Toxicology Letters 270 (2017) 72–79

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Full Length Article

Hexachlorobenzene alters cell cycle by regulating p27-cyclin E-CDK2 and c-Src-p27 protein complexes

C. Ventura[a], M. Núñez[a], V. Gaido[a], C. Pontillo[b], N. Miret[b], A. Randi[b], C. Cocca[a,c,*]

[a] Laboratorio de Radioisótopos, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina
[b] Laboratorio de Efectos Biológicos de Contaminantes Ambientales, Departamento de Bioquímica Humana, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina
[c] Instituto de Química y Fisicoquímica Biológicas "Prof. Alejandro C. Paladini", IQUIFIB UBA-CONICET, Buenos Aires, Argentina

HIGHLIGHTS

- HCB is a persistent organochlorine pollutant that acts as endocrine disruptor.
- HCB alters cell cycle progression and cell cycle regulating proteins in estrogen-sensitive MCF-7 breast cancer cell line.
- Cell cycle alterations exerted by HCB are related to the modulation of cyclin E-CDK2 and c-Src-p27 protein complexes.

ARTICLE INFO

Article history:
Received 22 November 2016
Received in revised form 31 January 2017
Accepted 7 February 2017
Available online 12 February 2017

Keywords:
Hexachlorobenzene
Cell cycle
Cyclin E
p27
c-Src

ABSTRACT

Hexachlorobenzene (HCB) is an organochlorine pollutant widely distributed in the environment around the entire world. Previous reports from our group and others have demonstrated that this compound is as an endocrine disruptor. We have also reported that HCB presents a co-carcinogenic effect in N-Nitroso-N-methyl-urea–induced mammary tumours in rats. In this work, we studied the effects of HCB on cell cycle progression and cell cycle regulating protein expression in the estrogen-sensitive breast cancer cell line, MCF-7. Here, we show that HCB alters cell cycle in a concentration-dependent way. The lowest assessed concentration (0.005 μM) promotes the cell cycle progression, enhances cyclin D_1 expression, and reduces the nuclear localization of p27 accompanied by an increased interaction between p27 and c-Src kinase. On the other hand, 5 μM HCB delays the cell cycle progression and promotes the formation of the cyclin E-CDK2-p27 protein complex. Our results show that HCB stimulates cell proliferation through cell cycle modulation and c-Src involvement in MCF-7 cells. Here, we report for the first time that differential mechanisms of action of HCB on mammary cell cycle progression are triggered at different concentrations of this pollutant.

1. Introduction

Breast cancer represents one of the most common malignancies among women worldwide (International Agency of Research on Cancer, 2014; Jemal et al., 2011). The study of the etiology of this illness is imperative to prevent the causes that promote this intricate pathology. It has been suggested that lipophilic environmental chemicals such as polycyclic aromatic hydrocarbons, contribute to increase breast cancer risk. Hexachlorobenzene (HCB) is a member of this chemical group and a persistent organic pollutant that was historically used as pesticide and fungicide (Mrema et al., 2013). Nowadays, the use of HCB is forbidden, but it is still produced as a byproduct of the manufacture of another chlorine compounds. Hence, HCB can be found present in the atmosphere, soil and water in many countries around the world (Nilsen et al., 2014; Robles-Molina et al., 2014; Wu et al., 2014). It has been reported that HCB is accumulated in adipose tissue of

Abbreviations: CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; E_2, estradiol; ERα, estrogen receptor alpha; HCB, hexachlorobenzene; IGF-I, insulin growth factor.

* Corresponding author at: Laboratorio de Radioisótopos, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina.
E-mail address: cmcocca@ffyb.uba.ar (C. Cocca).

http://dx.doi.org/10.1016/j.toxlet.2017.02.013
0378-4274/

breast cancer patients (Ociepa-Zawal et al., 2010). In our previous work, we demonstrated that HCB presents a co-carcinogenic effect in N-Nitroso-N-methylurea–induced mammary tumours in rats, enhancing their development and malignancy. Additionally, we have found that HCB induces a significant increase of the number of ducts in the rat mammary glands beside hyperplastic lobules in this tissue (Randi et al., 2006). Moreover, we have demonstrated that 0.005 and 0.05 μM HCB induce an overexpression of the insulin growth factor-I and insulin receptors in MCF-7 cells. At these concentrations, HCB also increases MCF-7 cell proliferation, which could be abolished by the estrogen receptor alpha (ERα) specific antagonist ICI 182,780 (García et al., 2010).

It is known that estrogen is one of the most important stimuli to induce mammary cell proliferation. Furthermore, estrogenic action is responsible for G1-S progression through the cell cycle (Castoria et al., 2012). Cyclins and cyclin-dependent kinases (CDKs) are also related to cell cycle progression. These proteins are regulated, in turn, by specific CDKs inhibitors (CDKI), such as p27 protein. Following anti-mitogenic signals, p27 binds to cyclin E–CDK2 complex to inhibit their catalytic activity and induce cell cycle arrest (Coqueret, 2003). p27 protein levels are modulated through transcriptional, translational and post-translational mechanisms. It has also been described that phosphorylation and polyubiquitination represent two regulatory devices for p27 modulation (Rodier et al., 2001).

c-Src activation has been shown a crucial response for ERα downstream signaling and it has been demonstrated that rapid estrogen actions are mediated by physical interaction between c-Src and ERα (Cheskis, 2004). c-Src activates many mitogenic signals to control proliferation, survival and metastasis in normal and transformed cells (González et al., 2006). In this sense, c-Src expression and activity have been reported increased in many human tumours compared to normal tissue. Additionally, c-Src phosphorylates p27 both *in vivo* as well as *in vitro* with consequences in cell cycle promotion (Chu et al., 2008).

Finally, c-Src regulates diverse physiological functions and promotes carcinogenesis through multiple pathways. We have previously demonstrated that the phophorylation of c-Src is enhanced by HCB which was accompanied by cell proliferation induction in MCF-7 cells (García et al., 2010). In addition, c-Src phosphorylation was also found induced in MDA-MB-231 cell line (Pontillo et al., 2010) and in rat mammary tumours (Peña et al., 2012). The aim of this work was to study the effects of HCB on cell cycle progression in ERα(+) breast cancer cells MCF-7, and the action of this pesticide on the proteins involved in cell cycle regulation.

2. Materials and methods

2.1. Cell culture

Human breast cancer cell line, MCF-7 (American Type Tissue Culture Collection, VA, USA), was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco BRL, NY, USA), 0.3 g/L glutamine and 0.04 g/L gentamicin (Gibco BRL, NY, USA). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and subcultured with 0.05% trypsin and 0.02% EDTA (Gibco BRL, NY, USA). Cells at 70–80% confluence were treated with HCB (>99% purity Aldrich-Chemie GmbH & Co., Steinheim, Germany) dissolved in absolute ethanol. Final ethanol concentration in each treatment was minor or equal than 0.5%, which had not influence on the analyzed parameters as we showed previously (García et al., 2010). Treatments were added to the cultures in phenol red free-RPMI medium (Gibco BRL, NY, USA) and 10% charcoal-treated fetal bovine serum. Ethanol 0.5% (vehicle) was added to the control cells.

2.2. Cell cycle analysis

2×10^5 cells were seeded into 6 wells plates. Adherent cells were serum-starved for 24 h to synchronize growth. Synchronized cells were treated for 6 h or 24 h with HCB at the concentrations indicated in each case, E_2 (10 nM) or vehicle immediately after release from the block. Cells were collected by trypsinization, fixed with ice-cold methanol, centrifuged, resuspended in 0.2 mg/mL of DNAse-free RNAse A (Sigma Chemical Co., MO, USA) and then were stained with propidium iodide (PI) staining solution (50 μg/mL PI in PBS; Sigma Chemical Co., MO, USA) and evaluated by flow cytometry (CyFlow Pas III, Partec; Görlitz, Deutsch-land, Germany). Cell cycle distribution was analysed using Cyflogic v1.2.1 software (Perttu Terho & © CyFlo Ltd, Turku, Finland).

2.3. BrdU incorporation

2×10^4 cells were seeded on cover slips into 24 wells plated. The culture was synchronized by serum-starvation during 24 h. Synchronized cells were treated with HCB (0.005 and 5 μM), E_2 (10 nM) or vehicle for 2, 6, 10, 18, 24 and 30 h. BrdU 30 μM (Sigma Chemical Co., MO, USA) was added for the last 2 h of exposure to the pesticide or vehicle and proceeded as described (García et al., 2010). Cells were finally exposed to anti-BrdU primary antibody (1:200, Sigma Chemical Co., MO, USA) and stained with FITC-conjugated anti-mouse IgG (1:200, Sigma Chemical Co., MO, USA) and 4′-6-diamidino-2-phenylindole (DAPI) (1:8000, Sigma Chemical Co., MO, USA). Cover slips were mounted with Fluor-Save™ Reagent (Calbiochem, Darmstadt, Germany) and fluorescence was observed by epifluorescence using an Olympus BX50 microscope (Center Valley, PA, USA). Images were acquired using a CoolSnap digital camera (Silver Spring, MD, USA). The percentage of BrdU-positive cells was calculated as BrdU-positive cells/total DAPI stained cells per field. At least 1000 cells were scored per sample in each experiment.

2.4. Doubling time

Starved cells were exposed to HCB (0.005 and 5 μM) or vehicle for 24, 48, 72, 96 and 120 h and counted by hemocytometer. The experiments were performed in the exponential phase of cell growth. Triplicate plates were analyzed for each treatment at each time. The following formula was used to calculate the doubling time: $N_t = N_0 \times e^{kt}$, where N_0 was the initial number of cells that increased exponentially with a rate constant, k. The doubling time (T_D) was calculated as: $T_D = \ln 2/k$. The GraphPad Prism 5.0 software was employed for equation fitting to data and parameter estimation.

2.5. Western blot

Cells were lysed using RIPA buffer supplemented whit protease and phosphatase inhibitors [50 mM Tris-HCl pH = 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EGTA, 0.1 mM NaF, 0.02 mg/mL leupeptin, 1 mM Na_3VO_4 and 1 mM phenylmethanesulfonyl fluoride (PMSF)]. Protein concentration was measured by Bradford assay (Bradford, 1976). 50 μg of protein were analyzed by SDS-PAGE and immunoassay as described (García et al., 2010). Membranes were probed overnight with primary monoclonal anti-p27 (1:200, Sigma Chemical Co., MO, USA), monoclonal anti-Cyclin E (1:500, Cell Signaling Technology, Beverly, USA), monoclonal anti-Cyclin D_1 (1:200, Cell Signaling Technology, Beverly, USA), monoclonal anti-CDK2 (1:200, Cell Signaling Technology, Beverly, USA), monoclonal anti-c-Src (1:500, Abcam Inc., Cambridge, England), polyclonal anti-Lamin B1 (1:500, Abcam Inc., Cambridge, England), monoclonal anti-α-Tubulin

(1:3000, Sigma Chemical Co., MO, USA) and monoclonal anti-β-Actin (1:1000, Sigma Chemical Co., MO, USA) antibodies as previously described (Pontillo et al., 2010).

2.6. Co-Immunoprecipitation

Total cellular protein lysates (300–500 μg) were suspended in immunoprecipitation buffer [200 mM Tris, pH 7.4, 1 M NaCl, 100 mM EDTA, 1% IGEPAL CA-630, 2%, Triton X-100, 0.02 mg/mL leupeptin, 1 mM PMSF, 1 mg/mL aprotinine, 1 mM Na_3VO_4 and 0.1 mM NaF]. Then, 4 μg of anti-cyclin E, anti-p27 or anti-c-Src antibodies were added and incubated for 2 h at 4 °C under constant shaking. After that, 30 μL of A/G plus agarose was added and incubated for others 2 h at 4 °C under constant shaking, in a final volume of 0.5 mL. Precipitates were washed four times with immunoprecipitation buffer, centrifuged at 12,000 × *g* and denatured in sample buffer at 95 °C for 5 min.

2.7. Subcellular fractionation

MCF-7 cells were washed three times with ice-cold PBS, and harvested in lysis buffer containing protease and phosphatase inhibitors [20 mM HEPES-KOH, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM PMSF, pH 7.5, 20 mg/mL aprotinin, 120 mM leupeptin, and 12 mM 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 control. The nuclear pellet was resuspended in RIPA buffer containing protease and phosphatase inhibitors, and the resulting suspension was used for the assay of nuclear proteins. Protein concentration was determined according to Bradford (Bradford, 1976) using bovine serum albumin (BSA) as a standard.

2.8. Immunofluorescence

5×10^4 cells were seeded on cover slips in RPMI complete growth medium followed by overnight incubation to allow the cells to attach. Cells were treated with HCB (0.005 and 5 μM) or vehicle for 1 h. After treatment, cells were washed with PBS and fixed with ice-cold methanol during 20 min. Cells were permeabilized with 1% Triton X-100 and blocked with 5% BSA for 1 h. After that, cell were incubated with monoclonal anti-p27 (1:50) and anti-c-Src (1:100) antibodies overnight at 4 °C. Finally, cells were incubated with Texas Red-conjugated anti-mouse IgG antibody (1:250) and FITC-conjugated anti-rabbit IgG (1:200). Cover slips were mounted with Fluor-Save™ Reagent and fluorescence was observed using a LSM510 confocal microscope. Photographs were taken with 400× magnification.

2.9. Statistical analysis

Normality of data and variance homogeneity were evaluated by Shapiro-Wilk and Levene tests, respectively. Then, ANOVA analysis

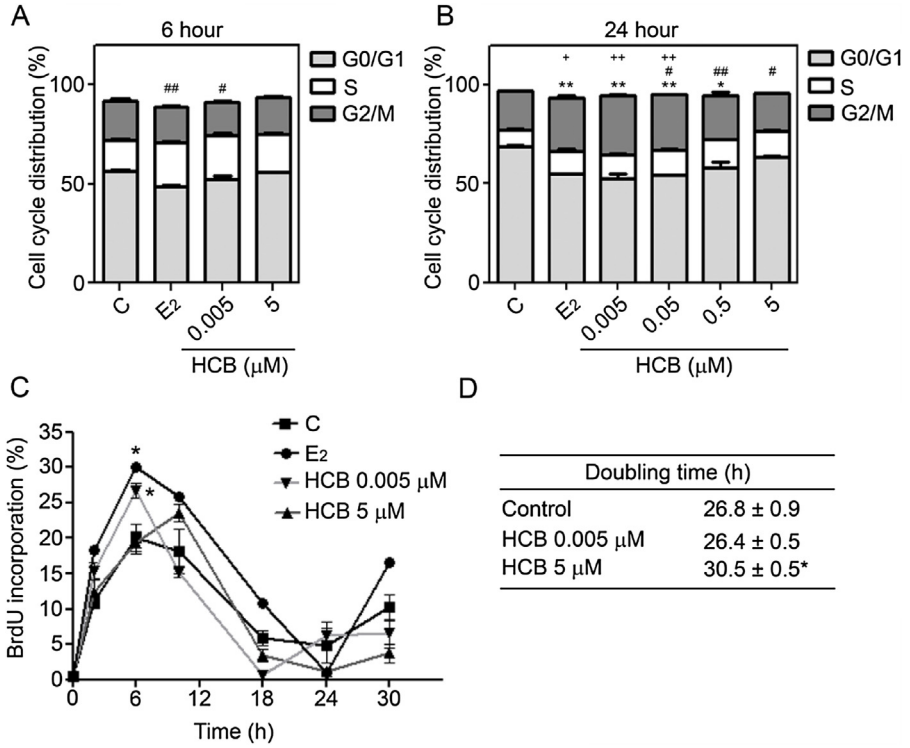

Fig. 1. HCB alters cell cycle parameters on MCF-7 cells. A) Cells were synchronized and treated with HCB (0.005 and 5 μM) or vehicle (C) for 6 h. 10 nM E_2 was employed as positive control. Percentage of cells in different phases of the cell cycle was monitored using PI by flow cytometry. Results represent the mean value ± SEM of two independent experiments, each one performed by duplicated. Percentage of cells at G0/G1 (p:ns), S ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. C), and G2/M (p:ns) phases were compared. One way ANOVA and Dunnett's Multiple Comparison Test. B) Cells were synchronized and treated with HCB (0.005, 0.05, 0.5 and 5 μM) or vehicle (C) for 24 h. 10 nM E_2 was employed as positive control. Percentage of cells in different phases of the cell cycle was monitored using PI by flow cytometry. Results represent the mean value ± SEM of three independent experiments, each one performed by duplicated. Percentage of cells at G0/G1 ($^{*}p < 0.05$, $^{**}p < 0.01$ vs. C), S ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. C), and G2/M ($^{+}p < 0.05$, $^{++}p < 0.01$ vs. C) phases were compared. One way ANOVA and Dunnett's Multiple Comparison Test. C) Starved cells were exposed to HCB (0.005 and 5 μM) or vehicle (C) for 2, 6, 10, 18, 24 and 30 h. 10 nM E_2 was employed as positive control. BrdU incorporation was evaluated by inmunofluoresence using a monoclonal specific antibody. The percentages of BrdU-positive cells are represented on the graph. Data are the mean value ± SEM of two independent experiments, each one performed by duplicated. $^{*}p < 0.05$ vs. its respective C. One way ANOVA and Dunnett's Multiple Comparison Test. D) Starved cells were exposed to HCB (0.005 and 5 μM) or vehicle (C) for 24, 48, 72, 96 and 120 h. Data of two independent experiments, each one performed by triplicated, were fitted to an exponential growth curve and doubling time was calculated. ($^{*}p < 0.05$). One-way ANOVA and Dunnett's Multiple Comparison Test.

was performed. Data were analyzed using the softwares GraphPad Prism 5.0 (GraphPad Software Inc., Philadelphia, USA) and InfoStat (InfoStat version 2016. InfoStat Group, Córdoba, Argentina). p-values less than 0.05 were considered statistically significant.

3. Results

3.1. HCB alters cell cycle progression on MCF-7 cell line

We have previously reported that HCB modifies cell proliferation in MCF-7 breast cancer cell line in a concentration-dependent way (García et al., 2010). In the present work, we studied the molecular bases involved in cell cycle progression induced by HCB. To this aim, DNA content was measured by flow cytometry. Our results indicate that 0.005 μM HCB significantly increments the percentage of cells in S phase after 6 h of exposure (21.9 ± 0.8% of the cells in S phase vs. 15.6 ± 1.3% found in control cells; $p < 0.05$ vs. C). No significant differences were detected when cells were exposed to 5 μM HCB in comparison with the control cells. After 24 h, we observed that 0.05, 0.5 and 5 μM HCB significantly increase the percentage of cells in S phase (13.1 ± 1.1%, $p < 0.05$; 14.0 ± 0.2%, $p < 0.01$ and 13.1 ± 0.2%, $p < 0.05$ respectively vs. 8.0 ± 0.2% of the control). Furthermore, 0.005 and 0.05 μM HCB have also induced an increment in G2/M cells in comparison with the control cells (30.4 ± 1.6% and 28.1 ± 0.5% respectively, vs. 19.9 ± 0.5% in control cells, $p < 0.01$). Estradiol was used as positive control of the experiment. Altogether, our data suggest that the lower concentration of HCB assayed alters the rate of cell division promoting the cell cycle (Fig. 1A and B).

We have also analyzed BrdU incorporation in HCB-exposed cells at different times (Fig. 1C). To this aim, cells were starved and their cell cycle initiated by the addition of serum to the culture. When the cells were treated with vehicle, we observed that 20.0 ± 1.9% of the cells entered S phase after 6 h of serum addition. On the other hand, 26.7 ± 1.0% of the cells progressed to S phase when the cells were exposed to 0.005 μM HCB at the same time ($p < 0.05$ vs. C). A similar result was observed when the cells were exposed to 10 nM E_2 (30.0 ± 3.2%, $p < 0.05$ vs. C). In contrast, 5 μM HCB induced a significant delay to enter S phase when it was compared to control or 0.005 μM HCB exposed cells. The highest percentage of BrdU-

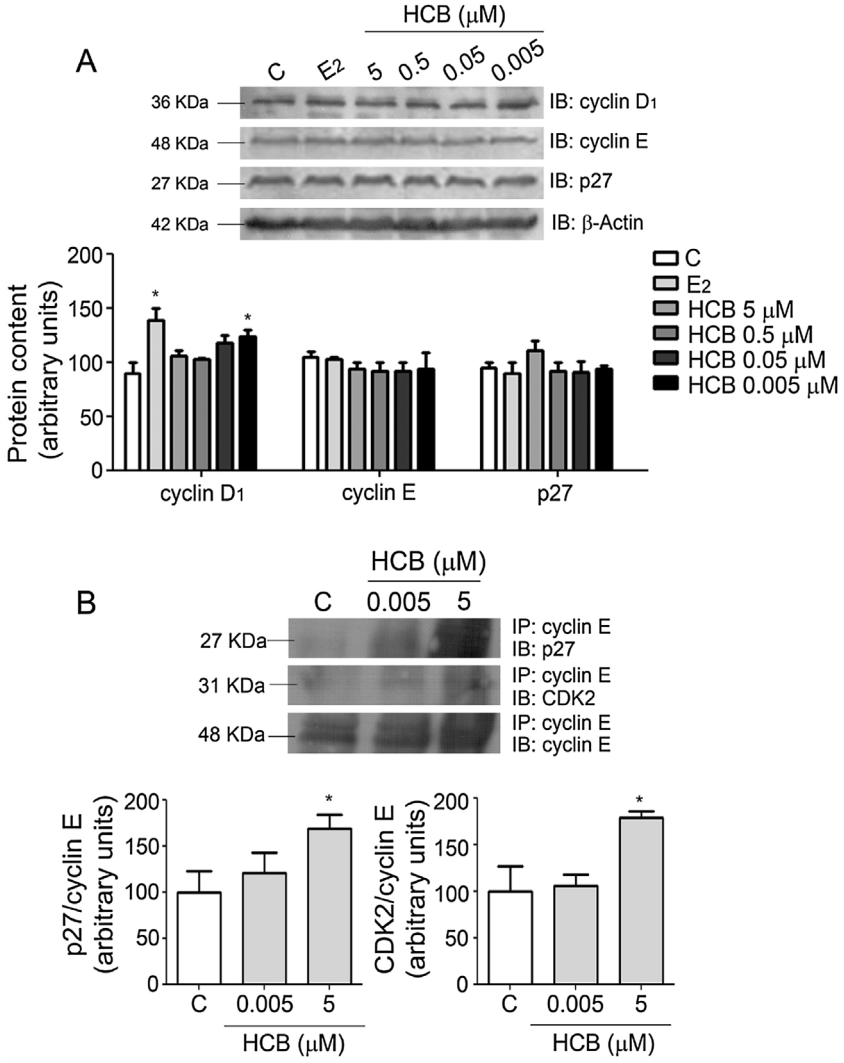

Fig. 2. HCB alters cell cycle regulating proteins in MCF-7 cells. A) MCF-7 starved cells were exposed to HCB (0.005, 0.05, 0.5 and 5 μM), or vehicle (Ethanol 0.5%) during 24 h. 10 nM E_2 was employed as positive control. Cyclin D_1, cyclin E and p27 levels were determined by western blot. Band densities were normalized with β-Actin content. A blot of a representative experiment is shown. Data were expressed as mean values ± SEM of three independent experiments, each performed by duplicate. *$p < 0.05$ vs. its respective C. One way ANOVA and Dunnett's Multiple Comparison Test. B) Cells were synchronized and treated with HCB (0.005 and 5 μM) or vehicle (C) for 24 h. Lysates were immunoprecipitated (IP) with anti-cyclin E antibody and probed (IB) with anti-p27, anti-CDK2 and anti-cyclin E antibodies. Band densities were normalized with the cyclin E content. A blot of a representative experiment is shown. Data were expressed as mean values ± SEM of two independent experiments, each performed by duplicate. *$p < 0.05$ vs. its respective C. One way ANOVA and Dunnett's Multiple Comparison Test.

positive cells was observed after 6 h of exposure to vehicle, 0.005 μM HCB and 10 nM E_2. However, this parameter was maximum after 10 h when 5 μM HCB was added to the culture.

To examine whether the differences observed in cell cycle progression and BrdU incorporation reflect changes in the doubling time of the HCB exposed cells, we quantified the number of cells along five consecutive days and the doubling time was calculated. We found significant differences in cell proliferation rates among control or 5 μM HCB-treated cells ($p < 0.05$), determined by estimating doubling times during exponential growth. No significant differences in the doubling time between cells exposed to 0.005 μM HCB and vehicle were found (Fig. 1D).

3.2. Cell cycle regulating proteins are modified by HCB

In order to study if the cell cycle regulating proteins may be altered by HCB, western blot analysis was carried out using specific antibodies. An increased level of cyclin D_1 (23.4% over control, $p < 0.05$) was found when the cells were exposed to 0.005 μM HCB (Fig. 2A). A similar effect was exerted by E_2, which was used as positive control (39.0% over control, $p < 0.05$). Higher concentrations of HCB did not alter the expression of cyclin D_1. Additional proteins such as cyclin E and p27 were also analyzed. As Fig. 2A shows, these proteins were not found regulated at any concentration of HCB neither E_2 after 24 h of exposure.

We observed an increased association between p27 and cyclin E after 5 μM HCB treatment (68.7% over control, $p < 0.05$). Moreover, its co-immunoprecipitation with cyclin dependent kinase CDK2 and cyclin E were also enhanced by 5 μM HCB (78.6% over control, $p < 0.05$), indicating that this concentration of pesticide increases the association of p27-cyclin E-CDK2 protein complex (Fig. 2B).

3.3. HCB alters c-Src-p27 interaction

The effects of p27 on cell cycle progression have been related to c-Src kinase activity. To determine whether this kinase was related to the cyclin E-p27 complex formation induced by HCB, we evaluated the interaction between c-Src and p27 in MCF-7 cells exposed to the pesticide. As is shown in Fig. 3A, c-Src was co-immunoprecipitated with p27. The immune complex was induced by 0.005 μM HCB (107.9 ± 11.1% over control, $p < 0.01$), 0.05 μM (95.3 ± 33.8% over control, $p < 0.001$) and 0.5 μM (55.6 ± 14.3% over control, $p < 0.05$). On the other hand, 5 μM HCB reduced the protein complex (18.4 ± 10.1%, p:ns). These results indicated that

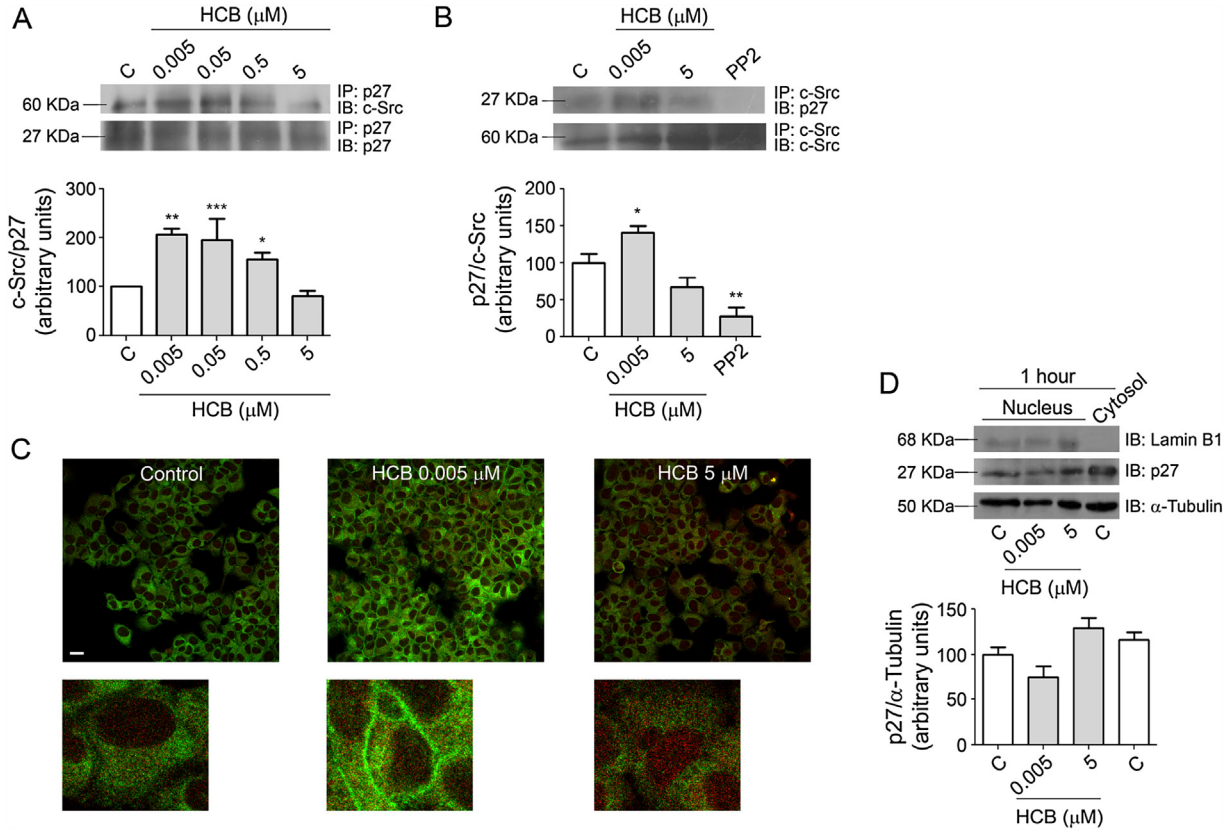

Fig. 3. HCB alters the interaction and intracellular localization of c-Src and p27 on MCF-7 cells. A) The cells were synchronized and treated with HCB (0.005, 0.05, 0.5 and 5 μM) or vehicle (C) for 1 h. Lysates were immunoprecipitated (IP) with anti-p27 antibody and probed (IB) with anti-c-Src and anti-p27 antibodies. Densities were normalized to the p27 content. A blot of a representative experiment is shown. Data were expressed as mean ± SEM of two independent experiments, each one performed by duplicate. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. C. One way ANOVA and Dunnett's Multiple Comparison Test. B) Starved cells were exposed to HCB (0.005 and 5 μM), vehicle or PP2 (10 μM) for 1 h. Lysates were IP with anti-c-Src antibody and probed with anti-p27 and anti-c-Src antibodies. Densities were normalized to the c-Src content. A blot of a representative experiment is shown. Data were expressed as mean ± SEM of two independent experiments, each one performed by duplicate. $^{*}p < 0.05$; $^{**}p < 0.01$ vs. C. One way ANOVA and Dunnett's Multiple Comparison Test. C) Starved cells were exposed to HCB (0.005 and 5 μM) or vehicle (Control) during 1 h. Cells were fixed and c-Src (green) and p27 (red) proteins were detected by immunofluorescence. Pictures are representative of three independent experiments. Magnification: 400×. Scale bar: 20 μm. A magnified section is shown under each photograph. D) Starved cells were exposed to HCB (0.005 and 5 μM) or vehicle (C) during 1 h. Nuclear fractions were prepared, and p27 levels were determined by western blot. Band densities were normalized with α-Tubulin content. Laminin B1 was used as nuclear marker. A cytosolic fraction was included as subcellular fractionation control. A blot of a representative experiment is shown. Data were expressed as mean ± SEM of two independent experiments, each one performed by duplicate. p:ns. One way ANOVA and Dunnett's Multiple Comparison Test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the lowest concentration of HCB induces the c-Src-p27 physical interaction but the highest concentration produced the opposite effect.

Moreover, c-Src-p27 interaction was evaluated in presence or absence of the c-Src inhibitor PP2 (1-*tert*-Butyl-3-(4-chlorophenyl)-1H-pyrazolo[3,4-*d*]pyrimidin-4-amine). As Fig. 3B shown, c-Src-p27 complex formation was prevented by PP2 indicating that c-Src activity is required for this interaction (Fig. 3B). These results suggest that 0.005 μM HCB enhances c-Src-p27 interaction by activation of c-Src protein. The phosphorylation of p27 mediated by c-Src regulates the localization of this protein to different intracellular compartments. Membrane localization of c-Src has been associated with its activation, and its ability to phosphorylate a large number of proteins, such as p27. To confirm if this modulation might be under HCB regulation, we evaluated the intracellular localization of c-Src and p27 by immunofluorescence studies employing specific antibodies. In concordance with our previous results, we observed an increase of membrane localized c-Src when the cells were exposed to 0.005 μM HCB. We have also observed a reduced nuclear localization of p27 in these culture conditions (Fig. 3C and D). In the other hand, 5 μM HCB conducted to a reduction of c-Src expression and a nuclear localization of p27. These results are consistent with the dual effect of HCB on cell cycle progression according to the concentration of the pesticide.

4. Discussion

In the present work we have analyzed the effect of HCB on cell cycle and the proteins that are related to the cycle control in ERα(+) MCF-7 breast cancer cell line. Concentrations assayed in our work are in the same range that can be found in the environment for the pesticide (Nilsen et al., 2014; Robles-Molina et al., 2014; Wu et al., 2014). Moreover, monitoring and epidemiological studies demonstrated that the concentration of HCB in serum of pregnant women of different countries, has been determined to be about 50 ng/g of lipid, which is equivalent to 0.001 μM (Arrebola et al., 2015; Artacho-Cordón et al., 2015; Guo et al., 2014; Lauritzen et al., 2016; Saoudi et al., 2014; Thomas et al., 2017). In the other hand, HCB levels in serum of exposed women increase to 15.1 ng/mL of serum, or its equivalent 0.05 μM (To-Figueras et al., 1997).

Our results show that 0.005 μM HCB significantly promotes cell cycle progression in MCF-7 cell line since a rapid increment of cells in S-phase was found. Moreover, the cell cycle analysis after 24 of 0.005 μM HCB exposure showed that a higher percentage of the cells could progress from S to G2/M phase. Because this effect was not observed when cells were exposed to 5 μM HCB, we postulate that it is due to the estrogenic action of the pesticide, which is only observed at low concentrations. In this way, it has been reported that HCB stimulates cell proliferation in estrogen-sensitive cells, without effect on cell proliferation on ERα(−) cells MDA-MB-231 (García et al., 2010). A recent study demonstrated that 5 μM HCB induces cell proliferation in HepG2 cells (de Tomaso Portaz et al., 2015); however this effect was mediated by the aryl-hydrocarbon receptor (AhR), which activation has been related to cell death in MCF-7 cells (García et al., 2010).

Cell cycle control is a key event in the normal development of tissues and cyclins proteins play a critical role in this process. We found that 0.005 μM HCB is able to induce cell cycle progression and, in concordance, an increased expression of cyclin D_1 was detected after 24 h of exposure. Analogous effects were produced by E_2 in this cell line as we showed. It is well known that cyclin D_1 is involved in early G1 transition, being activated by mitogenic signals (Muşat et al., 2004). In this way, other authors reported that β-hexachlorocyclohexane, an organochlorine pollutant, increases the expression of cyclin D_1 and other proto-oncogenes in MCF-10A cells (Wong and Matsumura, 2007). Furthermore, we evaluated cyclin E expression, which was not found altered by HCB at any concentration assessed.

The proper progression through the cycle includes a coordinated action of cyclins, CDKs, and their inhibitors as p27 whose expression were also evaluated in this work. Although we did not observe significant changes on p27 expression, 0.005 μM HCB enhanced its association with the oncoprotein c-Src and reduced its nuclear localization where p27 exert its inhibitor action on cell cycle progression. Additionally, p27-c-Src complex formation was prevented by the c-Src inhibitor, PP2, which indicates that c-Src activation is necessary for p27-cSrc complex arrangement. It has been described that p27 localization is finely regulated during G1 progression. A large number of studies suggested that p27 plays additional roles outside the nucleus. The nuclear export of p27 has to been reported as a mechanism needed not only to relieve cyclin E-CDK2 inhibition, but also to permit the c-Src-mediated Tyr-phophorylation of p27 to promote p27 ubiquitylation and degradation. This Tyr-phosphorylation of p27 is also required for the catalytic activation of p27-cyclin D_1-CDK4 complexes and cell cycle progression (Wander et al., 2011).

Interestingly, several reports showed that cytosolic localization as well as down-regulation of p27 are directly associated with poor survival for different types of cancer (Murray et al., 2005). Moreover, a large number of proliferative diseases are characterized by massively increase of p27 phosphorylation, which was always related to its cytoplasmic localization (Rodier et al., 2001).

In concordance with our results, it has been reported that c-Src-phosphorylated p27 is not able to negatively regulate cell cycle progression (Chu et al., 2008). Moreover, depletion of c-Src in MCF-7 cells increases the expression of p27 (González et al., 2006) evidencing the regulating role of this kinase on p27 activity. Based on this background, we propose that 0.005 μM HCB induces p27-c-Src kinase interaction, which, in turn, lead to cell cycle progression and promotes cell proliferation (Fig. 4). Because this effect was not observed when cells were exposed to 5 μM HCB, we postulate that it is due to the estrogenic action of the pesticide, which is only observed at low concentrations. It has been shown that estradiol-estrogen receptor complex interacts with c-Src and rapidly stimulates c-Src kinase in MCF-7 cells (Migliaccio et al., 1996). In addition, c-Src was found co-immunoprecipitated with p27 in MCF-7 cells. The complex formation precedes the loss of p27 from cyclin E-CDK2 complex prior to S phase entrance (Chu et al., 2008). These findings are in line with our results since mitogenic concentration of HCB was able to enhance c-Src-p27 complex formation.

Taken together, the experimental evidence presented in this study shows for the first time that HCB stimulates cell proliferation in estrogen-sensitive cells MCF-7 through cell cycle modulation with a key participation of c-Src and p27. Many reports agree that

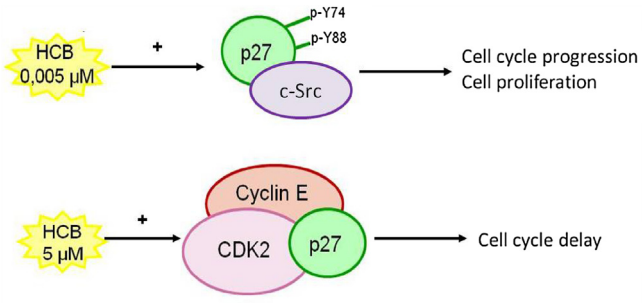

Fig. 4. Mechanisms of action triggered at different concentrations of HCB on MCF-7 cell. 0.005 μM HCB increased c-Src-p27 complex and induced cell cycle progression. In turn, 5 μM HCB induced cyclin E-CDK2-p27 complex that promoted a cell cycle delay.

cell cycle dysregulation plays a crucial role in breast cancer. As we demonstrated here, exposure to low concentration of HCB induces changes in cell cycle regulation so it is possible that this pollutant may induce an increasing breast cancer risk. Actually, a co-carcinogenic action of HCB was previously reported *in vivo* by our group (Randi et al., 2006).

The effects of higher concentrations of HCB on the cell cycle progression and the cell cycle regulatory proteins were also evaluated. In this work, we observed a delay of approximately four hours for the S phase entry when cells were exposed to 5 μM HCB. This effect may be related to the increment of the cyclin E-CDK2-p27 complex induced by 5 μM HCB. Dysregulation of cell cycle regulating proteins expression and its association with CDKIs such as p27 has been well described as a mechanism to inhibit cell cycle progression and it is also associated to many toxic compounds exposure (Shi et al., 2015; Simpkins et al., 2012; Zhang et al., 2015).

c-Src activity has been largely related with cell cycle regulation in diverse cell lines (Simpkins et al., 2012; Siriwardana and Seligman, 2015). We observed that 5 μM HCB reduces c-Src expression and increases nuclear localization of p27. Similar results have been reported to 3,3-diindolylmethane (DIM), the active derived of the chemopreventive agent indole-3-carbanol (I3C), which inhibits cell cycle progression by several mechanisms, including the induction of p27 expression, and its nuclear localization (Wang et al., 2008). Moreover, we observed a slight reduction of c-Src-p27 interaction after 5 μM HCB treatment. Our results indicate that 5 μM HCB retards the cell cycle progression by the inhibition of cyclin E-CDK2 complex activity, probably mediated by an interaction of this proteins with the CDKI, p27 (Fig. 4). Altogether, our results show for the first time that HCB presents differential mechanisms of action on mammary cell growth that are triggered at different concentrations of the pollutant.

Fundings

This work was supported by National Agency of Scientific and Technological Promotion [PICT-2012-1830], National Council of Scientific and Technological Research [CONICET, PIP112-201101-00654] and University of Buenos Aires [UBACYT 20020100100983]. Carolina Pontillo; Andrea Randi and Claudia Cocca are established researchers of the CONICET. Clara Ventura is postdoctoral fellow from the CONICET.

Conflict of interest

The authors declare that there are no conflicts of interest.

References

Arrebola, J.P., Belhassen, H., Artacho-Cordón, F., Ghali, R., Ghorbel, H., Boussen, H., Perez-Carrascosa, F.M., Expósito, J., Hedhili, A., Olea, N., 2015. Risk of female breast cancer and serum concentrations of organochlorine pesticides and polychlorinated biphenyls: a case-control study in Tunisia. Sci. Total Environ. 520, 106–113. doi:http://dx.doi.org/10.1016/j.scitotenv.2015.03.045.

Artacho-Cordón, F., Fernández-Rodríguez, M., Garde, C., Salamanca, E., Iribarne-Durán, L., Torné, P., Expósito, J., Papay-Ramírez, L., Fernández, M., Olea, N., Arrebola, J., 2015. Serum and adipose tissue as matrices for assessment of exposure to persistent organic pollutants in breast cancer patients. Environ. Res. 142, 633–643.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Ann. Biochem. 72, 248–254.

Castoria, G., Giovannelli, P., Lombardi, M., De Rosa, C., Giraldi, T., de Falco, A., Barone, M.V., Abbondanza, C., Migliaccio, A., Auricchio, F., 2012. Tyrosine phosphorylation of estradiol receptor by Src regulates its hormone-dependent nuclear export and cell cycle progression in breast cancer cells. Oncogene 31, 4868–4877. doi:http://dx.doi.org/10.1038/onc.2011.642.

Cheskis, B.J., 2004. Regulation of cell signalling cascades by steroid hormones. J. Cell. Biochem. 93, 20–27. doi:http://dx.doi.org/10.1002/jcb.20180.

Chu, I., Sun, J., Arnaout, A., Kahn, H., Hanna, W., Sun, P., Tan, C., Hengst, L., Slingerland, J., 2008. p27 phosphorylation by Src regulates inhibition of cyclin E-Cdk2 and p27 proteolysis. Cell 128, 281–294.

Coqueret, O., 2003. New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? Trends Cell Biol. 13, 65–70.

García, M.A., Peña, D., Alvarez, L., Cocca, C., Pontillo, C., Bergoc, R., de Pisarev, D.K., Randi, A., 2010. Hexachlorobenzene induces cell proliferation and IGF-I signaling pathway in an estrogen receptor alpha-dependent manner in MCF-7 breast cancer cell line. Toxicol. Lett. 192, 195–205. doi:http://dx.doi.org/10.1016/j.toxlet.2009.10.026.

González, L., Agulló-Ortuño, M.T., García-Martínez, J.M., Calcabrini, A., Gamallo, C., Palacios, J., Aranda, A., Martín-Pérez, J., 2006. Role of c-Src in human MCF7 breast cancer cell tumorigenesis. J. Biol. Chem. 281, 20851–20864. doi:http://dx.doi.org/10.1074/jbc.M601570200.

Guo, H., Jin, Y., Cheng, Y., Leaderer, B., Lin, S., Holford, T.R., Qiu, J., Zhang, Y., Shi, K., Zhu, Y., Niu, J., Bassig, B.A., Xu, S., Zhang, B., Li, Y., Hu, X., Chen, Q., Zheng, T., 2014. Prenatal exposure to organochlorine pesticides and infant birth weight in China. Chemosphere 110, 1–7. doi:http://dx.doi.org/10.1016/j.chemosphere.2014.02.017.

International Agency of Research on Cancer, 2014 GLOBOCAN 2012: Estimated cancer incidence mortality and prevalence worldwide in 2012 [WWW Document]. URL http://globocan.iarc.fr/ia/World/atlas.html (Accessed August 27 2014).

Jemal, A., Bray, F., Ferlay, J., Ward, E., Forman, D., 2011. Global cancer statistics. CA. Cancer J. Clin. 61, 69–90. doi:http://dx.doi.org/10.3322/caac.20107.

Lauritzen, H.B., Larose, T.L., Øien, T., Sandanger, T.M., Odland, J.Ø., van de Bor, M., Jacobsen, G.W., 2016. Maternal serum levels of perfluoroalkyl substances and organochlorines and indices of fetal growth: a Scandinavian case-cohort study. Pediatr. Res. 1–10. doi:http://dx.doi.org/10.1038/pr.2016.187.

Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E., Auricchio, F., 1996. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO J. 15, 1292–1300.

Mrema, E.J., Rubino, F.M., Brambilla, G., Moretto, A., Tsatsakis, A.M., Colosio, C., 2013. Persistent organochlorinated pesticides and mechanisms of their toxicity. Toxicology 307, 74–88. doi:http://dx.doi.org/10.1016/j.tox.2012.11.015.

Muşat, M., Vax, V.V., Borboli, N., Gueorguiev, M., Bonner, S., Korbonits, M., Grossman, A.B., 2004. Cell cycle dysregulation in pituitary oncogenesis. Front. Horm. Res. 32, 34–62.

Murray, S.A., Yang, S., Demicco, E., Ying, H., Sherr, D.H., Hafer, L.J., Rogers, A.E., Sonenshein, G.E., Xiao, Z.-X.J., 2005. Increased expression of MDM2, cyclin D1, and p27Kip1 in carcinogen-induced rat mammary tumors. J. Cell. Biochem. 95, 875–884. doi:http://dx.doi.org/10.1002/jcb.20414.

Nilsen, E., Zaugg, S., Alvarez, D., Morace, J., Waite, I., Counihan, T., Hardiman, J., Torres, L., Patiño, R., Mesa, M., Grove, R., 2014. Contaminants of legacy and emerging concern in largescale suckers (Catostomus macrocheilus) and the foodweb in the lower Columbia River, Oregon and Washington, USA. Sci. Total Environ. 484, 344–352. doi:http://dx.doi.org/10.1016/j.scitotenv.2013.04.012.

Ociepa-Zawal, M., Rubis, B., Wawrzynczak, D., Wachowiak, R., Trzeciak, W.H., 2010. Accumulation of environmental estrogens in adipose tissue of breast cancer patients. J. Environ. Sci. Health. A Tox. Hazard. Subst. Environ. Eng. 45, 305–312 Article.

Peña, D., Pontillo, C., García, M.A., Cocca, C., Alvarez, L., Chiappini, F., Bourguignon, N., Frahm, I., Bergoc, R., Kleiman de Pisarev, D., Randi, A., 2012. Alterations in c-Src/HER1 and estrogen receptor α signaling pathways in mammary gland and tumors of hexachlorobenzene-treated rats. Toxicology 293, 68–77. doi:http://dx.doi.org/10.1016/j.tox.2011.12.012.

Pontillo, C., García, M.A., Peña, D., Cocca, C., Chiappini, F., Alvarez, L., Kleiman de Pisarev, D., Randi, A., 2010. Activation of c-Src/HER1/STAT5b and HER1/ERK1/2 signalling pathways and cell migration by hexachlorobenzene in MDA-MB-231 human breast cancer cell line. Toxicol. Sci. 120, 284–296. doi:http://dx.doi.org/10.1093/toxsci/kfq390.

Randi, A.S., Cocca, C., Carbone, V., Nuñez, M., Croci, M., Gutiérrez, A., Bergoc, R., Kleiman de Pisarev, D.L., 2006. Hexachlorobenzene is a tumor co-carcinogen and induces alterations in insulin-growth factors signaling pathway in the rat mammary gland. Toxicol. Sci. 89, 83–92. doi:http://dx.doi.org/10.1093/toxsci/kfj023.

Robles-Molina, J., Gilbert-López, B., García-Reyes, J.F., Molina-Díaz, A., 2014. Monitoring of selected priority and emerging contaminants in the Guadalquivir River and other related surface waters in the province of Jaén, South East Spain. Sci. Total Environ. 479–480, 247–257. doi:http://dx.doi.org/10.1016/j.scitotenv.2014.01.121.

Rodier, G., Montagnoli, A., Di Marcotullio, L., Coulombe, P., Draetta, G.F., Pagano, M., Meloche, S., 2001. p27 cytoplasmic localization is regulated by phosphorylation on Ser10 and is not a prerequisite for its proteolysis. EMBO J. 20, 6672–6682. doi:http://dx.doi.org/10.1093/emboj/20.23.6672.

Saoudi, A., Fréry, N., Zeghnoun, A., Bidondo, M.L., Deschamps, V., Göen, T., Garnier, R., Guldner, L., 2014. Serum levels of organochlorine pesticides in the french adult population: the French National Nutrition and Health Study (ENNS), 2006–2007. Sci. Total Environ. 472, 1089–1099. doi:http://dx.doi.org/10.1016/j.scitotenv.2013.11.044.

Shi, M.-D., Shiao, C.-K., Lee, Y.-C., Shih, Y.-W., 2015. Apigenin, a dietary flavonoid, inhibits proliferation of human bladder cancer T-24 cells via blocking cell cycle progression and inducing apoptosis. Cancer Cell Int. 15, 1–12. doi:http://dx.doi.org/10.1186/s12935-015-0186-0.

Simpkins, F., Hevia-Paez, P., Sun, J., Ullmer, W., Gilbert, C. a., Da Silva, T., Pedram, A., Levin, E.R., Reis, I.M., Rabinovich, B., Azzam, D., Xu, X.X., Ince, T. a., Yang, J.Y.,

Verhaak, R.G.W., Lu, Y., Mills, G.B., Slingerland, J.M., 2012. Src inhibition with saracatinib reverses fulvestrant resistance in ER-positive ovarian cancer models in vitro and in vivo. Clin. Cancer Res. 18, 5911–5923. doi:http://dx.doi.org/10.1158/1078-0432.CCR-12-1257.

Siriwardana, G., Seligman, P., 2015. Iron depletion results in Src kinase inhibition with associated cell cycle arrest in neuroblastoma cells. Physiol. Rep. 3 doi:http://dx.doi.org/10.14814/phy2.12341 e12341–e12341.

Thomas, A., Leontjew Toms, L.-M., Harden, F.A., Hobson, P., White, N.M., Mengersen, K.L., Mueller, J.F., 2017. Concentrations of organochlorine pesticides in pooled human serum by age and gender. Environ. Res. 154, 10–18. doi:http://dx.doi.org/10.1016/j.envres.2016.12.009.

To-Figueras, J., Sala, M., Otero, R., Barrot, C., Santiago-Silva, M., Rodamilans, M., Herrero, C., Grimalt, J., Sunyer, J., 1997. Metabolism of hexachlorobenzene in humans: association between serum levels and urinary metabolites in a highly exposed population. Environ. Health Perspect. 105, 78–83. doi:http://dx.doi.org/10.2307/3433066.

Wander, S.A., Zhao, D., Slingerland, J.M., 2011. p27: A barometer of signaling deregulation and potential predictor of response to targeted therapies. Clin. Cancer Res. 17, 12–18. doi:http://dx.doi.org/10.1158/1078-0432.CCR-10-0752.

Wang, Z., Yu, B.W., Rahman, K.M.W., Ahmad, F., Sarkar, F.H., 2008. Induction of growth arrest and apoptosis in human breast cancer cells by 3, 3-diindolylmethane is associated with induction and nuclear localization of p27kip. Mol. Cancer Ther. 7, 341–349. doi:http://dx.doi.org/10.1158/1535-7163.MCT-07-0476.

Wong, P.S., Matsumura, F., 2007. Promotion of breast cancer by beta-hexachlorocyclohexane in MCF10AT1 cells and MMTV-neu mice. BMC Cancer 7, 130–138. doi:http://dx.doi.org/10.1186/1471-2407-7-130.

Wu, C., Luo, Y., Gui, T., Huang, Y., 2014. Concentrations and potential health hazards of organochlorine pesticides in shallow groundwater of Taihu Lake region. Chin. Sci. Total Environ. 470–471, 1047–1055. doi:http://dx.doi.org/10.1016/j.scitotenv.2013.10.056.

Zhang, Y., Zhou, S.-W., Jin, H., Hu, C., He, Z., Yu, Z., Ko, K.-M., Yang, T., Zhang, X., Pan, S.-Y., Zhou, S.-F., 2015. Schisandrin B inhibits cell growth and induces cellular apoptosis and autophagy in mouse hepatocytes and macrophages: implications for its hepatotoxicity. Drug Desing Dev. Ther. 9, 2001–2027.

de Tomaso Portaz, A.C., Caimi, G.R., Sánchez, M., Chiappini, F., Randi, A.S., Kleiman de Pisarev, D.L., Alvarez, L., 2015. 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. Toxicology 336, 36–47. doi:http://dx.doi.org/10.1016/j.tox.2015.07.013.