Chemosphere 251 (2020) 126426

Contents lists available at ScienceDirect

Chemosphere

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

Chlorpyrifos-induced cell proliferation in human breast cancer cell lines differentially mediated by estrogen and aryl hydrocarbon receptors and KIAA1363 enzyme after 24 h and 14 days exposure

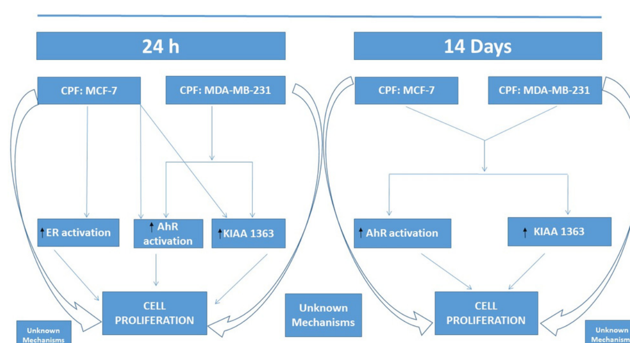
Paula Moyano [a], Jimena García [d], José Manuel García [a], Adela Pelayo [b], Pilar Muñoz-Calero [c], María Teresa Frejo [a], Maria Jose Anadon [b], Margarita Lobo [a], Javier Del Pino [a, *]

[a] Department of Pharmacology and Toxicology, Medicine School, Complutense University of Madrid, 28040, Madrid, Spain
[b] Department of Legal Medicine, Psychiatry and Pathology, Medicine School, Complutense University of Madrid, 28040, Madrid, Spain
[c] Alborada Foundation, 28690, Brunete, Spain
[d] Department of Pharmacology, Health Sciences School, Alfonso X University, 28691, Madrid, Spain

HIGHLIGHTS

- CPF and CPFO induced cell proliferation after acute and long-term treatment.
- CPF and CPFO induced cell proliferation in MCF-7 and MDA-MB-231 cells.
- CPF and CPFO alter differently KIAA1363, AhR and ERα expression at 24 h and 14 days.
- Cell proliferation was mediated by KIAA1363, AhR and ERα in MCF-7 cells.
- Cell proliferation was mediated by KIAA1363 and AhR in MDA-MB-231 cells.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 22 December 2019
Received in revised form
27 February 2020
Accepted 4 March 2020
Available online 6 March 2020

Handling Editor: Jian-Ying Hu

Keywords:
MCF-7
MDA-MB-231

ABSTRACT

Organophosphate biocide chlorpyrifos (CPF) is involved with breast cancer. However, the mechanisms remain unknown. CPF increases cell division in MCF-7 cells, by estrogen receptor alpha (ERα) activation, although it is a weak ERα agonist, suggesting other mechanisms should be involved. Aromatic hydrocarbon receptor (AhR) activation increases cell division in human breast cancer cells, and CPF strongly activates it. Finally, the KIAA1363 enzyme, which is regulated by CPF, is overexpressed in cancer cells. Accordingly, we hypothesized that CPF or its metabolite chlorpyrifos-oxon (CPFO) could induce cell viability promotion in MCF-7 and MDA-MB-231 cell lines, through mechanisms related to ERα, AhR, and KIAA1363, after 24 h and 14 days treatment. Results show that, after acute and long-term treatment, CPF and CPFO alter differently KIAA1363, AhR, ER and cytochrome P450 isoenzyme 1A1 (CYP1A1) expression. In addition, they induced cell proliferation through ERα activation after 24 h exposure in MCF-7 cells and

Abbreviations: AhR, aromatic hydrocarbon receptor; BSA, bovine serum albumin; CH22, CH-223191; CPF, chlorpyrifos; CPFO, chlorpyrifos-oxon; CYP1A1, cytochrome P450 isoenzyme 1A1; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethylsulphoxide; ER, estrogen receptor; E2, 17β-estradiol; FBS, fetal bovine serum; MTT, 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; PBS, phosphate-buffered saline; TMX, tamoxifen.

* Corresponding author. Department of Pharmacology and Toxicology, Medicine School Complutense, University of Madrid Avda, Puerta de Hierro s/n, 28040, Madrid, Spain.
E-mail address: jdelpino@pdi.ucm.es (J. Del Pino).

https://doi.org/10.1016/j.chemosphere.2020.126426
0045-6535/© 2020 Elsevier Ltd. All rights reserved.

Chlorpyrifos
ER
AhR
CYP1A1
KIAA163

through KIAA1363 overexpression and AhR activation in MCF-7 and MDA-MB-231 cells after acute and long-term treatment. The results obtained in this work provide new information relative to the mechanisms involved in the CPF toxic effects that could lead to breast cancer disease.

© 2020 Elsevier Ltd. All rights reserved.

1. Introduction

Chlorpyrifos (CPF) is an insecticide from the organophosphate (OPs) family, which applications ranging from industrial to home and agriculture use (Richardson and Chambers, 2005). OPs occupational exposure was related to increased leukemia, all lymphohematopoietic, brain, colorectal, lung and breast cancer risk (Engel et al., 2017; Lee et al., 2004, 2007; Weichenthal et al., 2010). Particularly, Engel et al. (2017) reported that breast cancer risk was elevated among women who had ever used the CPF with respect to women not exposed to it [HR = 1.4 (95% CI: 1.0, 2.0)]. This risk was increased even more when both the women and their husbands used this pesticide in relation to when it was only the women who used it [HR = 1.6 (95% CI: 0.9, 2.9)]. Chlorpyrifos was also related to the induction of breast cancer in rats chronically treated with small doses (Nishi and Hundal, 2013; Ventura et al., 2016, 2019). However, it remains unclear which mechanisms induce these effects.

Different *in vitro* studies have shown that CPF induces the promotion of cell division in the human estrogen-dependent breast cancer MCF-7 cell line after 10 days of repeated exposure from 0.05 μM concentration and that it reduces cell proliferation from 50 μM (Ventura et al., 2012, 2015). This effect was described to be produced either by estrogen receptor alpha (ERα) activation (Ventura et al., 2012, 2015). However, there is some controversy on whether CPF induces cell proliferation and the role of ER activation in this action. In this sense, CPF was reported to not produce cell proliferation and estrogen receptor activation after 9 days repeated exposure starting at 0.001 μM−10 μM (Vinggaard et al., 1999) or cell proliferation after single treatment starting at 0.01 μM−10 μM (Rich et al., 2012) in MCF-7 cells. However, it was reported to induce cell proliferation after 24 h single CPF treatment starting at 25 μM−50 μM in MCF-7 cells, producing from this concentration cell death and ERα weak activation (with a 25% response respect to estradiol) at 50 μM concentration (Andersen et al., 2002). Thus, ERα participation in CPF cell proliferation induction seems limited and another mechanism could be involved.

Previous works described the aromatic hydrocarbon receptor (AhR) participation in the induction of breast cancer (Vacher et al., 2018). MCF-7 and MDA-MB-231 cell proliferation was related to AhR overexpression and its silencing reduces tumor size increase and metastasis (Salisbury et al., 2014). Besides, AhR antagonists have been proposed as possible antitumoral agents since AhR agonists were found to induce tumor promotion and rescue breast cancer cells from apoptosis after toxic insults (Al-Dhfyan et al., 2017; Bekki et al., 2015; Chu et al., 2014; Romagnolo et al., 2015, 2017). Recently, AhR was also shown that could participate in the promotion, invasiveness, and migration of estrogen-dependent breast tumor cell lines compared to non-estrogen-dependent cell lines (D'Amato et al., 2015). CPF was shown to present concentration-dependent AhR agonist effects at concentrations above 1 μM in human and rat hepatoma cell lines (Long et al., 2003; Takeuchi et al., 2008). Therefore, CPF could mediate this effect through the AhR receptor in breast tumor cell lines.

Otherwise, after treatment with CPF of the non-hormone-dependent MDA-MB-231 cell line, no increase in promotion of cell division was observed after a single exposure at 48 h (Rich et al., 2012), although its reduction after 10 days of treatment has been described (Ventura et al., 2012). These studies were carried out only with CPF, which is extensively transformed aby liver action, mainly into chlorpyrifos-oxon (CPFO). Said metabolite, alongside with CPF, induces most of the toxic effects described (Eaton et al., 2008), so we cannot rule out that the observed effects are different after exposure to CPFO. In addition, it has been described that the enzyme KIAA1363, which influences the invasiveness and growth of tumor cells (Jessani et al., 2002) and participates in the detoxification of organophosphates (Eaton et al., 2008), shows high activity in the MDA-MB-231 cell line. Nevertheless, this enzyme has less activity in the MCF-7 cell line (Jessani et al., 2002), which suggests that there could be differences between both cell lines on cell proliferation susceptibility. In this sense, CPF was described to produce the proliferation of H508 colorectal adenocarcinoma human cell line, but not of other human cell lines from colon and liver (HT-29, CCD841, HepG2, and THLE-3), while CPFO can induce cell death in all of them (Suriyo et al., 2015). All commented above indicates that there may be different mechanisms between CPF and CPFO and between different cell lines or periods of exposure, leading to different results depending on the compound, cell line or exposure period used.

Considering the above, we hypothesized that CPF or its metabolite CPFO could induce the promotion of cell division in MCF-7 and MDA-MB-231 cell lines, by mechanisms related to ER and AhR in MCF-7 cells and through AhR and KIAA1363 enzyme in the MDA-MB-231 cells, with differential effect after acute and repeated exposure. This work is aimed at determining the mechanisms underlying cell proliferation induced in breast cancer cells by CPF, which could help to explain CPF participation as a factor in breast cancer induction.

2. Materials and methods

2.1. Chemicals

Chlorpyrifos (99.99%), chlorpyrifos-oxon (99.99%), tamoxifen, CH-223,191, dimethyl sulfoxide (DMSO), L-glutamine, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) compounds were purchased to Sigma (Madrid, Spain). The rest of the reagents were of the highest grade and purity available in laboratory.

2.2. Culture of MCF-7 and MDA-MB-231 cells

We used the human breast cancer MCF-7 cell line, extensively used to study ER activation effects on breast cancer cells, and the human breast cancer MDA-MB-231 cell line, as a non-estrogen-dependent cell line (Rich et al., 2012), as a standard of estrogen-dependent and independent breast cancer cells. Both cell lines express AhR (Salisbury et al., 2014).

The cells were gifted by Dr. Palacios (Ramon y Cajal Hospital, Spain). Dulbecco's Modified Eagle's Medium (DMEM)/F12 at 1:1 strengthened with 2 mM L-glutamine, 10% fetal bovine serum (FBS) and penicillin/streptomycin with or without 6 ng/mL insulin was used to maintain MCF-7 and MDA-MB-231 cells, respectively, at

37 °C and 5% CO_2. Cells (passages 7–15) were seeded in 6-wells (100,000–200000 cells/well) plates or 96-well (2000–4000 cells/well) plates in a complete medium for 24 h to attach. After that, we used only a phenol red-free with 2.5% charcoal-treated FBS as experimental medium for 24 h, and then we added the compounds that were going to be tested for either 24 h or repeatedly for 14 days, added daily to new media. The co-treatments with different compounds were performed at the same time and under the described conditions. Look Out Mycoplasma PCR Detection Kit (Sigma, Madrid, Spain) showed cells to be mycoplasma-free.

We determined ER and AhR gene expression and activation, KIAA1363 gene expression and the effects of KIAA1363 gene silencing and ER and AhR antagonist treatment on cell proliferation. Cells were incubated with CPF (from 0.01 μM to 100 μM) and CPFO (from 0.01 μM to 100 μM) concentrations during 24 h and recurrently during 14 days with or without the ER antagonist tamoxifen (TMX; 1 μM), with or without the AhR antagonist CH-223,191 (CH22; 20 nM), added daily to new media. Leastwise three repetitions wells per treatment were performed. Each experiment had a control vehicle group (0.1% DMSO) performed at the same time for comparison purposes.

According to several published works, CPF is transformed by liver action to CPFO. Systemic administered CPFO seems to be unavailable to tissues (Marty et al., 2012). In this sense, the CPFO produced is rapidly degraded at hepatic and plasma levels through the action of esterase, so the main cytotoxic effects induced by CPF may be the result of specific CPF to CPFO transformation in the tissues (Khokhar and Tyndale, 2012, 2014). Some of the principal cytochrome P450 (CYP450) isoforms that metabolize CPF to CPFO in humans (1A2, 2B6, 2C19, 2D6, 3A5 and 3A4) (Eaton et al., 2008) were described to be expressed in MCF-7 and MDA-MB-231 cells (Lo et al., 2010; Mitra et al 2011; Weissenstein et al., 2019), being able to metabolize CPF to CPFO. Thus, CPF effects in these cell lines could be similar to that produced after exposure *in vivo* and, if proven, may help explain the differences between CPF and CPFO toxic effects.

The range of 0.001 μM–50 μM concentrations is routinely used to study chlorpyrifos effect on cell proliferation in breast cancer cells after single and repeated treatment (Vinggaard et al., 1999; Rich et al., 2012; Ventura et al., 2012). Besides, animal studies showed that breast cancer is induced after repeated exposure from 0.01 mg/kg (Ventura et al., 2019), which would translate into 0.03 μM tissue concentration following the studies by Terry et al. (2003) where doses of 2.5–25.0 mg/kg/day CPF in rats were indicated to be equivalent to 7.0 and 8.0–70.0 and 80.0 μM tissue concentration. Thus, concentrations chosen for the present study would be outstanding to research cell proliferation. In addition, we selected CPF 10 μM and 1 μM concentrations, which were the highest concentrations observed to induced cell proliferation, ER and AhR activation after single and repeated exposure, respectively, to research the mechanisms by which CPF and CPFO induce cell proliferation. Finally, we chose TMX (1 μM) and CH22 (20 nM) concentrations since they were the minimum concentrations that block ER and AhR activation and did not present cytotoxicity.

2.3. Gene expression analysis

Trizol (Invitrogen, Madrid, Spain) was used to extract total RNA, following producer guidelines. We determined the final RNA concentration and quality with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Madrid, Spain) and an Experion Lab Chip (Bio-Rad, Madrid, Spain), respectively. We used an iScript™ cDNA Synthesis Kit (BIO-RAD, Madrid, Spain), according to the producer guidelines, to synthesize the first-strand cDNA from 1000 ng of cRNA. Prevalidated KiCqStart® SYBR® Green Primers sets (Sigma, Madrid, Spain) for mRNAs encoding ERα, AhR, KIAA1363, cytochrome P450 isoenzyme 1A1 (CYP1A1), and beta-actin (ACTB) (Table 1) were used to perform qPCR after reverse transcription. We used housekeeping gene ACTB to normalize results. We ran the reactions on a CFX96 using Fast Start Universal SYBR Green Master (Rox) (Sigma, Madrid, Spain). The thermocycler parameters were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 72 °C for 30 s, following the producer's guidelines. Ct (cycle threshold) method was performed to determine gene expression changes, and results are presented following Livak and Schmittgen (2001) method. $2^{-\Delta\Delta Ct}$ values were calculated by determining ΔΔCt $[(Ct_{target\ gene}\text{-}Ct_{ACTB})_{treatment} - (Ct_{target\ gene}\text{-}Ct_{ACTB})_{control}]$. $Ct_{target\ gene}$ and Ct_{ACTB} define the cycle thresholds for each target gene and ACTB of the different treatment and control groups.

We selected the CYP1A1 gene for the determination of CPF or CPFO action on AhR activation since CYP1A1 gene expression is specifically regulated by AhR and is widely used as a marker of AhR activity (Barouki et al., 2007).

2.4. Gene knockdown

MISSION siRNA Transfection reagent (Sigma, Madrid, Spain) was utilized to transfect MCF-7 cells with siRNAs in 6-well plates (1000000 cells/well), following producer guidelines. World-class siRNA Rosetta design algorithm (Rosetta Inpharmatics, Seattle, USA) was used to predesign the sequence of two siRNA groups (Sigma, Barcelona, Spain) homologous to human KIAA1363 (5-CAAUGAUCGUUAACAAUCAtt-3 and 5-UGAUUGUUAACGA-UCAUUGtt-3). MISSION siRNA Universal Negative Control siRNA (Sigma, Madrid, Spain) was utilized as a transfection control. The effectiveness of KIAA1363 silencing was analyzed 48 h after transfection by qPCR with primers encoding human KIAA1363 mRNA (Sigma, Madrid, Spain). We used the MTT test to evaluate KIAA1363 silencing effects on cell proliferation. PBS was used to wash cells after they had been incubated with siRNAs for one day. Afterward, cultures were treated, with CPF or CPFO with or without CH22 and/or TMX, during either one or 14 consecutive days.

2.5. ER activation assay

Nuclear proteins were obtained from cell homogenates using a Nuclear Extraction Kit (Abcam, Cambridge, UK; ab113474), following producer guidelines. Proteins were quantified and stored in a −80 °C freezer, before performing the rest of the experiments.

Table 1
Primers used for quantitative real-time PCR analyses.

Gene	Forward primer	Reverse primer
AhR	5-ATGGATCAATACTTCCACCTC-3	5-TTTGGCATCACAACCAATAG-3
CYP1A1	5-CATTAACATCGTCTTGGACC-3	5-TCTTGGATCTTTCTCTGTACC-3
ER1	5-GGAGTGTACACATTTCTGTC-3	5-CAAAGTGTCTGTGATCTTGTC-3
KIAA1363	5-GAATACAGGCTAGTTCCAAAG-3	5-TACTTCTGTAAGACTTCTGGC-3
ACTB	5-GACGACATGGAGAAAATCTG-3	5-ATGATCTGGGTCATCTTCTC-3

ER activity was determined on nuclear extracts by Estrogen Receptor Transcription Factor Assay Kit (Abcam, Cambridge, UK), according to producer guidelines. Optical density was determined with a Fluoroskan Ascent FL Microplate Fluorometer and Luminometer (Thermo Fisher Scientific, Madrid, Spain) at 450 nm and data were normalized with nuclear protein level values.

2.6. Protein analysis

After treatments, we washed cells with cold PBS, gathered them by scraping, and lysed them with RIPA buffer (Thermo Scientific, Madrid, Spain) plus a protease inhibitors mixture (Thermo Fisher Scientific, Madrid, Spain). Cell lysate supernatant was gatherer after being centrifuged at 10,000$\times g$ during 10 min at 4 °C. Total protein levels were assayed with a BCA kit (Thermo Fisher Scientific, Madrid, Spain).

CYP1A1, ERα, AhR, minichromosome maintenance complex component 2 (MCM2) and KIAA1363 protein concentrations were determined by commercial ELISA kits (MBS092945, MBS013925, MBS9500021, MBS2881669, and MBS9368767 MyBioSource, CA, USA) respectively, following producer guidelines. Interferences of CPF and CPFO with kits reagents were ruled out by performing a Negative control. We normalized the protein concentrations of each target gene with the total protein levels measured by BCA kit, to prevent possible interferences with the real value of the target proteins concentrations measured due to the induction of cell death or cell proliferation. Protein levels were expressed as ng/mg of protein and values were presented as percentage of control content.

2.7. Analysis of cell proliferation

MTT test was used to analyze CPF or CPFO action on cell proliferation after 24 h and 14 days incubation as previously described by Del Pino et al. (2016). Cell proliferation data was confirmed by the Crystal violet staining test. Cancer cells were incubated in 96-well plates during 24 h or 14 days. After medium removal, formalin (4%) and crystal violet (0.5%) were used to fix and stain cells. After several washes, 10% acetic acid was used to extract the staining. Finally, data were obtained through spectrophotometrically reading at 540 nm. Control viability values were set as a 100% response.

Furthermore, we measured MCM2 protein expression, as a marker of cell proliferation, in order to confirm CPF or CPFO action on cell division status. MCM2 was described to be a more sensitive proliferation marker in breast cancer cells (Yousef et al., 2017) and a more reliable marker of cell proliferation in different cancer cells (Maiorano et al., 2006).

2.8. Statistical testing

Each experimental condition was performed three separate times, each one in triplicate. Results are shown as mean ± standard error of the mean (SEM). Two-way analysis of variance (ANOVA) (treatment vs gene manipulation) or one-way ANOVA testing (analysis of diverse treatments) were used for comparing control and treatment groups, followed by the Tukey post-hoc analysis. Results were statistically analyzed with GraphPad software, and the data were considered statistically different when $p \leq 0.05$.

3. Results

3.1. ERα decreased expression in MCF-7 cells

After 24 h treatment with CPF or CPFO in MCF-7 cells, a decrease in ER expression starting at 100 μM and 10 μM concentrations, respectively, was observed (Fig. 1A). Nevertheless, ER expression was reduced from 0.1 μM concentration after CPF or CPFO 14 days treatment, this decrease was higher after CPFO exposure than after CPF exposure (Fig. 1B).

3.2. AhR induced expression in MCF-7 and MDA-MB-231 cells

AhR expression was not affected after 24 h CPF or CPFO treatment (Data not shown), but its expression was increased, in a concentration-dependent way, starting at 0.1 μM concentration after 14 days of treatment in both cell lines. This effect was more pronounced in MDA-MB-231 cells and after CPF exposure (Fig. 2A and B).

3.3. Cyp1A1 induced expression in MCF-7 and MDA-MB-231 cells

After 24 h exposure, CPF (0.1 μM−10 μM) and CPFO (1 μM−10 μM) induced, in a concentration-dependent way, CYP1A1 expression, in MCF-7 cells (Fig. 3A). Besides, CYP1A1 expression was induced after 24 h CPF and CPFO (0.1 μM−10 μM) exposure in MDA-MB-231 cells (Fig. 3B). After 14 days of exposure, CPF and CPFO (0.1 μM−1 μM) increased the expression of CYP1A1 in both cell lines (Fig. 3C and D). The effect on CYP1A1 induction was higher after CPF exposure than after CPFO exposure (Fig. 3). The increase in CYP1A1 expression observed was less pronounced after CPF and CPFO exposure in both cell lines, from 100 μM concentration after a single treatment and from 10 μM concentration after 14 days treatment (Fig. 3). After single and long-term treatment, CPF or CPFO co-treatment with CH22 reversed completely the induction of CYP1A1 gene expression in both cell lines (Fig. 3).

3.4. KIAA1363 induced expression in MCF-7 and MDA-MB-231 cells

After CPF (0.1 μM−10 μM) single treatment, KIAA1363 expression was increased, in a concentration-dependent way, in MCF-7 and MDA-MB-231 cell lines (Fig. 4A and B). After 24 h exposure to CPFO, KIAA1363 expression was increased in MCF-7 (1 μM−10 μM) and MDA-MB-231 cells (0.1 μM−10 μM) (Fig. 4A and B). After 14 days exposure to CPF or CPFO, KIAA1363 expression was induced in MCF-7 cells (0.1 μM−1 μM), and in MDA-MB-231 cells (0.1 μM−10 μM), but this induction started to fade from 1 μM concentration (Fig. 4C and D). The induction of KIAA1363 was more pronounced in MDA-MB-231 cells than in MCF-7 cells and after CPF treatment than after CPFO treatment (Fig. 4). After single and long-term treatment, CPF or CPFO co-treatment with CH22 did not show any effect on the induction of KIAA1363 gene expression observed in both cell lines; neither did TMX co-treatment in MCF-7 cells (Data not shown). Gene expression data was corroborated by protein expression analysis (Data not shown).

3.5. MCM2 disrupted expression in MCF-7 and MDA-MB-231 cells

CPF (0.1 μM−10 μM) single treatment induced MCM2 expression, in a concentration-dependent way, in MCF-7 and MDA-MB-231 cell lines (Fig. 5A and B). CPFO treatment for 24 h increased MCM2 in MCF-7 (1 μM−10 μM) and MDA-MB-231 cells (0.1 μM−10 μM) (Fig. 5A and B). CPF or CPFO treatment for 14 days increase MCM2 expression in MCF-7 cells (0.1 μM−1 μM), and in MDA-MB-231 cells (0.1 μM−10 μM), but this effect started to lessen from 1 μM concentration (Fig. 5C and D). CPF and CPFO treatment for 24 h or 14 days decreased the MCM2 expression from 1 μM concentration (Fig. 5). The induction of MCM2 was more pronounced in MDA-MB-231 cells than in MCF-7 cells and after CPF treatment than after CPFO treatment (Fig. 5).

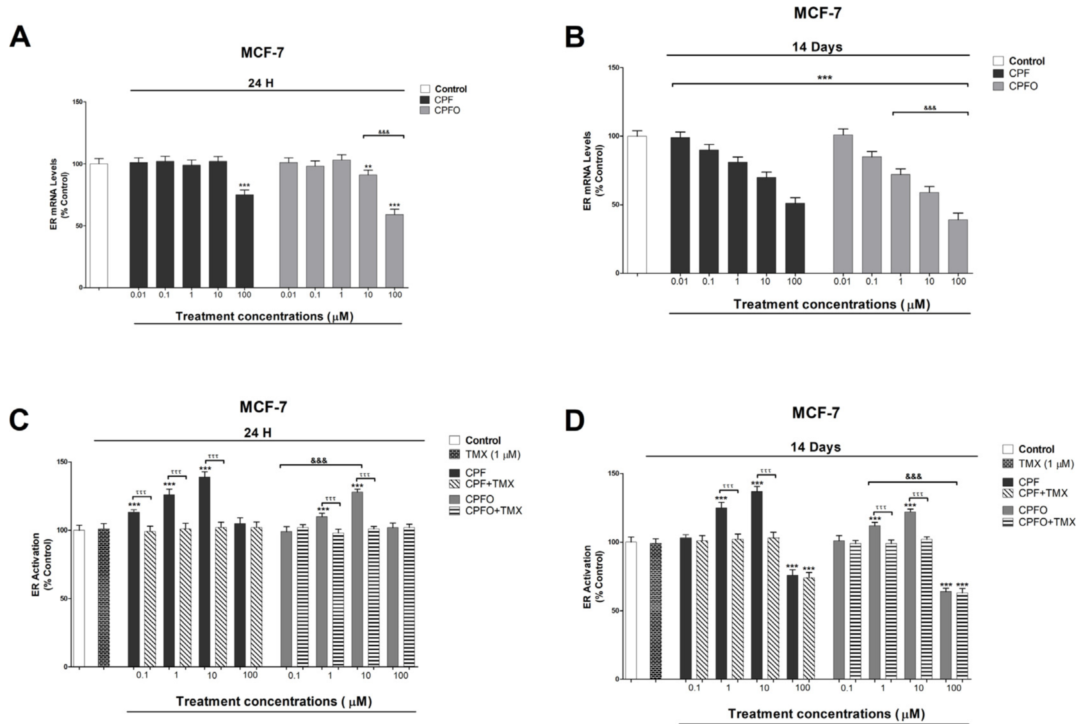


Fig. 1. Assessment of chlorpyrifos (CPF) and chlorpyrifos-oxon (CPFO) effect on estrogen receptor alpha (ERα) gene expression by semi-quantitative real-time PCR analysis after 24 h (A) and 14 days treatment (B) in MCF-7 cells. Assessment of CPF and CPFO effect on ERα activation by ELISA analysis after 24 h (C) and 14 days treatment (D) in MCF-7 cells. Data represent the mean ± SEM of three separate experiments from cells of different cultures, each one performed in triplicate. Data are presented as a percentage relative to the control group and were significantly different from the respective control group. ***p ≤ 0.001 and **p ≤ 0.01 compared to control; $^{\&\&\&}$p ≤ 0.001 compared to CPF treatment; ^{τττ}p≤0.001 compared to tamoxifen (TMX) treatment.

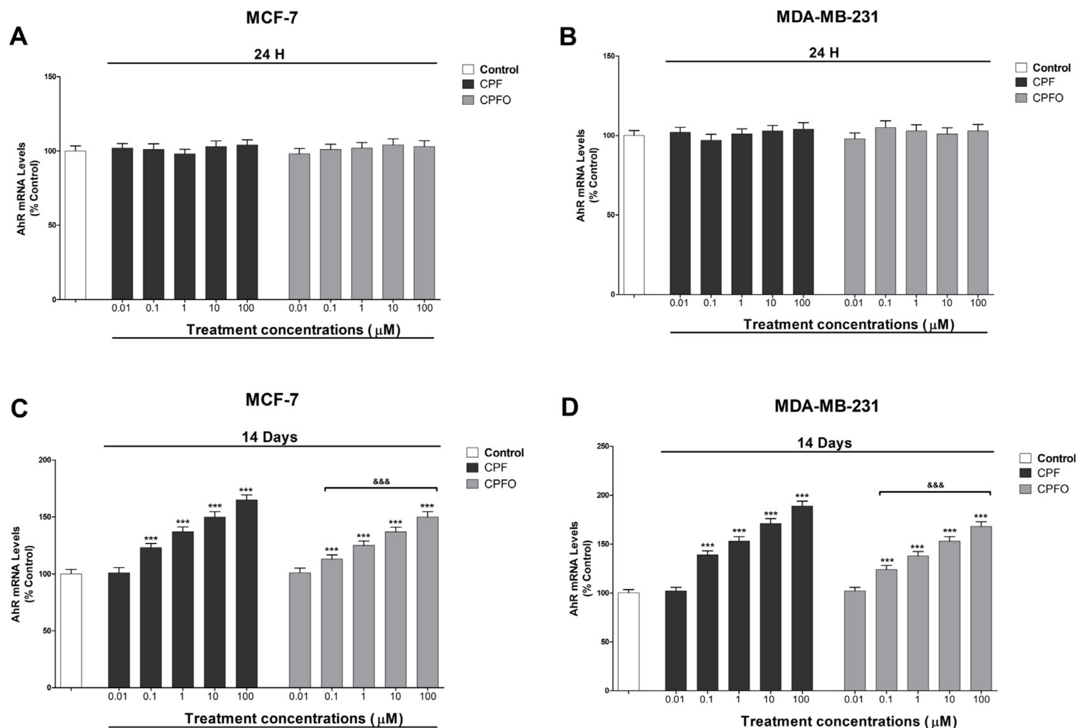


Fig. 2. Shows results from expression analysis targeting aromatic hydrocarbon receptor (AhR) after 14 days CPF and CPFO treatment in MCF-7 (A) and MDA-MB-231 cells (B). AhR gene expression was compared with controls. Data represent the mean ± SEM of three separate experiments from cells of different cultures, each one performed in triplicate. ***p ≤ 0.001, significantly different from controls; $^{\&\&\&}$p ≤ 0.001 compared to CPF treatment.

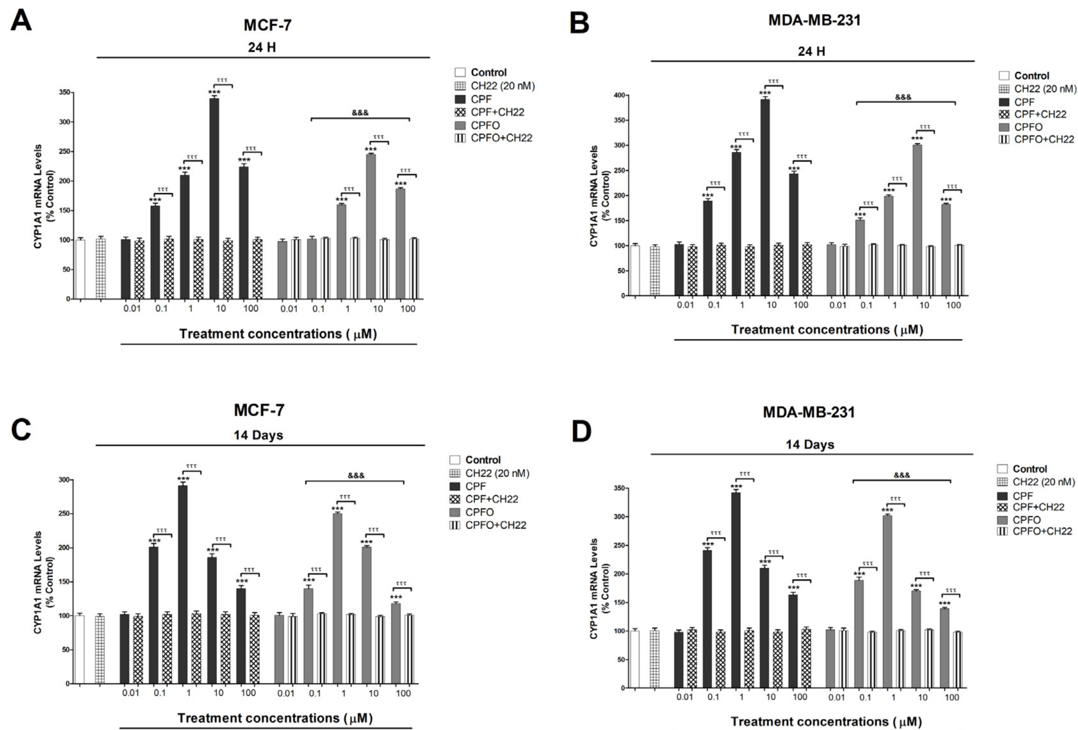


Fig. 3. Shows results from expression analysis targeting CYP1A1 after 24 h CPF and CPFO treatment in MCF-7 (A) and MDA-MB-231 cells (B) and after 14 days CPF and CPFO treatment in MCF-7 (C) and MDA-MB-231 cells (D). CYP1A expression was compared with controls. Data represent the mean ± SEM of three separate experiments from cells of different cultures, each one performed in triplicate. ***$p \le 0.001$, significantly different from controls; $^{\&\&\&}p \le 0.001$ compared to CPF treatment. $^{\tau\tau\tau}p \le 0.001$ compared to CH22 (CH-223,191) treatment.

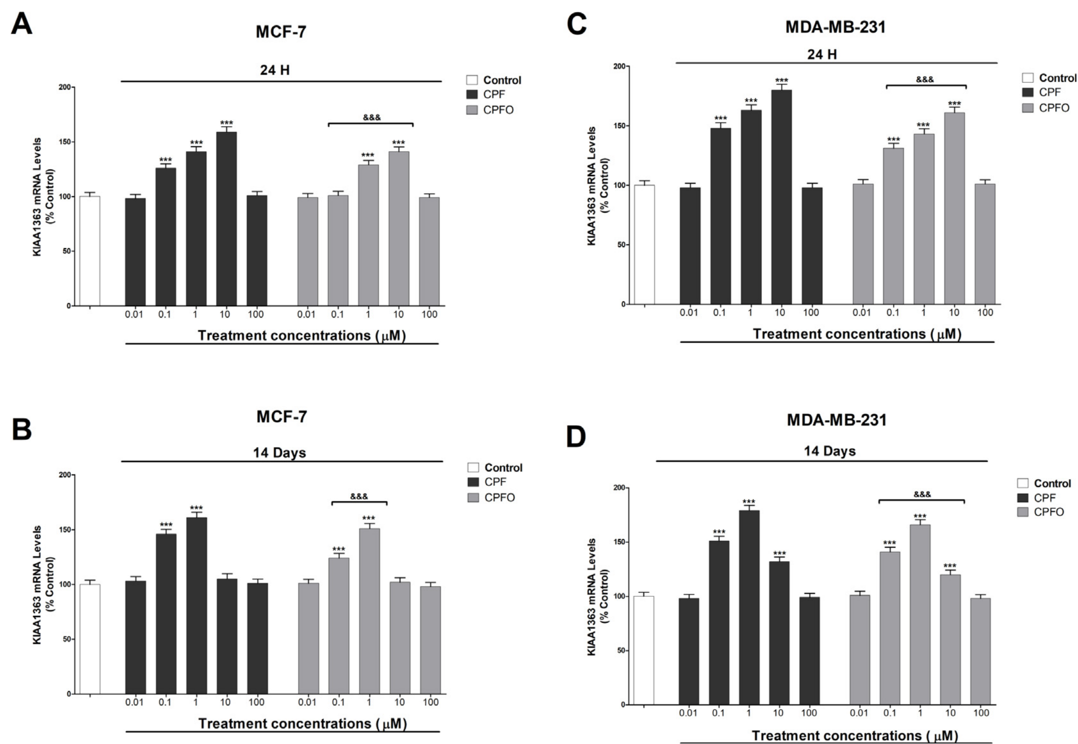


Fig. 4. Shows results from expression analysis targeting KIAA1363 after 24 h CPF and CPFO treatment in MCF-7 (A) and MDA-MB-231 cells (B) and after 14 days CPF and CPFO treatment in MCF-7 (C) and MDA-MB-231 cells (D). KIAA1363 expression was compared with controls. Data represent the mean ± SEM of three separate experiments from cells of different cultures, each one performed in triplicate. ***$p \le 0.001$, significantly different from controls; $^{\&\&\&}p \le 0.001$ compared to CPF treatment.

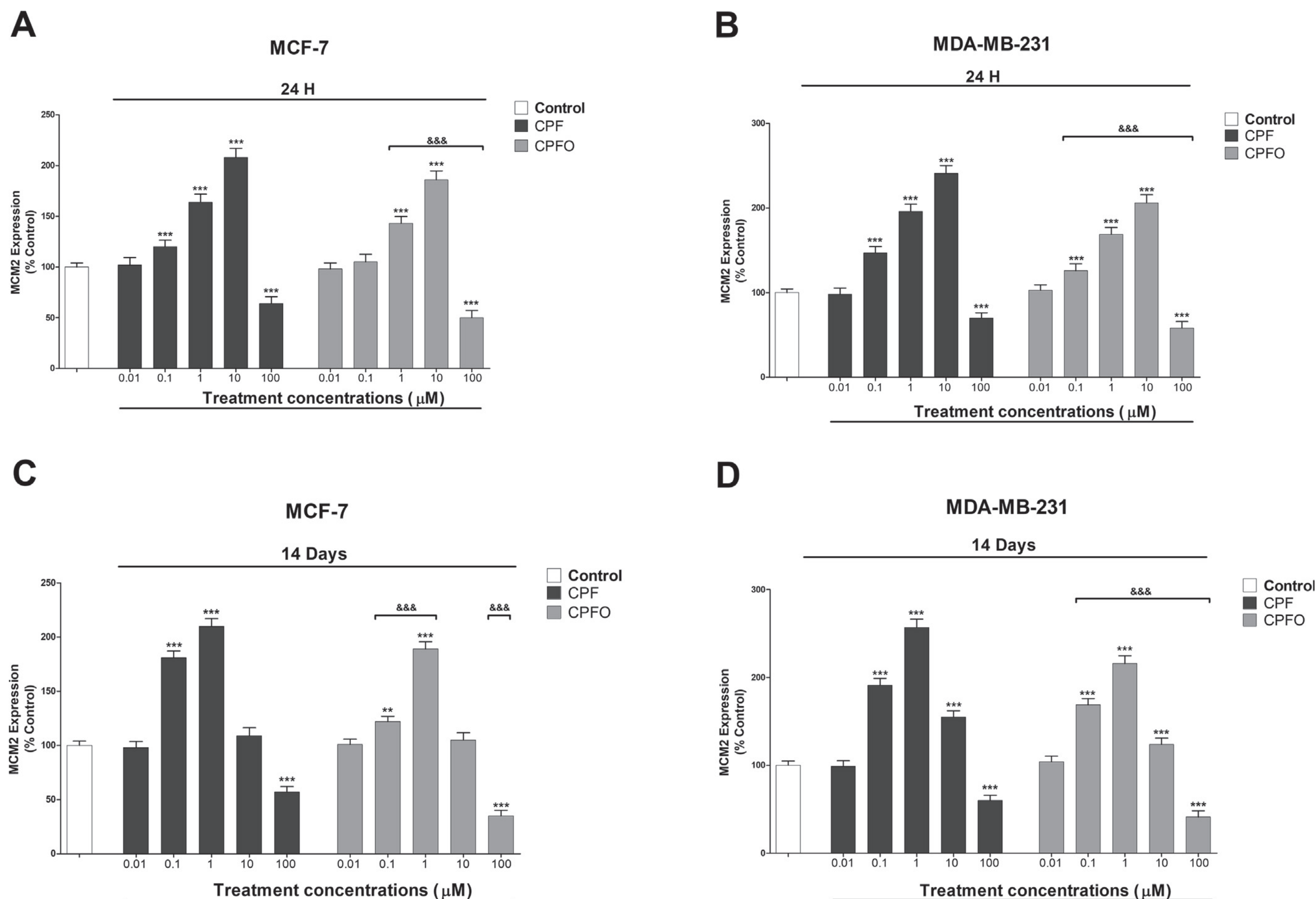


Fig. 5. Shows results from expression analysis targeting MCM2 after 24 h CPF and CPFO treatment in MCF-7 (A) and MDA-MB-231 cells (B) and after 14 days CPF and CPFO treatment in MCF-7 (C) and MDA-MB-231 cells (D). MCM2 expression was compared with controls. Data represent the mean ± SEM of three separate experiments from cells of different cultures, each one performed in triplicate. ***p ≤ 0.001, significantly different from controls; &&&p ≤ 0.001 compared to CPF treatment.

3.6. *Gene silencing*

To research the effect produced by the KIAA1363 silencing on cell viability, we inserted siRNA against KIAA1363 inside MCF-7 and MDA-MB-231 cells. Cells transfected with control siRNA presented no action on cell viability and KIAA1363 gene expression (Fig. 6). Results from vehicle and control treated cells showed no significant difference between them. Cultures transfected against KIAA1363 showed a large reduction on its gene expression (Fig. 6C and D), but without action on the viability of cells (Fig. 6A and B).

3.7. *ER activation analysis*

CPF (0.1 μM−10 μM) and CPFO (1 μM−10 μM) induced a concentration-dependent ER activation after a single treatment of MCF-7 cells (Fig. 1C). After 14 days of exposure to CPF and CPFO (0.1 μM−10 μM) a concentration-dependent ER activation was induced, although, in both compounds, a reduction in ER activation was observed starting at 100 μM concentration in the hormone-dependent cell line (Fig. 1D). The effect on ER activation was lower following CPFO treatment than after CPF treatment and was reversed completely after tamoxifen treatment except after 100 μM concentration (Fig. 1C and D).

3.8. *Measurement of cell proliferation*

MTT results showed, after a single exposure to CPF, an increase in cell proliferation starting at 0.1 μM−10 μM concentrations and a decrease in cell viability starting at 100 μM concentration in MCF-7 and MDA-MB-231 cells (Fig. 7A and B). CPFO induced cell proliferation after 24 h exposure in MCF-7 cells (1 μM−10 μM) and in MDA-MB-231 cells (0.1 μM−10 μM) and decreased cell proliferation from 100 μM concentration in both cell lines (Fig. 7A and B). After CPF and CPFO (0.1 μM−1 μM) 14 days treatment, an increase in cell proliferation was observed (Fig. 8A and B). However, the increase in cell proliferation started to fade from 10 μM concentration, producing finally a decrease of cell viability from 100 μM concentration (Fig. 8A and B). The reduction in cell proliferation was less pronounced in MDA-MB-231 cells than in MCF-7 cells after CPF and CPFO treatment (Fig. 8A and B). The induction of cell proliferation was more pronounced in MDA-MB231 cells than in MCF-7 cells with both treatments, and less pronounced after CPFO exposure in both cell lines after single and long-term treatment (Fig. 7A and B, 8A and B). The MTT results described above were confirmed by measuring the expression of the proliferation marker MCM2 (Fig. 5).

Single or simultaneous CH22 or tamoxifen exposure of KIAA1363 knockdown or wild type MCF-7 or MDA-MB-231 cells or MCF-7 cells, respectively, did not induce a significant effect on cell viability (Fig. 7C and D, 8C and D). CPF and CPFO (10 μM) effects on cell proliferation after 24 h treatment were partially reversed after co-treatment with tamoxifen or CH22 in wild type MCF-7 cells or after KIAA1363 silenced MCF-7 cells treatment (Fig. 7C), but was only partially reverse after single CHH22 co-treatment of wild type MDA-MB-231 cells or treatment of KIAA1363 silenced MDA-MB-213 cells (Fig. 7D). CPF or CPFO (10 μM) simultaneous co-treatment

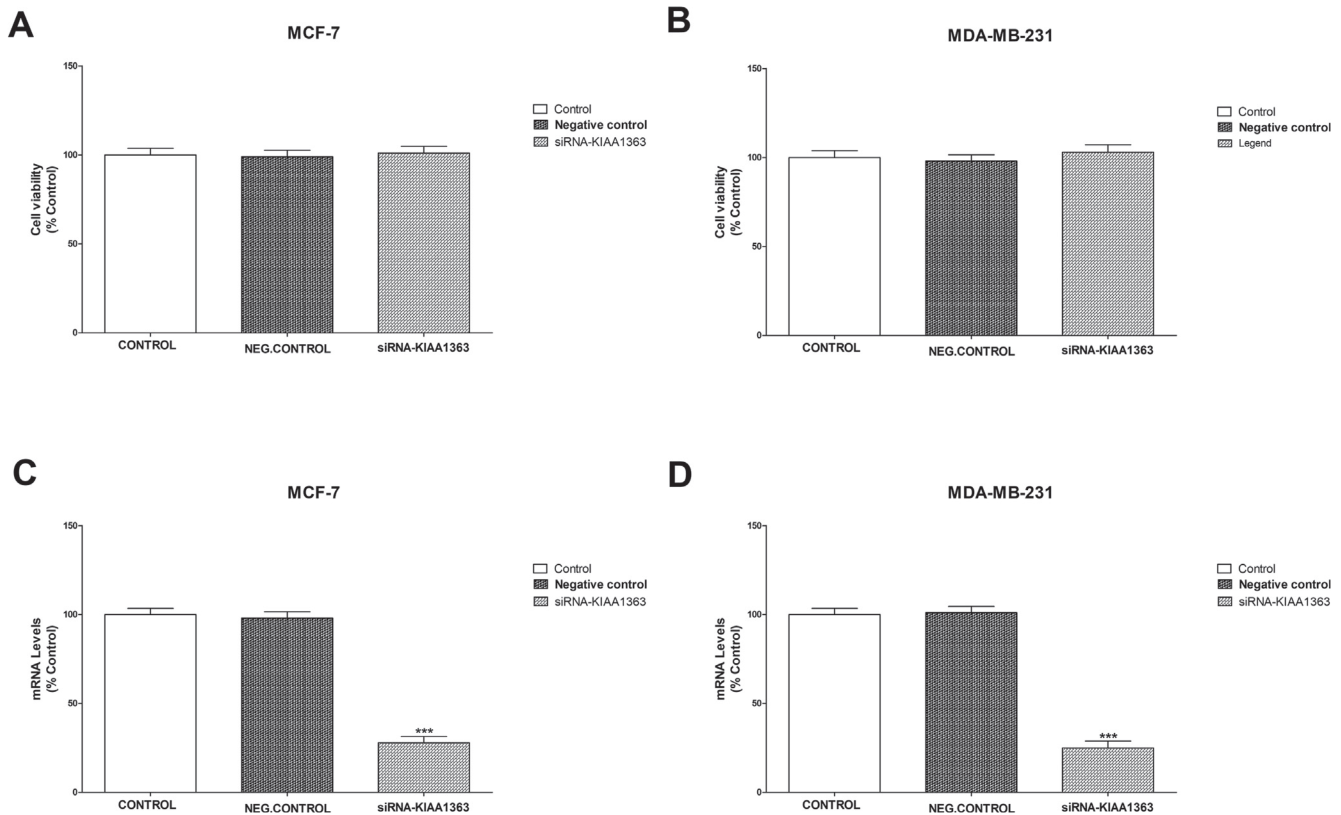


Fig. 6. **S**ilencing of KIAA1363 in MCF-7 and MDA-MB-231 cells and its impact on cell viability and KIAA1363 expression was determined. Control: cells transfected without siRNA. Negative (Neg.) control: cells transfected with scrambled siRNA. siRNA-KIAA1363: transfected with siRNA against KIAA1363. MTT test shows KIAA1363 down-regulation did not significantly induce cell damage after 48 h in MCF-7 (A) and MDA-MB-231 cells (B). KIAA1363 Single and down-regulation could be detected by RT-PCR and protein analysis 48 h after transfection in MCF-7 (C) and MDA-MB-231 cells (D). Values are given as mean ± SEM of three separate experiments from cells of different cultures, each one performed in triplicate. ***p ≤ 0.001 compared to control.

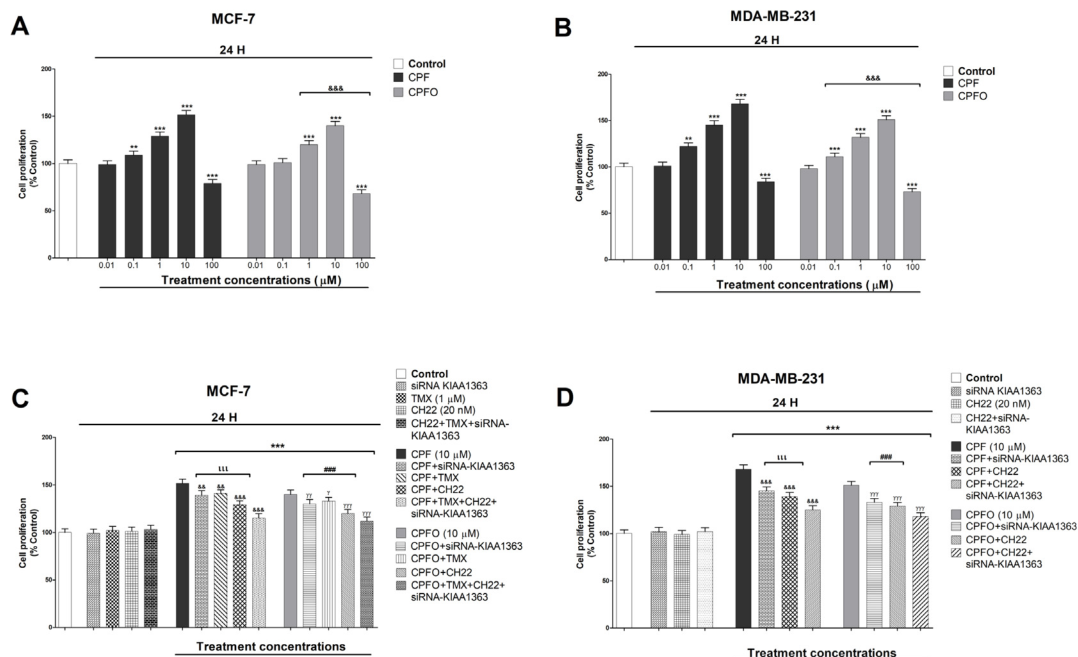


Fig. 7. CPF and CPFO (0.01 μM−100 μM) effect on cell proliferation in MCF-7 cells (A) and in MDA-MB-231 cells (B) after 24 h treatment. CPF and CPFO (10 μM) effect on cell proliferation in KIAA1363 silenced MCF-7 (C) and MBA-MB-231 cells (D) co-treated with or without TMX and with or without CH22 after 24 h treatment. Data represent the mean ± SEM of three separate experiments from cells of different cultures, each one performed in triplicate. Data are presented as a percentage relative to the control group and were significantly different from the respective control group. ***p ≤ 0.001 and **p ≤ 0.01 compared to control; $^{\&\&\&}$p ≤ 0.001 and $^{\&\&}$p ≤ 0.01 compared to CPF treatment; $^{\Upsilon\Upsilon\Upsilon}$p≤0.001, $^{\Upsilon\Upsilon}$p≤0.01 and $^{\Upsilon}$p≤0.05 compared to CPFO treatment; $^{\iota\iota\iota}$p≤0.001 compared to CPF co-treatment with CH22 and with or without TMX of KIAA1363 silenced cells. $^{\#\#\#}$p ≤ 0.001 compared to CPFO co-treatment with CH22 and with or without TMX of KIAA1363 silenced cells.

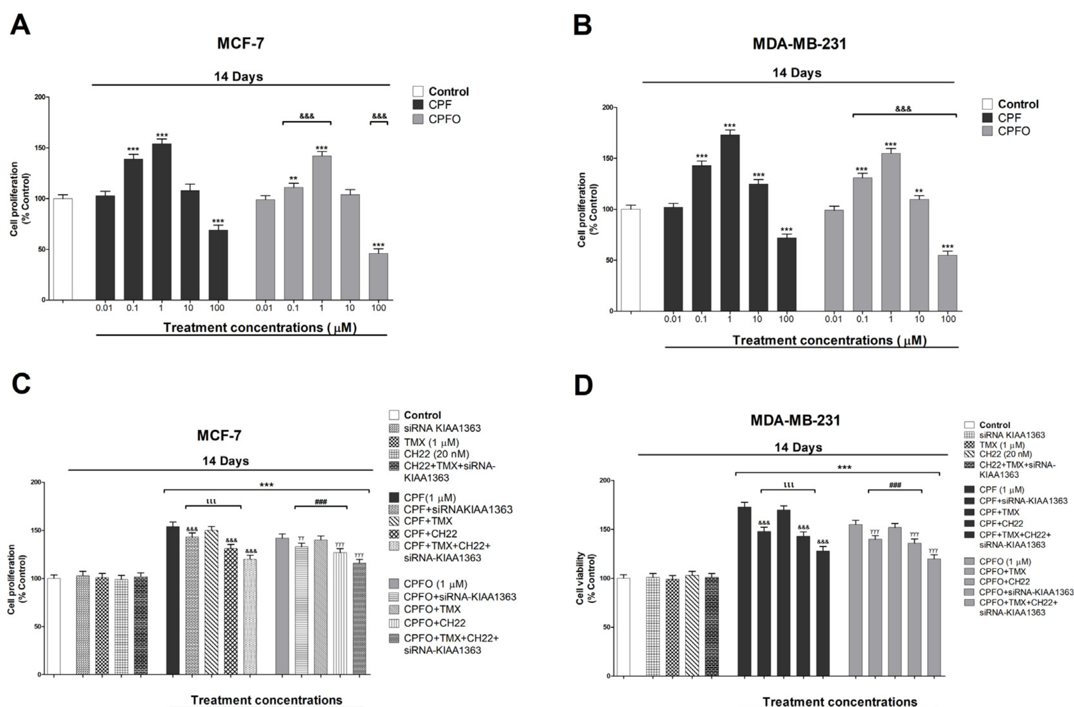


Fig. 8. CPF and CPFO (0.01 μM–100 μM) effect on cell proliferation in MCF-7 cells (A) and in MDA-MB-231 cells (B) after 14 days of treatment. CPF and CPFO (10 μM) effect on cell proliferation in KIAA1363 silenced MCF-7 (C) and MBA-MB-231 cells (D) co-treated with or without TMX and with or without CH22 after 14 days treatment. Data represent the mean ± SEM of three separate experiments from cells of different cultures, each one performed in triplicate. Data are presented as a percentage relative to the control group and were significantly different from the respective control group. $^{***}p \leq 0.001$ and $^{**}p \leq 0.01$ compared to control; $^{\&\&\&}p \leq 0.001$ and $^{\&\&}p \leq 0.01$ compared to CPF treatment; $^{\Upsilon\Upsilon\Upsilon}p \leq 0.001$, $^{\Upsilon\Upsilon}p \leq 0.01$ and $^{\Upsilon}p \leq 0.05$ compared to CPFO treatment; $^{\iota\iota\iota}p \leq 0.001$ compared to CPF co-treatment with CH22 and with or without TMX of KIAA1363 silenced cells. $^{\#\#\#}p \leq 0.001$ compared to CPFO co-treatment with CH22 and with or without TMX of KIAA1363 silenced cells.

with tamoxifen and CH22 of KIAA1363 silenced MCF-7 cells or with CH22 of KIAA1363 silenced MDA-MB-231 cells partially reversed the action on cell proliferation induced following CPF or CPFO single exposure, being this effect higher than that produced following single co-treatment with tamoxifen or CH22, or after CPF or CPFO treatment of KIAA1363 silenced MDA-MB-231 or MCF-7 cells (Fig. 7C and D). After 14 days exposure, CPF and CPFO (1 μM) effects on cell proliferation were only partially reversed after co-treatment with CH22 or after CPF and CPFO treatment of KIAA1363 silenced MCF-7 or MDA-MB-231 cells. (Fig. 7C and D, 8C and D). The reversion in the CPF and CPFO induced cell proliferation was higher in KIAAA1363 silenced MDA-MB231 cells than in KIAA1363 silenced MCF-7 cells (Fig. 7C and D, 8C and D). The cell proliferation induction shown with the MTT test was corroborated with the crystal violet staining assay.

4. Discussion

Our present results prove that CPF and CPFO induce, after 14 days repeated exposure (from 0.1 μM), a concentration-dependent AhR expression increase, in MCF-7 and MDA-MB-231 cell lines. Besides, following single exposure to CPF (starting at 100 μM) and CPFO (starting at 10 μM) and following 14 days repeated exposure to both compounds (starting at 0.1 μM), ERα expression was decreased in MCF-7 cells. CPF was shown to reduce ERα expression after repeated exposure (150 days) in rats (Ventura et al., 2019). Different studies showed the bi-directional inhibitory interaction between ERα and AhR, presenting antagonist effects. In this regard, AhR agonists have been described to induce ERα proteasome degradation, and AhR knockdown increased the expression of ERα (Madak-Erdogan and Katzenellenbogen, 2012; Ahmed et al., 2014). Nevertheless, AhR agonists were reported to reduce (Spink et al., 2003; Wormke et al., 2000) or not alter AhR expression in breast tumor cell lines (Vacher et al., 2018). Besides, the long-term activation of ERα through E2 exposure induces the overexpression of AhR in breast cancer cells (Wong et al., 2009). CPF is a strong AhR agonist and a weak ER agonist, so the activation of AhR could produce the decrease observed in the expression of ER, although it could not explain the AhR overexpression. In this regard, the activation of the ER receptor by CPF could contribute to the AhR overexpression observed both after single and repeated CPF and CPFO exposure. However, we cannot discard that CPF and CPFO could act directly in the expression of both receptors.

CPF and CPFO also induced a weak concentration-dependent ER activation, following single and 14 days exposure; which is supported by results of Andersen et al. (2002) showing CPF weak ER agonist effect in MCF-7 cells. CYP1A1A expression was also increased, in a concentration-dependent way, following single and repeated treatment to CPF and CPFO in both cell lines. In this sense, CPF was reported to induce CYP1A1 overexpression (Rudzok et al., 2009), supporting our results. Thus, CYP1A1 overexpression, as a marker of AhR activation (Barouki et al., 2007), indicates the agonist action of CPF and CPFO on AhR. In this sense, CPF was shown to present strong, concentration-dependent, AhR agonist effects *in vitro*, at concentrations above 1 μM (Long et al., 2003; Takeuchi et al., 2008), similarly to our results. Besides, our results show that after co-treatment with the AhR antagonist, the induction of CYP1A1 expression was completely reversed in both cell lines, indicating that the CPF and CPFO effect on CYP1A1 expression was mediated only through AhR activation, which confirms their AhR agonist action. Finally, CPF and CPFO decreased ER activation, following single and repeated exposure (from 100 μM) in MCF-7 cells, and CYP1A1 expression following single (from 100 μM) and repeated exposure (from 10 μM) in both cell lines. The reduction of

ER activation and CYP1A1 expression cannot be due to the decrease in the expression of ER or AhR since results were normalized with the total protein levels, indicating that other mechanisms could be involved. In this regard, CPF has been reported to produce reactive oxygen species (ROS) generation from 50 μM concentration (Ventura et al., 2012, 2015). ROS generation was reported to denaturalize proteins, altering their conformation and activity (Fawcett et al., 2002). Therefore, the ER and AhR activity decrease observed could respond to the denaturalization of some ER and AhR proteins from the total pool, induced by reactive oxygen species (ROS) generated after CPF and CPFO exposure. Further studies are necessary to corroborate this point.

Moreover, a concentration-dependent induction of KIAA1363 expression was observed, following single and repeated CPF and CPFO treatment of MCF-7 and MDA-MB-231 cell lines. After 14 days CPF or CPFO treatment, the increase observed in KIAA1363 expression was reduced from 1 μM concentration in both cell lines. The induction of KIAA1363 was less pronounced in MCF-7 than in MDA-MB-231 cells. This is the first report that describes the effect of CPF and CPFO on KIAA1363 expression after single and repeated treatment, being this action independent of ER and AhR receptors. Previously, KIAA1363 expression was described to be higher in MDA-MB-231 cells, showing lesser expression in the MCF-7 cell line (Jessani et al., 2002), which could explain the higher induction of KIAA1363 expression observed in MDA-MB-231 cells, following CPF and CPFO treatment.

Otherwise, CPF induced cell proliferation after 24 h exposure (0.1 μM−10 μM) in both cell lines. CPFO induced cell proliferation following single treatment in MCF-7 (1 μM−10 μM) and MDA-MB-231 cells (0.1 μM−10 μM). CPF and CPFO reduced cell proliferation starting at 100 μM concentrations following single and repeated treatment in both cell lines. In this sense, previous studies show that CPF induces cell proliferation in MCF-7 cells (Andersen et al., 2002), supporting our results. Conversely, CPF (0.01 μM−10 μM) was described not to induce cell proliferation after single exposure in MDA-MB-231 and MCF-7 cells (Rich et al., 2012), and induce cell proliferation after single treatment starting at 25 μM until 50 μM concentrations (Andersen et al., 2002), which does not agree with our results. Besides, CPF and CPFO induced cell proliferation after 14 days exposure (0.1 μM−1 μM) and started to reduce the increase of cell proliferation from 10 μM concentration, producing a decrease of cell viability from 100 μM concentration. CPF was also described to induce cell proliferation after 10 days of exposure from 0.05 μM concentration and reduce cell viability from 50 μM concentration in MCF-7 cells (Ventura et al., 2012), which is similar to our results. However, CPF (0.001 μM−10 μM) has also been described not to induce cell proliferation after 9 days exposure in MCF-7 cells (Vinggaard et al., 1999), or induce cell death from 0.05 μM concentration after 10 days exposure in MDA-MB-231 cells (Ventura et al., 2012). These differences could be due to the period of exposure time, the number of repeated treatments and the methods used to determine cell proliferation. Moreover, CPFO exposure induced a less pronounced cell proliferation and more cell viability decrease in both cell lines following single and repeated treatment. In this sense, CPFO has been reported to be more toxic in reducing cell viability than CPF (Suriyo et al., 2015), which is confirmed by our results. CPF was reported to induce cell death through induction of oxidative stress (Zhang et al., 2019) and the reduction of cell proliferation observed in our study could be due to the ROS generation as described previously in MCF-7 and MDA-MB-231 cells (Ventura et al., 2019). Cell proliferation results and cell viability decrease observed in our study were correlated with the increase or downregulation of MCM2 expression, respectively. In this sense, MCM2 participates in DNA replication initiation on the G1 phase, being highly expressed during cell division (Yousef et al., 2017), but downregulated in cell cycle arrest (Zhang et al., 2015), which supports our results.

CPF and CPFO effect on cell proliferation after 24 h treatment was partially reversed after treatment of KIAA1363 gene knockdown cells or after co-treatment with CH22 in both cell lines and after co-treatment with TMX in MCF-7 cells. After 14 days of exposure, cell proliferation effects were partially reversed after co-treatment with CH22 or after treatment of KIAA1363 silenced cells. These data confirm that the KIAA1363 enzyme and AhR participate in cell proliferation in both cell lines after a single and repeated treatment, and ER in MCF-7 cells after single treatment. In this sense, CPF was shown to produce cell proliferation in the MCF-7 cell line from 0.05 μM concentration mediated by the activation of the ERα (Ventura et al., 2012). The ER could induce cell proliferation through the activation of different signaling pathways including epidermal growth factor receptor (EGFR), phosphatidyl-inositol-3-kinase (PI3K)/AKT, G protein-coupled estrogen receptor 1 (GPER) or mitogen-activated protein kinase (MAPK) pathways, among others (Renoir et al., 2013). ERα expression was decreased after repeated CPF and CPFO treatment, which explains the lack of ER effect in the cell proliferation observed after 14 days of exposure.

AhR overexpression has been described to mediate the induction of cell division on MDA-MB-231 and MCF-7 cells and its silencing reduces tumor growth and metastasis (Salisbury et al., 2014). AhR agonists were also shown to promote cell division in breast cancer cells and rescue them from induced apoptosis after toxic insults (Al-Dhfyan et al., 2017; Bekki et al., 2015; Chu et al., 2014; Romagnolo et al., 2015, 2017), supporting our results. AhR could mediate cell proliferation through activation of different signaling pathways such as EGFR, transforming growth factor α (TGFα) or PI3K/AKT pathways, among others (Bekki et al., 2015). The activation of said pathways was suggested to be mediated through CYP1A1 induction, and its downregulation decreases their activation leading to cell death in breast cancer cells (Rodriguez and Potter, 2013). Besides, several AhR agonists that do not present toxicity, were shown to act as partial agonists of AhR, which block the promotion of cell division induced by AhR toxic agonist in a similar way as AhR antagonists do (Campbell et al., 2018). Conversely, multiple studies described that the exogenous AhR agonists induce dell death in estrogen-dependent and estrogen-independent breast cancer cells (Kolluri et al., 2017), which contradicts our results. A possible explanation in ERα-positive cells is that these compounds present antiestrogenic effects (Chen et al., 1998), but CPF and CPFO are ER agonist, which may be the cause of such differences due to the cross-talk between both receptors. Besides, we observed an AhR overexpression after CPF and CPFO treatment, which was not observed after treatment with AhR agonists that induce cell death. In this sense, AhR presents different variants in human cells (Celius and Matthews, 2010) and their differential overexpression could mediate the effect observed. Furthermore, AhR activation was not the main mechanism through which these compounds induced the apoptotic cell death and they continued to induce it after AhR silencing (Yoshioka et al., 2012). Finally, both antagonist and agonist of AhR were reported to present antitumoral actions in human breast cancer cells, suggesting a very complex regulation of AhR signaling pathway, which may depend on multiple factors (Narasimhan et al., 2018). According to all the above, probably the final effect of CPF or CPFO on AhR depends upon specific regulators that may be different from those of AhR agonists that induce cell death, leading to cell proliferation.

Ultimately, KIAA1363 is overexpressed in breast cancer, as well as other several cancers, and was related to cell proliferation and invasiveness mediated through alterations in lipid network (Chiang et al., 2006). KIAA1363 knockdown decreased tumor growth and cancer cell migration (Chiang et al., 2006), all these data support

that the KI11363 upregulation observed mediated the cell proliferation produced after acute and repeated exposure to CPF or CPFO. Besides, CPF was reported to alter lipid profile in prostate cancer cells, inducing the metastatic phenotype (Bedia et al., 2015). Thus, KIAA1363 could induce the observed effect through alteration of the lipid profile. The specific lipids that could mediate KIAA1363 effect on breast cancer cells' proliferation are unknown. However, the alkyl lysophosphatidic acid (LPA), a KIAA1363 metabolite network compound, was associated with breast cancer promotion (Panupinthu et al., 2010). LPA was reported to activate related G protein-coupled receptors (GPCRs) (Mills and Moolenaar, 2003) and mediate the induction of cell proliferation and migration through different pathways such as ER, MAPK or PI3K/AKT (Panupinthu et al., 2010). Otherwise, KIAA1363 was also described as the most important enzyme to degrade CPFO (Nomura et al., 2005, 2006). CPF and CPFO inhibit this enzyme, which could difficult its action on tumor promotion. However, KIAA1363 quickly spontaneously reactivates following phosphorylation (Nomura et al., 2005, 2006; Ross et al., 2016), so it could be overexpressed by these compounds and inhibited for a short period and then reactivated and mediate the observed effect. The reversion of cell proliferation induced by CPF and CPFO was higher after treatment of KIAAA1363 silenced MDA-MB231 cells than in KIAA1363 silenced MCF-7 cells. KIAA1363 was described to be less expressed in MCF-7 than in MDA-MB-231 cells (Jessani et al., 2002), and we show that CPF or CPFO induced a higher overexpression of this enzyme in MDA-MB-231 cells, which could explain the higher action in this cell line together with the AhR activation.

CPF or CPFO simultaneous co-treatment with tamoxifen and CH22 of KIAA1363 silenced MDA-MB-231 or MCF-7 cells lessened, in part, the effect on cell proliferation produced after CPF or CPFO single exposure, being this abatement higher than that observed after single co-treatment with tamoxifen or CH22, or after treatment of KIAA1363 silenced MDA-MB-231 or MCF-7 cells. Our data suggest another mechanism could be involved. In this sense, paraoxonase as well as KIAA1363 contributes to detoxifying CPFO and also participates in the regulation of lipid metabolism (Harel et al., 2004; Costa et al., 2006), which may also participate in the observed action. Heat shock proteins (HSPs) have been related to the etiology of breast cancer (Dong et al., 2016). HSP overexpression has been reported to induce cell proliferation (Ciocca et al., 1993; Lazaris et al., 1997; Vargas-Roig et al., 1998). CPF induces different HSPs (Reyna et al., 2017; Sachana et al., 2001), so this mechanism may also participate in the cell proliferation described.

Our present results prove that, CPF and CPFO, following single and repeated treatment, alter differently the expression of KIAA1363, AhR, ER and CYP1A1 and induce cell proliferation through ER activation after a single exposure in MCF-7 cells, and through an increase of KIAA1363 expression and AhR activation in MCF-7 and MDA-MB-231 cells following single and 14 days treatment. These results may assist in explaining the possible role of CPF as a factor in the induction of breast cancer. Further research should be taken on in order to elucidate the remaining mechanisms involved with the effect described, the percentage of CPF metabolized to CPFO in breast cancer cells and whether these mechanisms mediate cell proliferation *in vivo*. The presented data are valuable as they supply novel enlightenment on the mechanisms that produce cytotoxicity and cell proliferation induced following CPF and CPFO exposure that should be considered in this biocide's risk assessment.

Declaration of competing interest

There are no conflicts of interest to declare.

CRediT authorship contribution statement

Paula Moyano: Methodology, Investigation, Writing - original draft, Writing - review & editing. **Jimena García:** Investigation, Validation. **José Manuel García:** Validation, Writing - review & editing. **Adela Pelayo:** Resources, Supervision. **Pilar Muñoz-Calero:** Funding acquisition, Visualization. **María Teresa Frejo:** Formal analysis, Writing - review & editing. **Maria Jose Anadon:** Data curation, Writing - review & editing. **Margarita Lobo:** Formal analysis, Data curation. **Javier Del Pino:** Conceptualization, Methodology, Project administration, Supervision, Writing - review & editing.

Acknowledgments

This work was supported by research grants 172C126PMA from Alborada Foundation/Catedra Extradordinaria de Patologia y Medioambiente, UCM.

References

Al-Dhfyan, A., Alhoshani, A., Korashy, H.M., 2017. Aryl hydrocarbon receptor/cytochrome P450 1A1 pathway mediates breast cancer stem cells expansion through PTEN inhibition and β-Catenin and Akt activation. Mol. Canc. 16 (1), 14.
Ahmed, S., Wang, A., Celius, T., Matthews, J., 2014. Zinc finger nuclease-mediated knockout of AHR or ARNT in human breast cancer cells abolishes basal and ligand-dependent regulation of CYP1B1 and differentially affects estrogen receptor α transactivation. Toxicol. Sci. 138 (1), 89–103.
Andersen, H.R., Vinggaard, A.M., Rasmussen, T.H., Gjermandsen, I.M., Bonefeld-Jørgensen, E.C., 2002. Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity in vitro. Toxicol. Appl. Pharmacol. 179 (1), 1–12.
Barouki, R., Coumoul, X., Fernandez-Salguero, P.M., 2007. The aryl hydrocarbon receptor, more than a xenobiotic-interacting protein. FEBS Lett. 581, 3608–3615.
Bedia, C., Dalmau, N., Jaumot, J., Tauler, R., 2015. Phenotypic malignant changes and untargeted lipidomic analysis of long-term exposed prostate cancer cells to endocrine disruptors. Environ. Res. 140, 18–31.
Bekki, K., Vogel, H., Li, W., Ito, T., Sweeney, C., Haarmann-Stemmann, T., Matsumura, F., Vogel, C.F., 2015. The aryl hydrocarbon receptor (AhR) mediates resistance to apoptosis induced in breast cancer cells. Pestic. Biochem. Physiol. 120, 5–13.
Campbell, P.S., Mavingire, N., Khan, S., Rowland, L.K., Wooten, J.V., Opoku-Agyeman, A., Guevara, A., Soto, U., Cavalli, F., Loaiza-Pérez, A.I., Nagaraj, G., Denham, L.J., Adeoye, O., Jenkins, B.D., Davis, M.B., Schiff, R., Brantley, E.J., 2018. AhR ligand aminoflavone suppresses α6-integrin-Src-Akt signaling to attenuate tamoxifen resistance in breast cancer cells. J. Cell. Physiol. 234 (1), 108–121.
Celius, T., Matthews, J., 2010. Functional analysis of six human aryl hydrocarbon receptor variants in human breast cancer and mouse hepatoma cell lines. Toxicology 277 (1–3), 59–65.
Chu, X.T., De la Cruz, J., Hwang, S.G., Hong, H., 2014. Tumorigenic effects of endocrine-disrupting chemicals are alleviated by licorice (Glycyrrhiza glabra) root extract through suppression of AhR expression in mammalian cells. Asian Pac. J. Cancer Prev. APJCP 15 (12), 4809–4813.
Ciocca, D.R., Clark, G.M., Tandon, A.K., Fuqua, S.A., Welch, W.J., McGuire, W.L., 1993. Heat shock protein hsp 70 in patients with axillary lymph node-negative breast cancer: prognostic implications. J. Natl. Cancer Inst. 85, 570–574.
Chen, I., McDougal, A., Wang, F., Safe, S., 1998. Aryl hydrocarbon receptor-mediated antiestrogenic and antitumorigenic activity of diindolylmethane. Carcinogenesis 19 (9), 1631–1639.
Chiang, K.P., Niessen, S., Saghatelian, A., Cravatt, B.F., 2006. An enzyme that regulates ether lipid signaling pathways in cancer annotated by multidimensional profiling. Chem. Biol. 13 (10), 1041–1050.
Costa, L.G., Cole, T.B., Vitalone, A., Furlong, C.E., 2006. Paraoxonase Polymorphisms and Toxicity of organo phosphates. In: Gupta, R.C. (Ed.), Toxicology of Organophosphate and Carbamate Compounds. Elsevier Academic Press, London, pp. 247–255.
D'Amato, N.C., Rogers, T.J., Gordon, M.A., Greene, L.I., Cochrane, D.R., Spoelstra, N.S., Nemkov, T.G., D'Alessandro, A., Hansen, K.C., Richer, J.K., 2015. A TDO2-AhR signaling axis facilitates anoikis resistance and metastasis in triple-negative breast cancer. Cancer. Res. 75 (21), 4651–4664.
Dong, H., Zou, M., Bhatia, A., Jayaprakash, P., Hofman, F., Ying, Q., Chen, M., Woodley, D.T., Li, W., 2016. Breast cancer MDA-MB-231 cells use secreted heat shock protein-90 alpha (Hsp90α) to survive a hostile hypoxic environment. Sci. Rep. 6, 20605.
Del Pino, J., Zeballos, G., Anadón, M.J., Moyano, P., Díaz, M.J., García, J.M., Frejo, M.T., 2016. Cadmium-induced cell death of basal forebrain cholinergic neurons mediated by muscarinic M1 receptor blockade, increase in GSK-3β enzyme, β-

amyloid and tau protein levels. Arch. Toxicol. 90 (5), 1081–1092.
Eaton, D.L., Daroff, R.B., Autrup, H., Bridges, J., Buffler, P., Costa, L.G., Coyle, J., McKhann, G., Mobley, W.C., Nadel, L., Neubert, D., Schulte-Hermann, R., Spencer, P.S., 2008. Review of the toxicology of chlorpyrifos with an emphasis on human exposure and neurodevelopment. Crit. Rev. Toxicol. 38 (Suppl. 2), 1–125.
Engel, L.S., Werder, E., Satagopan, J., Blair, A., Hoppin, J.A., Koutros, S., Lerro, C.C., Sandler, D.P., Alavanja, M.C., Beane, Freeman, L.E., 2017. Insecticide use and breast cancer risk among farmers' wives in the agricultural health study. Environ. Health Perspect. 125 (9), 097002.
Fawcett, J.R., Bordayo, E.Z., Jackson, K., Liu, H., Peterson, J., Svitak, A., Frey, W.H., 2002. Inactivation of the human brain muscarinic acetylcholine receptor by oxidative damage catalyzed by a low molecular weight endogenous inhibitor from Alzheimer's brain is prevented by pyrophosphate analogs, bioflavonoids and other antioxidants. Brain Res. 950 (1–2), 10–20.
Harel, M., Aharoni, A., Gaidukov, L., Brumshtein, B., Khersonsky, O., Meged, R., Dvir, H., Ravelli, R.B., McCarthy, A., Toker, L., Silman, I., Sussman, J.L., Tawfik, D.S., 2004. Structure and evolution of the serum paraoxonase family of detoxifying and anti-atherosclerotic enzymes. Nat. Struct. Mol. Biol. 11 (5), 412–419.
Jessani, N., Liu, Y., Humphrey, M., Cravatt, B.F., 2002. Enzyme activity profiles of the secreted and membrane proteome that depict cancer cell invasiveness. Proc. Natl. Acad. Sci. U.S.A. 99 (16), 10335–10340.
Khokhar, J.Y., Tyndale, R.F., 2012. Rat brain CYP2B-enzymatic activation of chlorpyrifos to the oxon mediates cholinergic neurotoxicity. Toxicol. Sci. 126 (2), 325–335.
Khokhar, J.Y., Tyndale, R.F., 2014. Intracerebroventricularly and systemically delivered inhibitor of brain CYP2B (C8-Xanthate), even following chlorpyrifos exposure, reduces chlorpyrifos activation and toxicity in male rats. Toxicol. Sci. 140 (1), 49–60.
Kolluri, S.K., Jin, U.H., Safe, S., 2017. Role of the aryl hydrocarbon receptor in carcinogenesis and potential as an anti-cancer drug target. Arch. Toxicol. 91 (7), 2497–2513.
Lazaris, A.C.H., Chatzigianni, E.B., Panoussopoulos, D., Tzimas, G.N., Davaris, P.S., Golematis, B.C.H., 1997. Proliferating cell nuclear antigen and heat shock protein 70 immunolocalization in invasive ductal breast cancer not otherwise specified. Breast Canc. Res. Treat. 43 (1), 43–51.
Lee, W.J., Blair, A., Hoppin, J.A., Lubin, J.H., Rusiecki, J.A., Sandler, D.P., Dosemeci, M., Alavanja, M.C., 2004. Cancer incidence among pesticide applicators exposed to chlorpyrifos in the Agricultural Health Study. J. Natl. Cancer Inst. 96, 1781–1789.
Lee, W.J., Alavanja, M.C., Hoppin, J.A., Rusiecki, J.A., Kamel, F., Blair, A., Sandler, D.P., 2007. Mortality among pesticide applicators exposed to chlorpyrifos in the Agricultural Health Study. Environ. Health Perspect. 115, 528–534.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta DeltaC(T)) Method. Methods 25, 402–408.
Lo, R., Burgoon, L., Macpherson, L., Ahmed, S., Matthews, J., 2010. Estrogen receptor-dependent regulation of CYP2B6 in human breast cancer cells. Biochim. Biophys. Acta 1799 (5–6), 469–479.
Long, M., Laier, P., Vinggaard, A.M., Andersen, H.R., Lynggaard, J., Bonefeld-Jørgensen, E.C., 2003. Effects of currently used pesticides in the AhR-CALUX assay: comparison between the human TV101L and the rat H4IIE cell line. Toxicology 194 (1–2), 77–93.
Madak-Erdogan, Z., Katzenellenbogen, B.S., 2012. Aryl hydrocarbon receptor modulation of estrogen receptor α-mediated gene regulation by a multimeric chromatin complex involving the two receptors and the coregulator RIP140. Toxicol. Sci. 125 (2), 401–411.
Maiorano, D., Lutzmann, M., Méchali, M., 2006. MCM proteins and DNA replication. Curr. Opin. Cell Biol. 18 (2), 130–136.
Marty, M.S., Andrus, A.K., Bell, M.P., Passage, J.K., Perala, A.W., Brzak, K.A., Bartels, M.J., Beck, M.J., Juberg, D.R., 2012. Cholinesterase inhibition and toxicokinetics in immature and adult rats after acute or repeated exposures to chlorpyrifos or chlorpyrifos-oxon. Regul. Toxicol. Pharmacol. 63 (2), 209–224.
Mills, G.B., Moolenaar, W.H., 2003. The emerging role of lysophosphatidic acid in cancer. Nat. Rev. Canc. 3, 582–591.
Mitra, R., Guo, Z., Milani, M., Mesaros, C., Rodriguez, M., Nguyen, J., Luo, X., Clarke, D., Lamba, J., Schuetz, E., Donner, D.B., Puli, N., Falck, J.R., Capdevila, J., Gupta, K., Blair, I.A., Potter, D.A., 2011. CYP3A4 mediates growth of estrogen receptor-positive breast cancer cells in part by inducing nuclear translocation of phospho-stat 3 through biosynthesis of (±)-14,15-Epoxyeicosatrienoic acid (EET). J. Biol. Chem. 286 (20), 17543–17559.
Narasimhan, S., Stanford, Z.E., Novikov, O., Parks, A.J., Schlezinger, J.J., Wang, Z., Laroche, F., Feng, H., Mulas, F., Monti, S., Sherr, D.H., 2018. Towards resolving the pro- and anti-tumor effects of the aryl hydrocarbon receptor. Int. J. Mol. Sci. 19 (5), E1388 pii.
Nishi, K., Hundal, S.S., 2013. Chlorpyrifos induced toxicity in reproductive organs of female Wistar rats. Food Chem. Toxicol. 62, 732–738.
Nomura, D.K., Leung, D., Chiang, K.P., Quistad, G.B., Cravatt, B.F., Casida, J.E., 2005. A brain detoxifying enzyme for organophosphorus nerve poisons. Proc. Natl. Acad. Sci. U.S.A. 102 (17), 6195–6200.
Nomura, D.K., Durkin, K.A., Chiang, K.P., Quistad, G.B., Cravatt, B.F., Casida, J.E., 2006. Serine hydrolase KIAA1363: toxicological and structural features with emphasis on organophosphate interactions. Chem. Res. Toxicol. 19 (9), 1142–1150.
Panupinthu, N., Lee, H.Y., Mills, G.B., 2010. Lysophosphatidic acid production and action: critical new players in breast cancer initiation and progression. Br. J. Canc. 102 (6), 941–946.
Renoir, J.M., Marsaud, V., Lazennec, G., 2013. Estrogen receptor signaling as a target for novel breast cancer therapeutics. Biochem. Pharmacol. 85 (4), 449–465.
Reyna, L., Flores-Martín, J., Ridano, M.E., Panzetta-Dutari, G.M., Genti-Raimondi, S., 2017. Chlorpyrifos induces endoplasmic reticulum stress in JEG-3 cells. Toxicol. In. Vitro 40, 88–93.
Rich, J.D., Gabriel, S.M., Schultz-Norton, J.R., 2012. In vitro effects of herbicides and insecticides on human breast cells. ISRN. Toxicol 2012, 232461.
Richardson, J.R., Chambers, J.E., 2005. Effects of repeated oral postnatal exposure to chlorpyrifos on cholinergic neurochemistry in developing rats. Toxicol. Sci. 84, 352–359.
Rodriguez, M., Potter, D.A., 2013. CYP1A1 regulates breast cancer proliferation and survival. Mol. Canc. Res. 11 (7), 780–792.
Romagnolo, D.F., Papoutsis, A.J., Laukaitis, C., Selmin, O.I., 2015. Constitutive expression of AhR and BRCA-1 promoter CpG hypermethylation as biomarkers of ERα-negative breast tumorigenesis. BMC Canc. 15, 1026.
Romagnolo, D.F., Donovan, M.G., Papoutsis, A.J., Doetschman, T.C., Selmin, O.I., 2017. Genistein prevents BRCA1 CpG methylation and proliferation in human breast cancer cells with activated aromatic hydrocarbon receptor. Curr. Dev. Nutr. 1 (6), e000562.
Ross, M.K., Pluta, K., Bittles, V., Borazjani, A., Allen, C.J., 2016. Interaction of the serine hydrolase KIAA1363 with organophosphorus agents: evaluation of potency and kinetics. Arch. Biochem. Biophys. 590, 72–81.
Rudzok, S., Schmücking, E., Graebsch, C., Herbarth, O., Bauer, M., 2009. The inducibility of human cytochrome P450 1A by environmental-relevant xenobiotics in the human hepatoma derived cell line HepG2. Environ. Toxicol. Pharmacol. 28, 370–378.
Sachana, M., Flaskos, J., Alexaki, E., Glynn, P., Hargreaves, A.J., 2001. The toxicity of chlorpyrifos towards differentiating mouse N2a neuroblastoma cells. Toxicol. In. Vitro 15 (4–5), 369–372.
Salisbury, T.B., Tomblin, J.K., Primerano, D.A., Boskovic, G., Fan, J., Mehmi, I., Fletcher, J., Santanam, N., Hurn, E., Morris, G.Z., Denvir, J., 2014. Endogenous aryl hydrocarbon receptor promotes basal and inducible expression of tumor necrosis factor target genes in MCF-7 cancer cells. Biochem. Pharmacol. 91 (3), 390–399.
Spink, D.C., Katz, B.H., Hussain, M.M., Pentecost, B.T., Cao, Z., Spink, B.C., 2003. Estrogen regulates Ah responsiveness in MCF-7 breast cancer cells. Carcinogenesis 24 (12), 1941–1950.
Suriyo, T., Tachachartvanich, P., Visitnonthachai, D., Watcharasit, P., Satayavivad, J., 2015. Chlorpyrifos promotes colorectal adenocarcinoma H508 cell growth through the activation of EGFR/ERK1/2 signaling pathway but not cholinergic pathway. Toxicology 338, 117–129.
Takeuchi, S., Iida, M., Yabushita, H., Matsuda, T., Kojima, H., 2008. In vitro screening for aryl hydrocarbon receptor agonistic activity in 200 pesticides using a highly sensitive reporter cell line, DR-EcoScreen cells, and in vivo mouse liver cytochrome P450-1A induction by propanil, diuron and linuron. Chemosphere 74 (1), 155–165.
Terry Jr., A.V., Stone, J.D., Buccafusco, J.J., Sickles, D.W., Sood, A., Prendergast, M.A., 2003. Repeated exposures to subthreshold doses of chlorpyrifos in rats: hippocampal damage, impaired axonal transport, and deficits in spatial learning. J. Pharmacol. Exp. Therapeut. 305, 375–384.
Vacher, S., Castagnet, P., Chemlali, W., Lallemand, F., Meseure, D., Pocard, M., Bieche, I., Perrot-Applanat, M., 2018. High AHR expression in breast tumors correlates with expression of genes from several signaling pathways namely inflammation and endogenous tryptophan metabolism. PloS One 13 (1), e0190619.
Vargas-Roig, L.M., Gago, F.E., Tello, O., Aznar, J.C., Ciocca, D.R., 1998. Heat shock protein expression and drug resistance in breast cancer patients treated with induction chemotherapy. Int. J. Canc. 79 (5), 468–475.
Ventura, C., Núñez, M., Miret, N., Martinel, L.D., Randi, A., Venturino, A., Rivera, E., Cocca, C., 2012. Differential mechanisms of action are involved in chlorpyrifos effects in estrogen-dependent or -independent breast cancer cells exposed to low or high concentrations of the pesticide. Toxicol. Lett. 213 (2), 184–193.
Ventura, C., Venturino, A., Miret, N., Randi, A., Rivera, E., Núñez, M., Cocca, C., 2015. Chlorpyrifos inhibits cell proliferation through ERK1/2 phosphorylation in breast cancer cell lines. Chemosphere 120, 343–350.
Ventura, C., Nieto, M.R., Bourguignon, N., Lux-Lantos, V., Rodriguez, H., Cao, G., Randi, A., Cocca, C., Núñez, M., 2016. Pesticide chlorpyrifos acts as an endocrine disruptor in adult rats causing changes in mammary gland and hormonal balance. J. Steroid Biochem. Mol. Biol. 156, 1–9.
Ventura, C., Zappia, C.D., Lasagna, M., Pavicic, W., Richard, S., Bolzan, A.D., Monczor, F., Núñez, M., Cocca, C., 2019. Effects of the pesticide chlorpyrifos on breast cancer disease. Implication of epigenetic mechanisms. J. Steroid Biochem. Mol. Biol. 186, 96–104.
Vinggaard, A.M., Breinholt, V., Larsen, J.C., 1999. Screening of selected pesticides for estrogen receptor activation in vitro. Food Addit. Contam. 16 (12), 533–542.
Weichenthal, S., Moase, C., Chan, P., 2010. A review of pesticide exposure and cancer incidence in the Agricultural Health Study cohort. Environ. Health Perspect. 118 (8), 1117–1125.
Weissenstein, U., Kunz, M., Oufir, M., Wang, J.T., Hamburger, M., Urech, K., Regueiro, U., Baumgartner, S., 2019. Absence of herb-drug interactions of mistletoe with the tamoxifen metabolite (E/Z)-endoxifen and cytochrome P450 3A4/5 and 2D6 in vitro. BMC Compl. Alternative Med. 19, 23.
Wong, P.S., Li, W., Vogel, C.F., Matsumura, F., 2009. Characterization of MCF mammary epithelial cells overexpressing the Aryl hydrocarbon receptor (AhR). BMC Canc. 9, 234.

Wormke, M., Stoner, M., Saville, B., Safe, S., 2000. Crosstalk between estrogen receptor alpha and the aryl hydrocarbon receptor in breast cancer cells involves unidirectional activation of proteasomes. FEBS Lett. 478 (1–2), 109–112.

Yoshioka, H., Hiromori, Y., Aoki, A., Kimura, T., Fujii-Kuriyama, Y., Nagase, H., Nakanishi, T., 2012. Possible aryl hydrocarbon receptor-independent pathway of 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced antiproliferative response in human breast cancer cells. Toxicol. Lett. 211 (3), 257–265.

Yousef, E.M., Furrer, D., Laperriere, D.L., Tahir, M.R., Mader, S., Diorio, C., Gaboury, L.A., 2017. MCM2: an alternative to Ki-67 for measuring breast cancer cell proliferation. Mod. Pathol. 30 (5), 682–697.

Zhang, X., Teng, Y., Yang, F., Wang, M., Hong, X., Ye, L.G., Gao, Y.N., Chen, G.Y., 2015. MCM2 is a therapeutic target of lovastatin in human non-small cell lung carcinomas. Oncol. Rep. 33 (5), 2599–2605.

Zhang, Q., Zheng, S., Wang, S., Wang, W., Xing, H., Xu, S., 2019. Chlorpyrifos induced oxidative stress to promote apoptosis and autophagy through the regulation of miR-19a-AMPK axis in common carp. Fish Shellfish Immunol. 93, 1093–1099.