type cyclins in association with CDK4/6. Because of the effects of the pesticide on cell cycle progression reported before, we examined cyclin D1 protein levels by immunoblotting. FRTL-5 cells were incubated with HCB (5 μM) or ETOH, for 2, 4, 6, 24 and 30 h. As seen in Fig. 5A, a time-dependent decrease (40, 50, 60 and 80%) of nuclear cyclin D1 protein levels, was observed at 4, 6, 24 and 30 h, respectively.

Analysis of cyclin D1 protein levels in total cell lysates after HCB (5 μM) or ETOH treatment, for 24 h, showed that the pesticide decreased total cyclin D1 protein levels (72%) (Fig. 5B).

3.5. P27 and cyclin D1 protein levels, depend on TGF-β1 SMAD signaling pathway

Considering that HCB increases TGF-β1 mRNA levels, and alters p27 and cyclin D1 cell cycle regulatory proteins, we sought to determine whether HCB-induced alterations were dependent on TGF-β1 SMAD pathway.

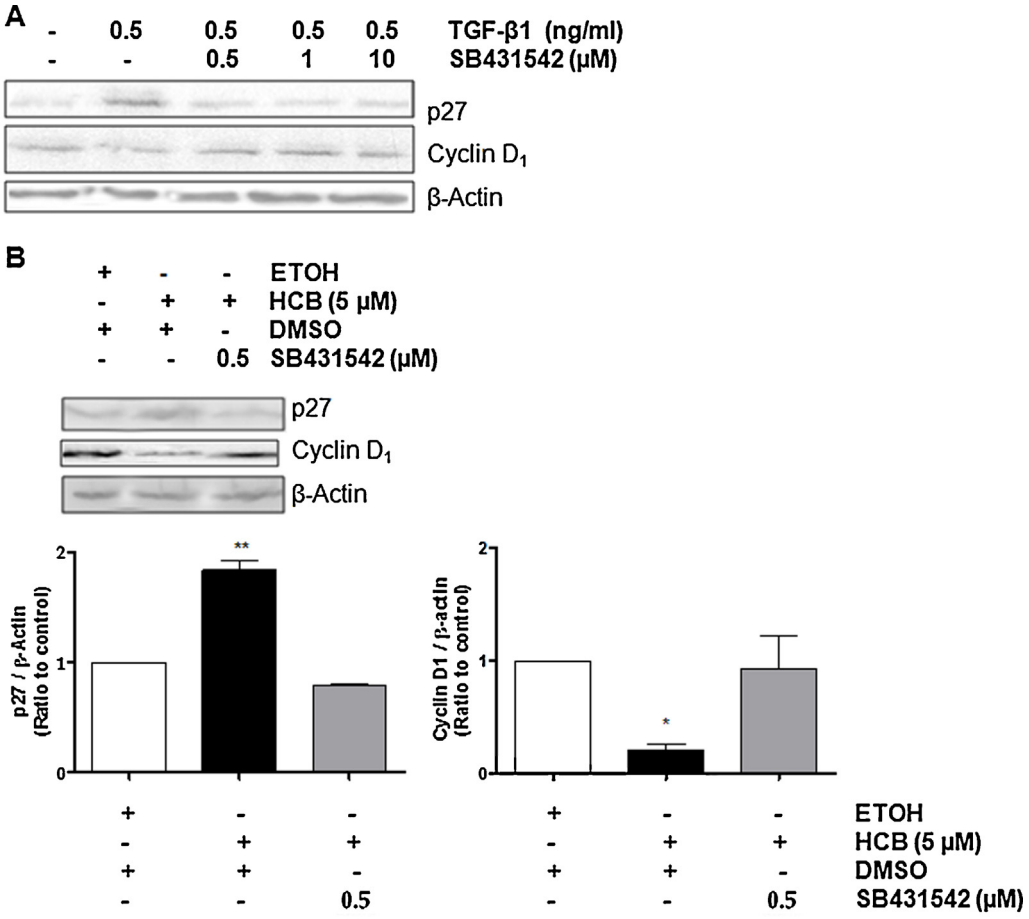

Fig. 6. Effects of TGF-β1 signaling on expression of p27 and cyclin D1. (A) Protein levels of p27 and cyclin D1 in total lysate of FRTL-5 cells pretreated with 0.5, 1 and 10 μM SB431542, for 1 h, followed by 24 h of exposure to 0.5 μM TGF-β1. β-actin was used as a loading control. (B) p27 and cyclin D1 protein levels, in total lysate, of FRTL-5 cell, pretreated with with 0.5 μM SB431542 or DMSO, for 1 h, followed by 24 h of exposure to 5 μM HCB or ETOH. Western blots from one representative experiment are shown in the upper panels. Quantification by densitometric scanning of the immunoblots is shown in the lower panels. Data are expressed as means ± SEM of three independent experiments. Asterisks indicate significant differences versus ETOH-treated cells. ($^{*}p < 0.05$, $^{**}p < 0.01$). Statistical comparisons were made by one-way ANOVA with a 95% confidence interval followed by Tukey post hoc test to identify significant differences between mean values and indicated controls.

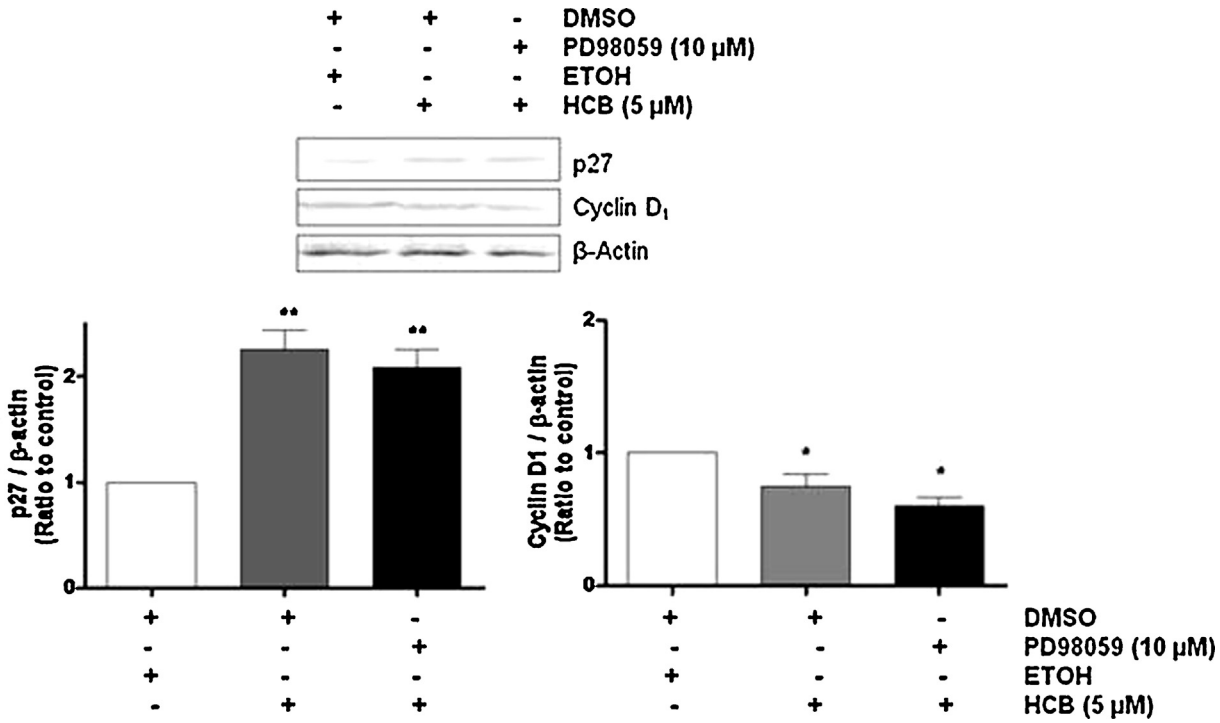

Fig. 7. Role of ERK1/2 on HCB-induced modulation of p27 and cyclin D1 protein levels. FRTL-5 cells were pretreated with 10 μM PD98059 or DMSO and further treated with 5 μM HCB or ETOH for 24 in the presence of the inhibitor. Protein levels were determined by western blotting. Protein expression was relativized to β-actin. Quantification by densitometric scanning of the immunoblots of three independent experiments is shown in the lower panels. Data are expressed as means ± SEM of three independent experiments. Asterisks indicate significant differences versus ETOH-treated cells. ($^{*}p < 0.05$, $^{**}p < 0.01$). Statistical comparisons were made by one-way ANOVA with a 95% confidence interval followed by Tukey post hoc test to identify significant differences between mean values and indicated controls.

In order to determine the effective inhibitory dose of SB431542, a specific inhibitor of TGF-β1 receptor 1 (TGFβR1) tyrosine kinase, on TGF-β1 SMAD-dependent regulation of p27 and cyclin D1 levels, FRTL-5 cells were pretreated with (0.5, 1 and 10 μM SB431542), for 1 h, followed by 24 h of exposure to 0.5 μM TGF-β1. As shown in Fig. 6A, all assayed doses of SB431542, reversed TGF-β1 effect on p27 and cyclin D1.

We further used western blot analysis, to evaluate the involvement of TGF-β SMAD pathway on HCB-induced alterations of p27 and cyclin D1 proteins. FRTL-5 cells were pretreated with 0.5 μM SB431542, followed by 24 h of exposure to 5 μM HCB. Our results showed that a significant decrease of p27 protein levels was observed in the presence of the inhibitor, compared to HCB-treated cells. On the other hand decreased cyclin D1 protein levels returned to ETOH levels, in cells pretreated with the inhibitor (Fig. 6B). Altogether, these findings suggest that TGF-β SMAD pathway might be involved in HCB-induced arrest of cell cycle progression in FRTL-5-cells, through regulating the expression of p27 and cyclin D1 proteins.

3.6. Role of ERK1/2 on HCB-induced modulation of cell cycle regulatory proteins

As it has been demonstrated that MAPK signaling cascades regulate not only cell growth, but also cell cycle arrest (Miyoshi et al., 2004), and we have previously reported that ERK1/2 is a critical mediator in HCB-induced loss of cell viability (Chiappini et al., 2013), we investigated whether ERK1/2 is involved in HCB-induced alterations of cell cycle regulatory proteins.

FRTL-5 cells were pretreated during 1 h, with 10 μM PD98059, a specific MEK1 inhibitor, followed by 24 h exposure to 5 μM HCB. p27 and cyclin D1 protein levels were evaluated by immunoblot. Our results showed that HCB-induced alterations in p27 and cyclin D1 protein levels were not further affected, when cells were pretreated with MEK inhibitor. These results indicate that ERK1/2 signaling cascade is not required for HCB-induced decrease of cyclin D1 (Fig. 7).

4. Discussion

Our recent studies demonstrated that HCB exerted a potent cytotoxic activity and induced apoptosis by reactive oxygen species (ROS)-mediated ERK1/2 activation, in FRTL-5 rat thyroid cells (Chiappini et al., 2013). Because apoptosis and the cell cycle are closely coupled, most compounds that are cytotoxic are also cytostatic (Vermeulen et al., 2003). It has also been demonstrated that the abnormal production of ROS may trigger cell cycle arrest and apoptosis (Sauer et al., 2001).

Herein, we have demonstrated that HCB induced loss of cell viability after 24 h of cell exposure.

TGF-β1 regulates cell proliferation and differentiation of diverse cell types, including epithelial, endothelial and hematopoietic cells, by autocrine and paracrine mechanisms (Massague et al., 2000). In a previous work, we have demonstrated that doses of HCB that do not disrupt thyroid economy induced an increase in TGF-β1 mRNA content in the rat thyroid gland (Chiappini et al., 2009). Similarly, in this study we have demonstrated that HCB increases TGF-β1 mRNA content in FRTL-5 cells. As TGF-β1 is a negative regulator of cell growth in thyroid epithelial cells, we hypothesized that TGF-β1 could play a physiological role in the control of thyroid growth through the modification of cell cycle progression.

In the present study, we observed that treatment of FRTL-5 cells with HCB resulted in a prominent and long term G0/G1 cell cycle arrest, as well as a moderate arrest in G2/M phase. It is also known that other xenobiotics induce G1 and/or G2/M phase arrest depending on cell type (Jin et al., 2004).

In normal cells, the amount of p27 is large during the quiescent G0 phase of the cell cycle, while it is rapidly decreased on reentry of

cells from G0 to G1 phase (Susaki and Nakayama, 2007). Our results have shown that in response to growth stimuli, HCB increased both cytosolic and nuclear p27 protein levels at early times of the pesticide treatment, suggesting that HCB induced cells arrest in G0/G1 transition. The translocation of p27 from the nucleus to the cytoplasm in response to growth stimuli is necessary for the ubiquitin-dependent proteolysis of this protein in the cytoplasm. Our results have shown that after longer times of HCB-exposure (24–30 h) nuclear p27 content returned to control levels suggesting that p27 might have been translocated from nuclei to cytoplasm. We have also demonstrated that HCB decreased the percentage of cells in S-phase. In this respect it has been reported that cell cycle progression, particularly the G1/S transition, is negatively controlled by p27, which inhibits CDK2 kinase activity, depending on the cell line and proliferating state (Depoortere et al., 2000; Carneiro et al., 1998). In the present study, we have also observed that HCB induced cell cycle arrest, not only at G1 phase but at G2 phase. Similarly, Hashimoto et al. (2003) reported that TGF-β1 induced the cell cycle arrest not only at G1 phase but at G2 phase in some hepatoma cells. It has also been reported that the polyaromatic hydrocarbon benzo[*a*]pyrene (B*a*P) exerts an anti-proliferative effect through a p53-dependent pathway involving cell cycle arrest at G2/M (Drukteinis et al., 2005).

Cyclin D expression is the converging point at which diverse mitogenic and signaling from growth factors cascades integrates to mediate G1-S phase cell cycle progression through activation of specific cdks. In the present study we have demonstrated that HCB induced a time-dependent decrease in nuclear cyclin D1, as early as 4 h after HCB treatment that was consistent with a corresponding inhibition in cells entry into S-phase. In this respect, it has been reported that targeting cyclin D1 for proteasomal degradation is sufficient to induce G1 cell cycle arrest in ovarian cancer cell lines (Masamha and Benbrook, 2009). Consistent with our observation, a decrease in the protein levels of cyclin D1 was also observed in 2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB-153)-treated MCF-10A human non-tumorigenic mammary epithelial cells (Venkatesha et al., 2010). Bates et al. (1994) proposed that TGF-β1 disrupts cyclin D1-CDK4/6 association, leaving free cyclin D1 that is rapidly degraded. Cyclin D1-CDK4/6 complexes can be disrupted decreasing CDK4 levels. Although we have not investigated CDK4 protein levels, we cannot disregard a direct inhibition of CDK4 translation in HCB-treated cells. Further experiments are required to clarify this point.

It has been proposed that TGF-β1 regulates cell cycle progression, through upregulation of cell cycle inhibitors, such as p27, and through down-regulation of cyclins and CDK (Massague et al., 2000). As in our study, we have demonstrated that HCB induced TGF-β1 mRNA expression, at very early times of pesticide exposure, we hypothesized that HCB-induced cell cycle arrest and TGF-β1 might be related, through the modification of cell cycle regulatory proteins. Our results have shown that TGF-β1 Smad pathway, is involved in HCB-induced alterations of p27 and cyclin D1, suggesting that TGF-β1 might be a critical mediator of HCB-induced cell cycle arrest.

Considering that TGF-β1 transduces signals to the nucleus through Smad and non-Smad pathways, we explored the possibility that MAPK signaling pathway was involved in HCB-induced cell cycle arrest. Considerable evidence indicates that MAPK signaling cascades regulate not only cell growth, development and differentiation, but also apoptosis and cell cycle arrest (Miyoshi et al., 2004). Our results have shown that HCB-induced abnormal expressions of p27 and cyclin D1 was not restored to normal values as a result of MEK inhibition. Undoubtedly, these results show that ERK1/2 pathway is not involved in HCB-induced alterations of cell cycle regulatory proteins. Other authors have also reported that TGF-β1 is able to modulate cellular proliferation by a signaling pathway that is totally independent of the MAPK cascade (Chatani et al., 1995). Conversely, we have previously demonstrated that HCB induces apoptosis via ERK1/2 signaling in FRTL-5 cells (Chiappini et al., 2013). Finally, it has been reported that many intracellular signaling pathways are activated upon stimulation with xenobiotic AHR ligands, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and benzo(*a*) pyrene (B(*a*)P). As HCB is a weak ligand of AHR, the possibility exists that the pesticide effect on cell cycle regulatory proteins would be AHR-mediated.

In conclusion we have demonstrated that HCB-induced arrest of cell cycle progression, occurs during G0/G1 and G2/M phase, and involves alterations in p27 and cyclin D1, which are mediated by TGF-β1 Smad pathway. On the other hand, the activation of ERK1/2 is not a requirement for the alteration of cell cycle regulatory proteins.

Conflict of interest

The authors declare that there is no conflict of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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