comportamiento diferencial de CONTAMINANTES QUÍMICOS en cáncer de mama EN LAS ISLAS CANARIAS

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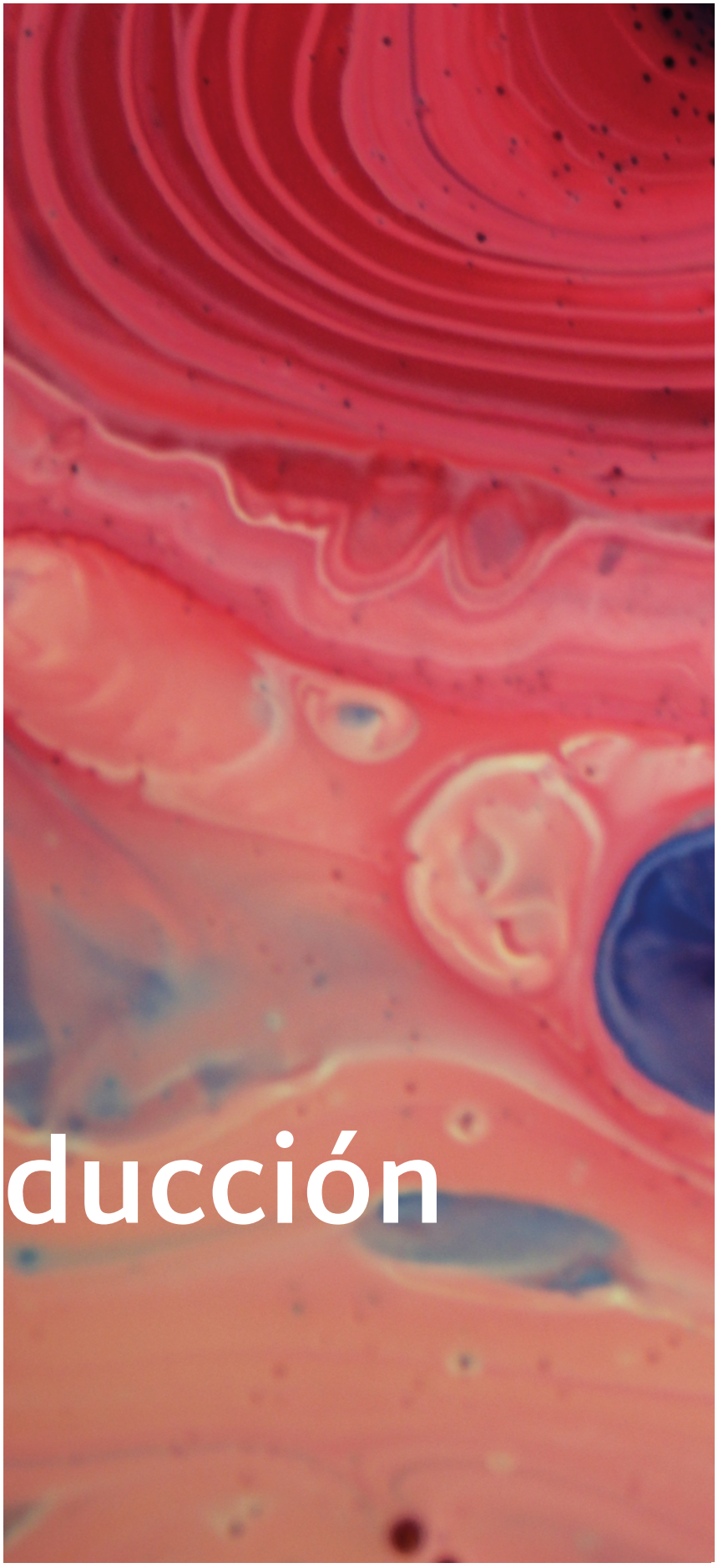
Contenido



Introducción

El cáncer de mama es el cáncer más diagnosticado en mujeres alrededor del mundo. Además, es la mayor causa de mortalidad de cáncer femenino en la Unión Europea y el de mayor malignidad en la mayoría de los países industrializados. Así, mientras la mortalidad y la incidencia del cáncer de mama en la Península Ibérica son más bajas que en otros países europeos, los ratios de mortalidad en las Islas Canarias y, concretamente, en Gran Canaria, han sido alarmantes en los últimos años.

La etiología del cáncer de mama es compleja, a la cual se asocian factores genéticos, epigenéticos y ambientales. De estos, los que más contribuyen a la aparición del mismo están relacionados con altos niveles de estrógenos en plasma y con altos niveles de expresión de los receptores de estrógenos en el tejido mamario. No obstante, también se han propuesto otros factores de riesgo ambientales para el cáncer de mama durante las últimas décadas como el tabaco, el consumo de alcohol, la obesidad, la dieta y la exposición a contaminantes ambientales.

Por esto, y dado el papel de los estrógenos en la patogénesis del cáncer de mama, se ha planteado que la exposición a xenobióticos con propiedades estrogénicas, también llamados xenoestrógenos, podrían explicar el incremento en la incidencia de cáncer de mama de las últimas décadas en los países industrializados. En este aspecto, algunos estudios in vitro han revelado que la exposición a xenoestrógenos puede modular el control del ciclo celular y otros parámetros moleculares regulados por estrógenos en células de cáncer de mama hormono-dependiente y en células epiteliales de mama normales.

De esta forma, se ha asociado la exposición a compuestos organohalogenados, especialmente a pesticidas organoclorados (OCs), como factor etiológico en el cáncer de mama debido a sus propiedades como disruptores endocrinos (son considerados xenoestrógenos) y a sus características químicas (alta lipofilia y resistencia a la biotransformación), las cuales favorecen su acumulación en el medio ambiente, alimentación, biota y seres humanos alrededor del mundo.

Los OCs son químicos sintéticos que se han utilizado ampliamente en todo el mundo por sus aplicaciones en la industria química y en agricultura. Estos se clasifican en tres grupos diferenciados: los derivados del etano, como el diclorodifeniltricloroetano (DDT) y sus metabolitos, el diclorodifenildicloroetileno (DDE) y el diclorodifenildicloroetano (DDD); los ciclodienos, como el clordano, aldrín, dieldrín, heptaclor, endrín y toxafeno; y los hexaclorociclohexanos, como el lindano.

A este respecto, y a pesar de que la mayoría de los pesticidas OCs fueron prohibidos en la década de los 70 en los países desarrollados, una gran parte de la población occidental presenta niveles basales de contaminación por estos compuestos.

No obstante, contrariamente a las asociaciones teóricas establecidas entre el cáncer de mama y los pesticidas OCs, existen controversias en los estudios epidemiológicos que se han realizado en las últimas décadas. Estas diferencias en las asociaciones positivas y negativas que se han descrito podrían ser debidas a que la mayoría de los estudios están enfocados al análisis de compuestos aislados, mientras que la población está expuesta a numerosas combinaciones de OCs, las cuales podrían presentar efectos agonistas, sinérgicos o aditivos.

Otro de los factores que podrían explicar la ausencia de relación entre el cáncer de mama y la exposición a OCs es que la mayoría de los estudios no considera que algunos xenoestrógenos puedan interactuar también con otros receptores de hormonas esteroideas como el receptor de andrógenos, lo cual les permite mostrar efectos androgénicos o anti-androgénicos, constituyendo de esta forma otra clase de disruptores endocrinos que han recibido gran interés en los últimos años.

Así, el Grupo de Investigación en Medio Ambiente y Salud (GIMAS) de la Universidad de Las Palmas de Gran Canaria (ULPGC) ha estudiado extensivamente el patrón de contaminación por OCs de la población de las Islas Canarias, mostrando altos niveles de exposición de la misma a varios de estos compuestos (Luzardo y col., 2006; Luzardo y col., 2009; Zumbado y col., 2005). Además, se han estudiado los efectos de determinados OCs, individualmente y en combinación, en células epiteliales de mama humanas (HMEC), a las concentraciones encontradas previamente en humanos, demostrando que los efectos biológicos producidos variaban claramente entre los OCs individuales y las diferentes combinaciones de OCs del ensayo (Valerón y col., 2009).

Esta tesis doctoral surge como consecuencia de un estudio publicado por el GIMAS, en el cual se analizaron los perfiles de pesticidas OCs presentes en suero sanguíneo en pacientes con cáncer de mama y en mujeres sanas, determinando que las concentraciones séricas de estos compuestos eran mayores en pacientes con cáncer de mama (Boada y col., 2012). Estos hallazgos sugirieron que ciertas combinaciones de OCs podrían haber jugado un papel importante como factor de riesgo en la aparición del cáncer de mama, lo que impulsó el desarrollo del presente trabajo con el objetivo de profundizar en el conocimiento de los mecanismos subyacentes que conducen a la transformación celular.

En la *Figura 1* se muestra la relación de pesticidas OCs estudiados en el desarrollo de la tesis, así como las concentraciones de cada uno de ellos en las mezclas de ensayo, las cuales se corresponden con las encontradas en el estudio previo, mencionado anteriormente, y representan la concentración 1X de cada una de las mezclas.

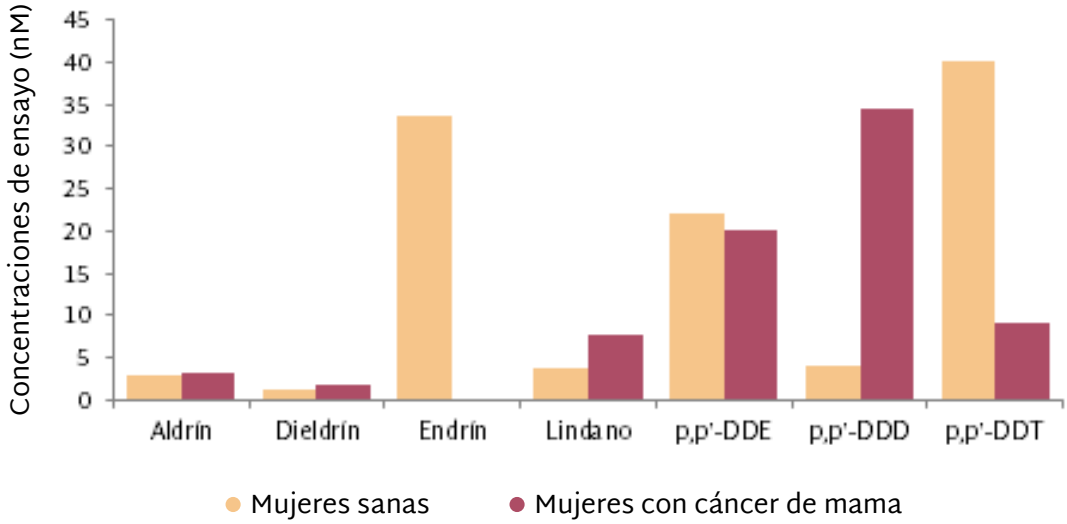


Figura 1. Concentraciones finales de ensayo (nM) de los pesticidas OCs en las mezclas de mujeres sanas y mujeres con cáncer de mama. Estas se corresponden con la concentración 1X establecida en cada estudio para cada una de las mezclas.

De este modo, se propusieron diferentes líneas de trabajo para evaluar los efectos de estas mezclas de OCs. Por un lado, se analizó el potencial estrogénico y androgénico de las mezclas de OCs a diferentes dosis en líneas celulares tumorales de mama. Además, se estudiaron los patrones de expresión génica, en células epiteliales normales de mama tratadas con las diferentes mezclas de OCs, de una amplia familia de proteínas, las proteína-quinasas, las cuales incluyen receptores de factor de crecimiento, reguladores del ciclo celular y oncoproteínas. Por último, y una vez obtenidos los resultados anteriores, se valoró el uso de la Simvastatina como modulador de la expresión de estos genes presuntamente implicados en la transformación celular.

Con este trabajo se pretende apoyar las teorías preliminares sobre el papel que juegan los pesticidas OCs como factor de riesgo en la aparición del cáncer de mama, así como dar respuesta a cómo estos compuestos, y sus múltiples combinaciones, son capaces de producir una respuesta celular diferencial, induciendo su transformación y dando lugar a un escenario que favorece el desarrollo de la enfermedad.

Objetivos

OBJETIVO 1

Evaluar in vitro los efectos citotóxicos/proliferativos ejercidos por las mezclas de OCs encontradas en pacientes con cáncer de mama y en pacientes sanas.

OBJETIVO 2

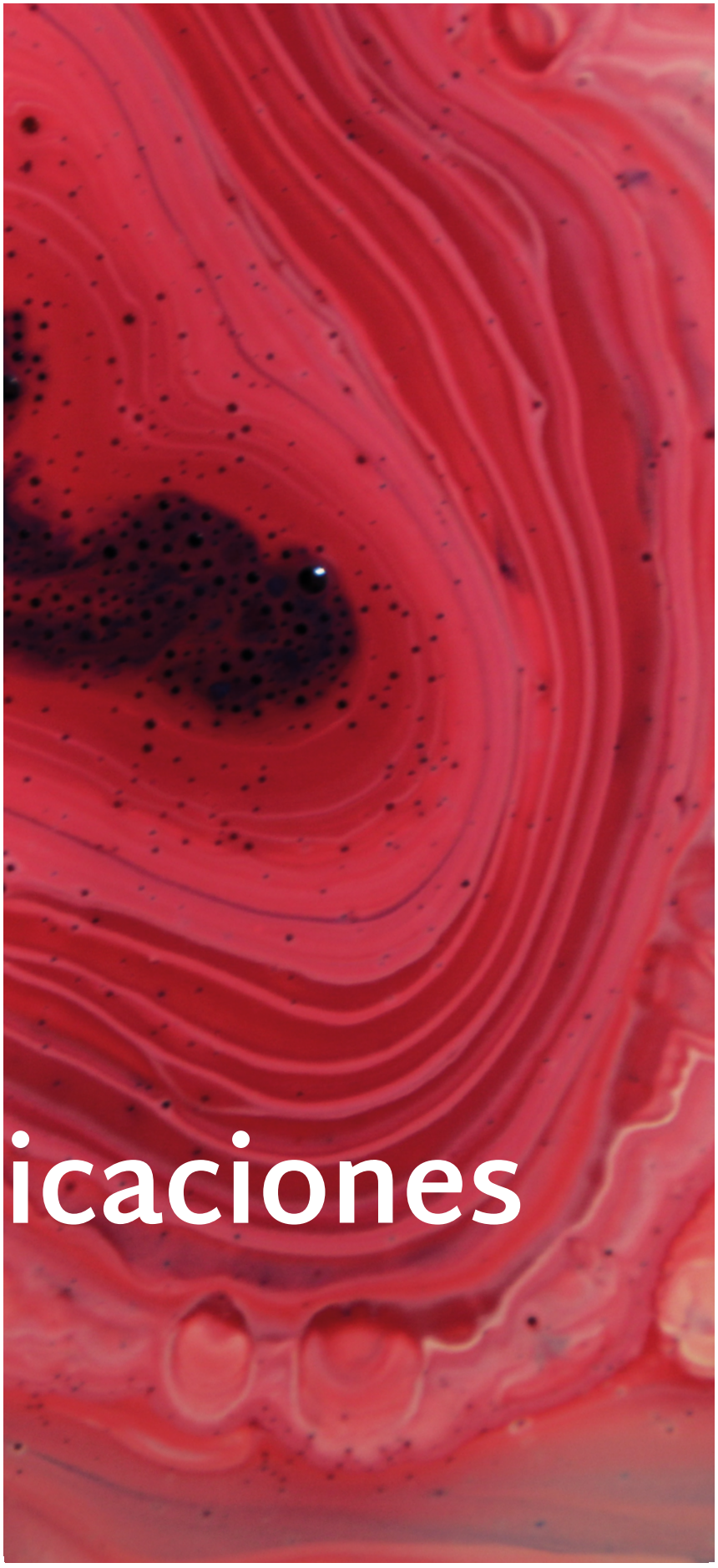
Una vez encontrada la relación de las dos mezclas con sus efectos proliferativos, valorar la capacidad de disrupción endocrina de estas a concentraciones no citotóxicas.

OBJETIVO 3

Analizar los perfiles de expresión génica diferenciales en células inducidas por las mezclas de OCs objetos de estudio.

OBJETIVO 4

Evaluar los efectos de la Simvastatina en los patrones de expresión génica inducidos por la mezcla de OCs encontrada en pacientes con cáncer de mama.



Publicaciones

ARTÍCULO 1

In vitro evaluation of oestrogenic/androgenic activity of the serum organochlorine pesticide mixtures previously described in a breast cancer case–control study

Evaluación in vitro de la actividad estrogénica/androgénica de las mezclas séricas de pesticidas organoclorados descritas previamente en un estudio de casos-control en cáncer de mama

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In vitro evaluation of oestrogenic/androgenic activity of the serum organochlorine pesticide mixtures previously described in a breast cancer case–control study

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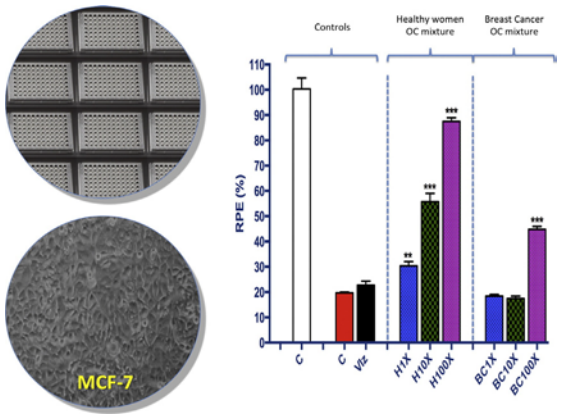
HIGHLIGHTS

- E-screen and A-screen of two mixtures of organochlorine pesticides (OCP)
- Assay concentrations based on a previous breast cancer case–control study
- Only non-cytotoxic concentrations assayed
- Both OCP mixtures induce proliferation mediated by oestrogen receptor.
- OCP mixture of breast cancer patients exhibits additional androgenic activity.

GRAPHICAL ABSTRACT



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ABSTRACT

Some organochlorine pesticides (OCs) have been individually linked to breast cancer (BC) because they exert oestrogenic effects on mammary cells. However, humans are environmentally exposed to more or less complex mixtures of these organochlorines, and the biological effects of these mixtures must be elucidated. In this work we evaluated the in vitro effects exerted on human BC cells by the OC mixtures that were most frequently detected in two groups of women who participated in a BC case–control study developed in Spain: healthy women and women diagnosed with BC. The cytotoxicity, oestrogenicity, and androgenicity of the most prevalent OC mixtures found in healthy women (H-mixture) and in BC patients (BC-mixture) were tested at concentrations that resembled those found in the serum of the evaluated women. Our results showed that both OC mixtures presented a similar oestrogenic activity and effect on cell viability, but BC-mixture showed an additional anti-androgenic effect. These results indicate that although the proliferative effect exerted by these mixtures on human breast cells seems to depend mainly on their oestrogenic action, the BC-mixture might additionally induce cell proliferation due to its anti-androgenic activity, therefore increasing the carcinogenic potential of this mixture. The findings of this study demonstrate that subtle variations in the composition of a mixture may induce relevant changes in its biological action.

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1. Introduction

Breast cancer (BC) is the largest cause of female cancer mortality in the European Union with around 90,000 deaths in 2013 (Maruthappu et al., 2015), and the most common female malignancy in most industrialized countries (Lacroix et al., 2006). While the BC mortality and incidence are lower in mainland Spain than in other European countries, the rates of BC mortality are alarming in the archipelago of the Canary Islands, specifically in Gran Canaria Island (Cabanes et al., 2009; Lopez-Abente et al., 2004).

Risk factors for the disease include high plasma oestrogen levels (Missmer et al., 2004) and high levels of oestrogen receptor (ER) expression in mammary tissue (Kurebayashi et al., 2000). In fact, the administration of anti-oestrogens constitutes one of the most useful treatments for hormone-dependent breast cancer (Mustonen et al., 2014). Given the pivotal role of oestrogens in the pathogenesis of breast cancer, exposure to xenobiotics with oestrogenic properties, also known as xeno-oestrogens, has been suggested to explain the increase in the incidence of BC recorded over the last four decades in industrialized countries. In vitro studies revealed that exposure to xeno-oestrogens may modulate cell cycle control and other oestrogen-sensitive molecular parameters in hormone-dependent BC cells and in normal human mammary cells (Fucic et al., 2012; Valeron et al., 2009). A number of organochlorine pesticides (OCs), such as dichlorodiphenyltrichloroethane (DDT), aldrin, dieldrin, and endrin, as well as their metabolites, are considered xeno-oestrogens (Colborn et al., 1993; Snedeker, 2001) and have been linked to environmentally induced BC (Jaga and Dharmani, 2003; Snedeker, 2001; Wolff et al., 2000; Zumbado et al., 2005). Because dichlorodiphenyldichloroethylene (DDE, the major metabolite of DDT) is the most prevalent organochlorine residue found in human tissues (Jaga and Dharmani, 2003; Snedeker, 2001; Zumbado et al., 2005), most studies have focused on the potential role played by DDE as a risk factor for BC. However, the overall evidence from several epidemiological studies that evaluated the relationship between BC and exposure to OCs does not clearly support a link between these compounds and BC risk (Lopez-Cervantes et al., 2004).

A number of circumstances may help to explain this absence of a relationship between BC and exposure to OCs. First, most studies did not consider that a number of xeno-oestrogens may also interact with other steroid hormone receptors, such as the androgen receptor (AR), which allow them to exhibit androgenic or anti-androgenic actions. In fact, because androgens control the proliferation of breast cancer cell lines and seem to be effective in complementing the treatment of hormone-dependent BC (Coss et al., 2014; Ortmann et al., 2002), environmental compounds that bind the AR constitute another class of endocrine disruptors that have received growing interest in recent years (Aube et al., 2008; Kortenkamp et al., 2014). On the other hand, most studies did not evaluate the fact that human beings are simultaneously exposed to multiple OCs that might exhibit agonistic, synergistic or additive actions. Therefore, it has been reported that the biological effects exerted by a mixture of OCs clearly differ from those exerted by each one of the OCs individually considered (Aube et al., 2011; Boada et al., 2012; Valeron et al., 2009). Comprehensive toxicological and biological data on the OC mixtures found in humans are scarce in the literature. In this picture, evaluating the role played by an isolated environmental contaminant does not seem to be the most adequate model to study the putative role exerted by OCs on breast cancer. As a consequence, the endocrine-disrupting properties of the most prevalent OC mixtures found in human samples need to be evaluated.

As mentioned earlier, most published studies have focused only on DDT and its metabolites (mainly DDE). However, humans are exposed to mixtures of pesticides that include many compounds, including non-DDT derivatives, as it has been widely reported by our research group and many other authors (Almeida-González et al., 2012; Arrebola et al., 2015; Boada et al., 2012, 2014; Luzardo et al., 2009, 2014; Zumbado et al., 2005). The objective of this study was to test the hypothesis that slight changes in the mixture of pollutants to which the cells are exposed to may induce different responses in them, especially in relation with a relevant end-point such as cell proliferation. Thus, we investigated the biological activities of the most prevalent OC mixtures described in healthy women and BC patients from a region showing high rates of BC mortality, such as the Spanish Archipelago of the Canary Islands, with the design of a set of in vitro experiments in human breast cancer cells.

2. Materials and methods

2.1. Reagents

Pesticides (lindane, aldrin, dieldrin, endrin, *p,p'*-DDE, *p,p'*-DDD, and *p,p'*-DDT) were purchased from Dr Ehrenstorfer, Reference Materials (Augsburg, Germany). Vinclozolin was purchased from Riedel de Haën (Sigma-Aldrich Laborchemikalien, Germany). Bicalutamide (ICI 176,334) was obtained from Enzo Life Sciences (Lausen, Switzerland). Dimethylsulfoxide (DMSO), sulforhodamine B (SRB), Fulvestrant (ICI 182,780), 5α-dihydrotestosterone (DHT), 17β-Oestradiol (E_2) and all other common products cited throughout this work were purchased from Sigma (Saint Louis, MI, USA). The OCs were dissolved in ultrapure DMSO to prepare stock solutions (1 mg/mL), whereas steroids (DHT, E_2, ICI 182,780) were prepared in ethanol at 10 mM. Once completely dissolved, the compounds were stored at −20 °C until use. Prior to each experiment, to reach the assay concentrations the stocks were diluted in phenol red-free Eagle's medium (DMEM).

2.2. OC mixtures

Given that the hypothesis to check in this research was whether small differences in the mixtures of pollutants may cause different effects on their potential as endocrine disruptors, our experimental design departed from a previous case–control study of BC in Gran Canaria, Spain (Boada et al., 2012), in which it was described that cases and control exhibited different mixtures of pollutants. Thus, we assayed in this research the effect exerted by the two OC mixtures more frequently detected by Boada et al. (2012) in both, controls (103 healthy women) and cases (121 women diagnosed with BC). In order to test environmentally relevant concentrations (as close as possible to the levels found in human beings) for this study we selected as 1× mixture the upper bound (95th percentile) combination of prevalent OCs (lindane, aldrin, dieldrin, endrin, *p,p'*-DDE, *p,p'*-DDD, and *p,p'*-DDT) found in the serum of women diagnosed with BC (BC-mixture) and healthy women (H-mixture) (Boada et al., 2012) (Table 1).

2.3. Cell lines and culture conditions

MDA-MB-231 cells were obtained from ATCC (American Type Culture Collection, LGC Standards, UK). MCF-7 BUS and MCF-7 AR1 cell sub-lines were kindly provided by N. Olea (Granada, Spain) from an MCF7 AR1 line stock (C. Sonnenschein, Boston, MA, USA). The cells were maintained in 75-cm^2 culture flasks (Nunclon, PA, USA) and re-seeded at a 1:3 split ratio in maintenance Dulbecco's modified Eagle's medium (DMEM, Lonza, Basel, Switzerland) containing 10% foetal bovine serum (FBS) (Biowest, Nuaillé, France) supplemented with 1× Glutamax (GLx; Gibco, Life Technologies, NY, USA), 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Lonza, Basel, Switzerland) and 54 μg/L gentamicin sulfate (GS, Lonza, Basel, Switzerland). The medium was changed every 2–3 days before reaching confluence. The cultures were regularly tested for mycoplasma contamination and were found to be mycoplasma-free. To test the cytotoxicity or proliferative response of all cell lines to chemical mixtures the cells were grown in phenol red-free DMEM supplemented with 10% charcoal-dextran (CD)-treated FBS (DMEMcs) plus PenStrepto and Glutamax.

Table 1
Median levels of organochlorine compounds (ng/g lipid) previously described in the serum of the studied women (Boada et al., 2012) and their corresponding final concentrations assayed.

	Healthy women			Breast cancer patients			
Compound	pM	Median (p5th–p95th)	1× (nM)	100× (μM)	Median (p5th–p95th)	1× (nM)	100× (μM)
Aldrin	364.90	0.0 (0.0–100.1)	2.8	0.28	75.8 (0.0–116.4)	3.2	0.32
Dieldrin	380.90	0.0 (0.0–46.2)	1.2	0.12	0.0 (0.0–72.0)	1.9	0.19
Endrin	380.90	29.1 (0.0–1279.0)	33.6	3.36	–	0	0
Lindane	290.80	0.0 (0.0–111.4)	3.8	0.38	0.0 (0.0–220.0)	7.6	0.76
p,p'-DDE	318.04	167.7 (45.0–706.0)	22	2.2	300.1 (106.1–653.3)	20	2.0
p,p'-DDD	320.04	0.0 (0.0–129.2)	4	0.4	551.1 (0.0–1108.2)	34.6	3.46
p,p'-DDT	354.50	217.0 (0.0–1428.6)	40	4.0	153.0 (0.0–327.9)	9.2	0.92

Abbreviations: p5th–p95th, percentiles 5 and 95 of the distribution.

2.4. Cell proliferation assays

We investigated the potentially oestrogenic compounds and the competitive AR antagonism of pesticide mixtures following previously published recommendations (Szelei et al., 1997), with slight modifications. Briefly, for each one of the experiments performed the cells were trypsinized on the first day, plated in 96-well plates at an initial density of 5000 cells per well, and allowed to attach. After 24 h, the medium was carefully aspirated, and the wells were washed with 100 μL of oestrogen-free medium and filled with 200 μL of oestrogen-free medium. Then, DMSO (pesticides) or ethanol (hormones) stocks of the compounds to be tested were diluted in DMEM and added to the wells to reach the desired concentrations (final DMSO concentration ≤0.001% in all experiments; final ethanol concentration ≤0.01% in all experiments).

To assess the cytotoxic effect of the two OC mixtures, we used ER-negative human breast carcinoma cells (MDA-MB-231), in which the cell proliferation was measured in the presence of increasing concentrations of H-mixture and BC-mixture. This assay compared the cell proliferative responses both, in the presence of 17β-Oestradiol (positive control) and in the presence of the pesticide mixtures.

ER-positive MCF-7 BUS cells were used to assess the oestrogenicity of the evaluated OC mixtures (E-screen). In the E-screen assay the proliferation of MCF7-BUS cells in the absence of oestrogens (negative control), in the presence of 17β-Oestradiol (positive control), and in the presence of a range of concentrations of H- and BC-mixtures was compared.

Finally, an AR-positive human breast carcinoma cell line (MCF7-AR1) was used to assess the androgenicity of the OC mixtures (A-screen). In the A-screen assay the cell proliferation is induced using 17β-Oestradiol. Androgens (DHT) are known to inhibit the cell proliferation in this line, and androgen antagonists reverse this effect. In this context, the potential actions of OC mixtures on androgen receptors were analysed in relation to two synthetic androgen antagonists with different anti-androgenic potency (bicatulamide and vinclozolin).

2.5. Sulforhodamine B assay

Cell proliferation was assessed after 120 h (MDA-MB-231 cells) or 144 h (MCF-7 cells) using the Sulforhodamine B (SRB) colorimetric assay (Skehan et al., 1990). These time points were selected according to the different growth characteristics of the cell line in order to optimize their proliferation promoted by the hormones (over the control). The medium was then carefully aspirated to avoid cell detachment and the cells were subsequently incubated at 4 °C for 60 min in 10% (w/v) trichloroacetic acid (TCA). Following incubation, the TCA was discarded, and the wells were washed four times under a gentle stream of tap water and air-dried completely. The TCA-fixed cells were stained in a shaker for 10 min with 100 μL of 0.4% (w/v) SRB dissolved in 1% acetic acid. Following incubation, the supernatant was discarded, and the unbound dye was removed by rinsing the wells four times with 1% acetic acid. After complete drying, the bound dye was solubilized with 10 mM Tris base, pH 10.5, for 20 min on a shaker, and the dye intensity was read at 490 nm in PowerWave HT microtiter plate reader (Biotek Instruments, USA).

2.6. Statistical analysis

At least two independent experiments for each assay were performed to establish the relationship between the concentrations of OC mixtures and biological responses. Within each experiment, three culture wells per concentration were used to establish mean values. The cell proliferation was normalized to its specific control to correct for differences in the initial seeding density. Statistically significant differences between OC mixture concentrations were calculated with Student's t-test or one-way analysis of variance (ANOVA). All statistical analyses were performed using the SPSS for Windows software (v.17.0; SPSS Inc., Chicago, IL).

3. Results and discussion

In the present study, we have used the E-screen and A-screen assays to evaluate the endocrine-disrupting properties of two different OC mixtures at concentrations which resembled those that were previously described as highly prevalent in healthy women and in women diagnosed with BC (Boada et al., 2012). E-screen and A-screen assays are commonly used as screening methods to evaluate potential endocrine-disrupting chemicals (Aube et al., 2008). In addition, the cytotoxicity of the OC mixtures was also evaluated. As reported by Boada et al. (2012), the different profile of contamination between BC cases and controls could be attributed to differences due to life style, dietary habits, or occupation among healthy women and BC patients, and the possibility existed that this differential exposure to contaminants could exert a relevant role in BC development. Our findings indicate that the OC mixtures currently found in these two groups of women might be able to alter the androgen–oestrogen balance in breast cells.

3.1. Cytotoxic and proliferative effect of the H- and BC-mixtures

Before testing the effect of both OC mixtures on the oestrogen–androgen balance, we first assessed their cytotoxic/proliferative response in MDA-MB-231 cells, which is a cell line where ER is lacking. This experiment allowed us to evaluate the effect of the mixtures independently of their potential oestrogenic action. We evaluated the proliferative effect of the two OC mixtures compared to cells treated with 100 pM of 17β-Oestradiol (as a control) in the cell culture medium over a 120 h period. As shown in Fig. 1, the two OC mixtures showed similar viability. Thus, the H-mixture elicited a proliferative response of 89.75% and 45.49% at concentrations of 300× and 500×, respectively compared with the control. For the BC-mixture, the proliferative responses at 300× and 500× were 93.76% and 58.64%, respectively compared with the control. These differences were only significant in the case of 500× concentration for both mixtures with respect to the control ($p < 0.001$ for both mixtures). These findings suggest that the evaluated mixtures are clearly cytotoxic at concentrations of 500-fold or

higher than the concentrations chosen as 1× for the study population in this research (Table 1) (Boada et al., 2012). Our results agree with those reported by other authors in studies in breast cancer cell lines (Aube et al., 2011) and with the findings of cytotoxicity studies of OC mixtures on normal human mammary epithelial cells (HMEC) reported previously by our group (Valeron et al., 2009).

Based on this result, the subsequent experiments aimed to evaluate the potential endocrine disrupting capacity of these two mixtures were performed at non-cytotoxic concentrations (ranging between 0.1× and 300×).

3.2. Oestrogenicity of the evaluated OC mixtures

To determine the oestrogen-like effects of OC mixtures, the ability of these mixtures to promote the dose-dependent growth of MCF-7 BUS cells was determined. ER is expressed in MCF-7 BUS, which makes this cell line appropriate to determine the oestrogenic effects of chemicals. The cell proliferation was measured using the SRB assay. The relative proliferative effects (RPEs, %) compare the maximal proliferation of MCF-7 BUS cells induced by OC mixtures with the proliferation induced by 100 pM of E_2 (Fig. 2). The proliferative effect exerted by the H-mixture (Fig. 2A) was similar to that exerted by the BC-mixture (Fig. 2B). In both cases, the highest RPEs were observed at 300× (94.2% for the H-mixture and 86.2% for the BC-mixture) compared with the maximal E_2 response (RPE = 100%). Nevertheless, at two non-cytotoxic concentrations (100× and 10×), the H-mixture exerted a stronger proliferative effect than the BC-mixture. Thus, the RPEs for the H-mixture were 84.9% and 42.4% at 100× and 10×, respectively, while the RPE values elicited by the BC-mixture were 59.6% and 20.2% at 100× and 10×, respectively. The MCF-7 BUS cell line is highly sensitive to oestrogenic stimulation and for this reason has been extensively used to characterize the oestrogenic potential of xenoestrogens (Schiliro et al., 2012). Our results suggest that the H-mixture would be more oestrogenic than the BC-mixture. These findings agree with previously published results that showed that the combination of OCs described in the plasma of women living in Arctic areas also exerted a proliferative effect on breast cancer cell lines (Aube et al., 2011). In addition, these findings are also coincident with those previously published by our group, which demonstrated that a mixture of OCs, which is highly prevalent in the population of the Canary Islands (*p,p'*-DDD, plus *p,p'*-DDE, plus *o,p'*-DDE, plus aldrin, plus dieldrin) exerted a clear proliferative effect on HMEC (Valeron et al., 2009).

Taking into account these findings, we treated MCF-7 BUS cells with the anti-oestrogen ICI 182,780 to determine if the observed cell proliferation depended on the activation of the ER. This anti-oestrogen is a well-known pure ER antagonist that can bind to ERα and ERβ to impair the dimerization of this receptor and accelerate its degradation. Consequently, ER-mediated transcription is completely abolished, leading to the suppression of oestrogen-dependent gene expression (Nuttall et al., 2001; Robertson, 2001). As expected, our results showed that the maximum proliferative effect of E_2 (100%) was drastically reduced when MCF-7 BUS cells were co-treated with 1 μM of ICI 182,780 (RPE = 8.4%). Similarly, co-treatment with OC mixtures and the oestrogen antagonist ICI 182,780 completely abolished the induced cell growth at all assayed concentrations (Fig. 2). Thus, ICI 182,780 was found to inhibit the cell proliferation induced by both the H-mixture and BC-mixture. These findings indicated that both OC mixtures evaluated exhibit oestrogenic activity related to ER activation.

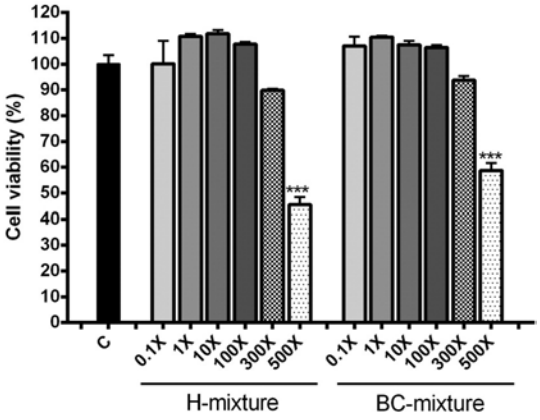

Fig. 1. The cell growth inhibition of oestrogen-receptor negative (MDA-MB-231) human mammary carcinoma cells was tested after 120 h of incubation. Cells were treated with 17β-Oestradiol (Control, $[E_2] = 100$ pM) or each OC mixture in phenol red-free DMEM supplemented 10% charcoal-dextran (CD) foetal bovine serum (FBS). After 5 days, the number of viable cells was measured using the SRB assay (mean ± SD, n = 3).

3.3. Androgenicity of the evaluated OC-mixtures

The oestrogen and androgen signalling pathways are well known to exert opposite influence on breast cells, with oestrogens inducing mitogenic effects. Although it has been reported that several pesticides are able to exerting anti-androgenic effects (Wilson et al., 2008), the possibility that OC mixtures act as agonists or antagonists of the AR has not been explored in depth. In this work, we evaluated the possible androgenic/anti-androgenic action of these two OC mixtures using the A-screen assay. This assay measures the androgen-dependent inhibition of the proliferation of the AR-positive human mammary carcinoma cell line MCF7-AR1. These cells over-express the AR and contain approximately five times more AR than wild-type MCF7 cells. Thus, MCF7-AR1 cells a) retain their capacity to proliferate when they are exposed to oestrogens, b) do not acquire the ability to proliferate when they are treated with androgens, c) are prevented from proliferating in response to oestrogens by natural and synthetic androgens, and d) proliferate in response to androgen antagonists in the presence of oestrogens and androgens because the anti-androgens antagonise the androgen-induced proliferative shut off in MCF7-AR1 cells (Szelei et al., 1997).

The A-screen assay showed that both, H- and BC-mixture induced a dose-dependent increase in MCF7-AR1 proliferation in the presence of 100 pM E_2 and 100 pM DHT (Fig. 3A). As expected, dihydrotestosterone (DHT) clearly inhibited the cell proliferation induced by E_2. OC mixtures at a concentration of 100× caused a 1.9-fold (H-mixture) and 2-fold (BC-mixture) increase in cell proliferation compared with the E_2 + DHT treatment ($p < 0.001$). This increase suggests that these mixtures may exert an anti-androgenic effect on MCF7-AR1 cells. To clarify whether the reversion of the inhibited proliferation by DHT might be due to an AR antagonistic action or to an activation of the oestrogenic pathway, we evaluated the effect of the exposure of MCF7-AR1 cells to OC mixtures in the absence of E_2 and DHT. Under these conditions it is expected that only compounds with oestrogenic activity will induce the proliferation of MCF7-AR1 cells. Our results demonstrated that all tested concentrations of the H-mixture induced a significant proliferative response (1.5-, 2.8- and 4.4-fold RPE increase at 1×, 10× and 100×, respectively) (Fig. 3B). In contrast, only the highest concentration of the BC-mixture induced a significant proliferative response (2.2-fold induction), while lower concentrations of this mixture showed an effect similar to that found in MCF7-AR1 cells treated with 10 μM of the anti-androgenic drug vinclozolin (Fig. 3B). To the best of our knowledge, this study is the first to demonstrate that two similar OC mixtures seem to exert different endocrine effects, specifically with respect to androgenic actions, which suggests that subtle changes in the proportion or concentration of the chemicals involved in the mixture may strongly affect its biological activity.

Our findings seem to indicate that both OC mixtures exert a proliferative effect on breast cancer cells, which is likely due to their reported oestrogen-like properties. However, the fact that BC-mixture did not induce the proliferation of MCF7-AR1 cells in the absence of sex hormones suggests that the BC-mixture, unlike the H-mixture, shows greater

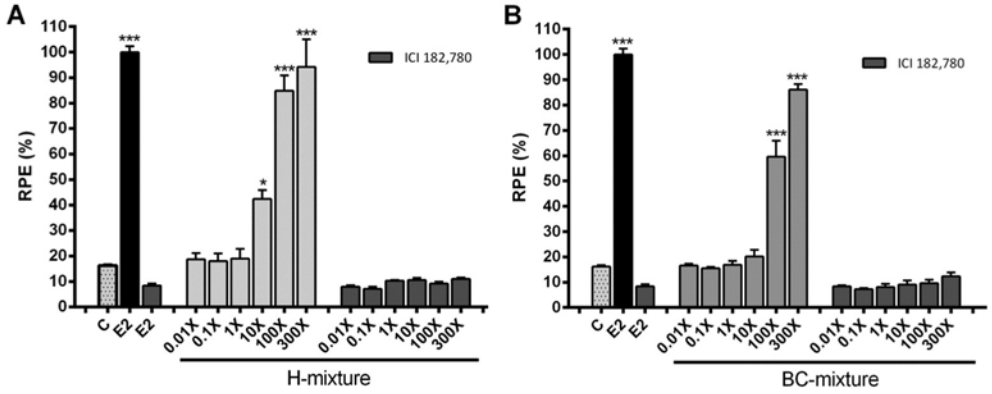

Fig. 2. Relative proliferative effect percentage (RPE%) induced by the different concentrations of the mixtures tested with and without co-incubation with 1 μM ICI 182,780 in MCF-7 BUS. A) Increasing concentrations of the H-mixture, and B) Increasing concentrations of the BC-mixture. Cell proliferation was assessed after 5 days of treatment. Each bar represents the mean ± SE of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, versus the negative control (untreated cells). Differences were assessed by an analysis of variance followed by one-tailed Bonferroni post hoc test.

affinity for AR than for ER resulting in the predominance of the AR signalling pathway, even at the lower concentrations assayed. It is noteworthy that this effect was observed even at the real concentrations found in the women affected by breast cancer (1×) (Boada et al., 2012). As a consequence, our results suggest that the BC-mixture of OC pesticides may increase BC cell proliferation by interfering with both oestrogenic and androgenic pathways, while the H-mixture might be less carcinogenic to breast tissue due to the absence of anti-androgenic activity. The fact that *p,p'*-DDD is a major constituent of the BC-mixture should be highlighted and could explain the reported anti-androgenic effect because this DDT metabolite displays a greater affinity for AR than for the ER (Maness et al., 1998). Consequently, the additional anti-androgenic effect of the OC mixture found in women diagnosed with BC might be due to the presence of high levels of *p,p'*-DDD in this mixture. In this sense, the present results may help to explain why *p,p'*-DDD has been described as a risk factor for BC in our previously reported population-based case–control study (Boada et al., 2012).

The agonistic and antagonistic activities of hormone-mimicking chemicals are early molecular events that can lead to the disruption of normal endocrine system function. Chemicals that act as ER or AR receptor agonists or antagonists can affect human development and reproductive health (Andersson et al., 1999; Gray et al., 2006; WHO, 2002). These effects are a particular matter of concern because humans are reportedly exposed to these mixtures during specific sensitive times of foetal development (Luzardo et al., 2009), and this early exposure can lead to molecular alterations that can cause reproduction-related diseases later in adulthood.

4. Conclusion

The results of our study suggest that the evaluated OC mixtures exert differential endocrine-disrupting activities, which could explain why similar, but not identical, OC mixtures can exert different biological effects on breast tissue. Nevertheless, further in vivo toxicological and biological studies of the most prevalent OC mixtures are necessary. In addition, on the light of these results we consider that other environmentally relevant mixtures of pollutants capable of inducing breast cell proliferation should be evaluated regarding their androgenic/oestrogenic properties.

Conflict of interest

The authors declare that there are no conflicts of interest.

Competing financial interests declaration

There are no actual or potential conflicts of interest to declare for any author.

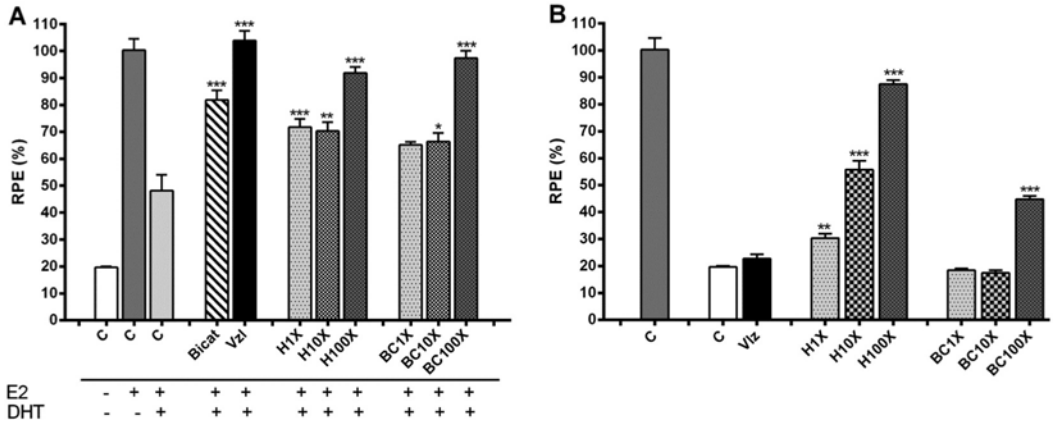

Fig. 3. Relative proliferative effect percentage (RPE%) induced by the different concentrations of the H- and BC-mixtures in MCF7-AR1. Cell proliferation was assessed after 5 days of treatment. Each bar represents the mean ± SE of three independent experiments. A) Proliferative effects of the mixtures co-incubated with 17β-Oestradiol (E_2; 100 pM) and dihydrotestosterone (DHT; 100 pM). B) Proliferative effects of the mixtures versus the negative control (untreated cells). Final concentration of vinclozolin = 10 μM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, Differences were assessed by an analysis of variance followed by a one-tailed Bonferroni post hoc test.

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ARTÍCULO 2

Differential gene expression pattern in human mammary epithelial cells induced by realistic organochlorine mixtures described in healthy women and in women diagnosed with breast cancer

Patrón de expresión génica diferencial en células epiteliales inducidas por mezclas reales de organoclorados descritas en mujeres sanas y mujeres diagnosticadas con cáncer de mama

OBJETIVO 1

Evaluar in vitro los efectos citotóxicos/ proliferativos ejercidos por las mezclas de OCs encontradas en pacientes con cáncer de mama y en pacientes sanas.

OBJETIVO 3

Analizar los perfiles de expresión génica diferenciales en células inducidas por las mezclas de OCs objetos de estudio.

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Differential gene expression pattern in human mammary epithelial cells induced by realistic organochlorine mixtures described in healthy women and in women diagnosed with breast cancer

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HIGHLIGHTS

- Different organochlorine mixtures exert different effects on HMEC.
- Different organochlorine mixtures modify the expression of different genes.
- Subtle changes in the composition and levels of pollutants induce different biological effects.
- The expression of oncogenes and tumor suppressor genes is modified by organochlorine mixtures.

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ABSTRACT

Organochlorine pesticides (OCs) have been associated with breast cancer development and progression, but the mechanisms underlying this phenomenon are not well known. In this work, we evaluated the effects exerted on normal human mammary epithelial cells (HMEC) by the OC mixtures most frequently detected in healthy women (H-mixture) and in women diagnosed with breast cancer (BC-mixture), as identified in a previous case-control study developed in Spain. Cytotoxicity and gene expression profile of human kinases ($n = 68$) and non-kinases ($n = 26$) were tested at concentrations similar to those described in the serum of those cases and controls. Although both mixtures caused a down-regulation of genes involved in the ATP binding process, our results clearly indicate that both mixtures may exert a very different effect on the gene expression profile of HMEC. Thus, while BC-mixture up-regulated the expression of oncogenes associated to breast cancer (*GFRA1* and *BHLHB8*), the H-mixture down-regulated the expression of tumor suppressor genes (*EPHA4* and *EPHB2*). Our results indicate that the composition of the OC mixture could play a role in the initiation processes of breast cancer. In addition, the present results suggest that subtle changes in the composition and levels of pollutants involved in environmentally relevant mixtures might induce very different biological effects, which explain, at least partially, why some mixtures seem to be more carcinogenic than others. Nonetheless, our findings confirm that environmentally relevant pollutants may modulate the expression of genes closely related to carcinogenic processes in the breast, reinforcing the role exerted by environment in the regulation of genes involved in breast carcinogenesis.

1. Introduction

Breast cancer (BC) is a complex disease with a heterogeneous etiology involving genetic, epigenetic and environmental factors (Anderson et al., 2014). The role of genetics in the origin of BC is limited since over 70% of the diagnosed tumors are not associated with inheritance of any of the major high risk genes identified to

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date. The best established factors that contribute to BC development are related to cumulative exposure of the breast tissue to endogenous estrogens, such as early menarche, late age at first pregnancy, nulliparity, lactation, years of reproductive life, hormonal contraception and hormone replacement therapy (Chen, 2008). However, epidemiological data suggest an important contribution of several environmental factors as responsible for the increase of incidence of BC from 1940 onwards (Harris et al., 1992). The fact that daughters of women who migrate from low-incidence to high-incidence countries acquire the breast cancer risk prevailing in the new country (Buell, 1973) reinforces the hypothesis that aspects of lifestyle or the environment are major determinants of breast cancer.

Organochlorine pesticides (OCs) are persistent pollutants, which were widely used worldwide. Although they began to be banned in developed countries in the decade of 1970s, due to their persistence and resistance to degradation they are still frequently detected in the environment, food, biota, and human beings worldwide. The ubiquity and universal presence of OCs in the environment is a matter of public health concern because they have been linked with several deleterious effects on human health (Trasande et al., 2015), including breast cancer (Snedeker, 2001; Lopez-Cervantes et al., 2004). Several OCs and some of their metabolites are considered endocrine disruptors and have been found to induce estrogen-like effects in exposed humans (Gellert et al., 1972). The pattern of contamination by OCs in the Spanish population of the Canary Islands has been extensively studied (Zumbado et al., 2005; Luzardo et al., 2006, 2009; Almeida-González et al., 2012), showing relatively high levels of exposure of these people to OCs. In parallel, this Spanish population also exhibits alarming rates of mortality because of BC (Cabanes et al., 2009), and the possibility exists that the high exposure to environmental pollutants could play a role in the development of this disease in that specific region.

Despite the theoretical association between BC and OCs (mainly referred to DDT-derivative pesticides), epidemiologic studies have been controversial, with positive (Wolff et al., 2000; Wolff and Toniolo, 1995; Wolff et al., 1993; Fenton, 2006) and negative (Lopez-Cervantes et al., 2004; Stellman et al., 2000) associations reported during the last decades. This lack of association might be attributed to the fact that people are exposed to countless combinations of OCs, while most of these studies are focused in the study of isolated compounds. It is well known that biological effects exerted by a mixture of OCs may be considerably different from those exerted by any OC taken individually (Rajapakse et al., 2002; Valeron et al., 2009).Thus, greater emphasis should be given to examine the effects exerted by mixtures of OCs on human health. In that sense, we have recently published that BC patients show a different profile of OC mixtures than healthy women, suggesting that OC mixtures may play a relevant role in breast cancer risk (Boada et al., 2012). In addition, we have reported that the OC-mixture described in BC patients, unlike the OC-mixture showed by healthy women, clearly exerted an anti-androgenic activity, which may facilitate cell proliferation. Consequently, the OC-mixture described in BC patients might facilitate the initiation of carcinogenic processes in the breast (Rivero et al., 2015).

At a molecular level, certain changes have to occur in epithelial cells to convert normal cells into tumor cells. These changes must alter the DNA to facilitate cell proliferation, avoid apoptosis and achieve invasive characteristics (Calaf and Russo, 1993; Calaf et al., 2000). These changes may be conditioned by individual compounds, or mixtures of compounds (Valeron et al., 2009). It is well known that the activation of membrane receptors and/or regulation of gene expression are the main molecular mechanisms and signaling pathways underlying the action of chemicals involved in BC development (Bulayeva and Watson, 2004; Frigo et al., 2004; Lemaire et al., 2005). In this context, a number of protein kinases – key regulators of cell function – have been shown to be critical for disease development and malignancy (Cance and Liu, 1995; Anneren et al., 2003). The knowledge of these genetic changes is critical for understanding the molecular basis of breast carcinogenesis. Furthermore, genetic studies about the expression of key genes in tumor development may offer additional information about the early steps of carcinogenesis potentially induced by xenoestrogens.

We have designed an *in vitro* study aimed to explore the molecular actions and biological activities of the most prevalent OC mixtures described in healthy women and BC patients from a case-control study previously developed in Spain, with special emphasis in the regulation of gene expression of protein kinases.

2. Materials and methods

2.1. Chemicals

Pesticides (lindane, aldrin, dieldrin, endrin, p,p′-DDE, p,p′-DDD and p,p′-DDT) were purchased from Dr Ehrenstorfer, Reference Materials (Augsburg, Germany). Dimethylsulfoxide (DMSO) and all other common products cited throughout this work were purchased from Sigma (Saint Louis, MI, USA) unless stated otherwise. The OCs were dissolved in ultrapure DMSO to prepare stock solutions (1 mg/mL) and stored at −20 °C until use.

2.2. OC mixtures

Since people are exposed to mixtures of OCs and not to isolated compounds, we assayed the effect exerted by the mixtures detected in a population-based study of 103 healthy women and 121 women diagnosed with BC from the Canary Islands (Boada et al., 2012). In order to test environmentally relevant concentrations (as close as possible to the levels found in human beings), for this study we selected the 1× mixture observed in the upper bound (95th percentile) of prevalent OCs found in the serum of women diagnosed with BC (BC-mixture: lindane plus aldrin plus dieldrin plus endrin plus p,p′-DDE plus p,p′-DDD plus p,p′-DDT) and healthy women (H-mixture: lindane plus aldrin plus dieldrin plus p,p′-DDE plus p,p′-DDD plus p,p′-DDT) (Table 1). The low levels of p,p′-DDD and the high levels of p,p′-DDT described in healthy women, and the absence of endrin together with the high levels of p,p′-DDD residues in BC patients were especially taken into account for the calculation of the mixtures. The OC mixtures were dissolved in DMSO and diluted in phenol red-free Mammary Epithelial Basal Medium (MEBM) purchased from Lonza (Walkersville, MD, USA). Final DMSO concentration was ≤0.001% in all experiments.

Table 1
H-mixture and BC-mixture 1× Final concentrations (nM) calculated from the median levels of organochlorine pesticides (ng/g lipid) previously described in a population-based case-control study (Boada et al., 2012).

Compound	Healthy women (H-mixture)	Breast cancer patients (BC-mixture)
Aldrin	2.8	3.2
Dieldrin	1.2	1.9
Endrin	33.6	0.0
Lindane	3.8	7.6
p,p'-DDE	22	20
p,p'-DDD	4	34.6
p,p'-DDT	40	9.2

2.3. Cell lines and culture conditions

The primary human breast epithelial cell line (HMEC) was purchased from Lonza (Walkersville, MD, USA). This cell line has all the characteristics of the normal epithelium, including genetic characteristics, as guaranteed by the provider. HMEC cells were cultured in phenol red-free MEBM containing the following growth supplements: bovine pituitary extract (BPE), 0.4%; epidermal growth factor (hEGF), 0.5 ml; hydrocortisone, 0.5 ml; GA-1000, 0.5 ml; insulin, 0.5 ml. HMEC were incubated in 5% CO_2 atmosphere at 37 °C. In each experiment, the OC mixture was added to the fresh medium. All assays were performed at least in triplicate and each dose-group within an experiment was assayed using triplicate wells.

2.4. Cell proliferation assays

For cell proliferation assays, 4750 cells were seeded in 24-welldishes. 72 h later, the indicated OC mixtures at 1×, 10×, 50×, or 100× concentrations, were added to the fresh medium. At the indicated time intervals, cells were washed, fixed on 1% glutaraldehyde (500 μl) for 30 min, and washed three times with 1× phosphate-buffered saline (PBS). Once all dishes of the time intervals were collected, 500 μl of 0.1% crystal violet was added to the cells for 30 min and then washed as described previously (Valeron et al., 2009). Cell confluence when crystal violet was added depends on the time course: after five days, cell confluence was 85–90%. To obtain the incorporated crystal violet, 500 μl of 10% acetic acid was added for 10 min and the dishes were later collected and read at a wavelength of 595 nm.

2.5. Protein kinases transcriptional regulation arrays

Total RNA from HMEC exposed to the 10× OC mixtures for five days was isolated using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) in accordance with the instructions of the manufacturer. The required quantities of RNA were measured with a NanoDrop® ND-1000 UV–Vis Spectrophotometer (NanoDrop, Wilmington, DE, USA). The ratio quality/integrity was verified in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). First-strand cDNAs were generated from 0.5 μg of the total RNA using the High-Capacity cDNA Archive kit (Applied Biosystems).

Quantitative PCR was performed using TaqMan® Low Density Array (Applied Biosystems, Foster City, CA, USA). Each mix reaction containing 5 μl of reverse transcribed RNA and 50 μl of "Taqman Universal PCR Master Mix" (Applied Biosystems) was done on an ABI Prism7900HT (Applied Biosystems) in a final volume of 100 μl. Conditions for amplification were as follows: 1 cycle of 94.5 °C for 2 min, followed by 40 cycles of 97 °C for 30 s and 59.7 °C for 1 min. The raw data were recorded with the SDS 2.2.2 software of the instrument. Differential expression was determined by delta–delta Ct method that shows the fold changes of each sample relative to reference sample. *18S* expression was performed for each sample as housekeeping gene. All assays were performed at least in quadruplicate. Mean values for each probe were $\log_2$ transformed to have an escalated interpretation of the expression ratios. The TaqMan Low Density array contains assays for 68 human kinases and 26 non-kinases genes. Fifty-three of the 68 kinases were from the protein-tyrosine kinase receptor (RPTK) families: *EGFR*, Insulin receptor, *PDGFR*, *VEGFR*, *FGFR*, *CCK*, *NGFR*, *HGFR*, *EPHR*, *AXL*, *TIE*, *RYK*, *DDR*, *RET*, *ROS*, *LTK*, *ROR*, and *MUSK*. The remaining 15 kinases were Ser/Thr kinases from the kinase families: *CAMKL*, *IRAK*, *Lmr*, *RIPK*, and *STKR*. The remaining non-kinase genes were also involved in signal transduction and mediate protein–protein interaction, transcriptional regulation, neural development, or cell adhesion.

2.6. Statistical analysis

The data were expressed as the means ± SD. The significance of differences between the groups was tested by ANOVA test. GraphPad Prism 6 program (GraphPad Software, San Diego, CA)

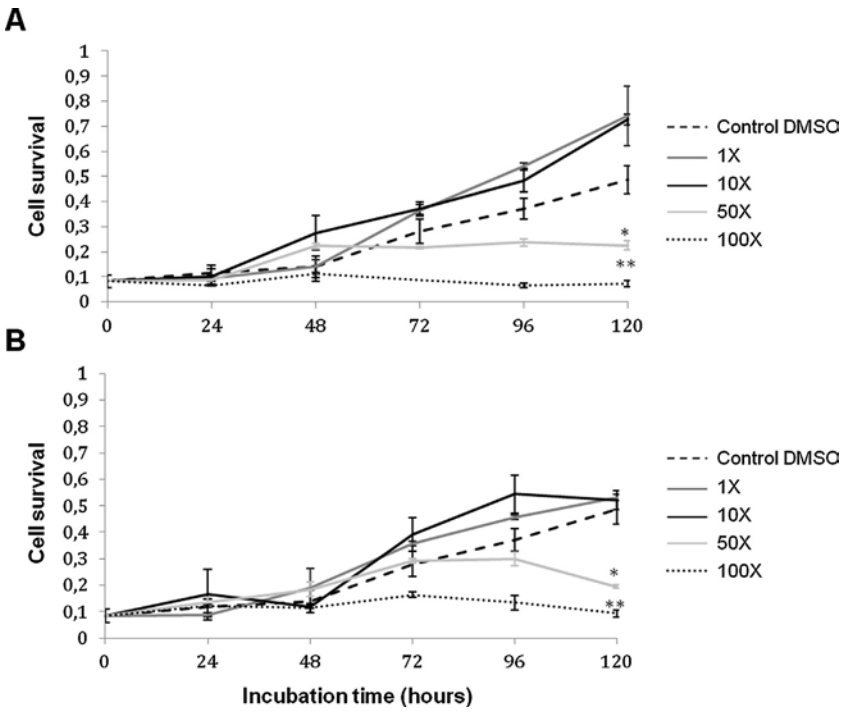

Fig. 1. Dose- and time-dependent proliferative effects of OCs mixtures on the viability of HMEC. HMEC were exposed to increasing concentrations of H-mixture (A), and BC-mixture (B). Each experiment was performed at least three times. In the figure, each data point represents the mean ± SD of three replicates in one representative experiment. $^{*}p < 0.05$; $^{**}p < 0.01$. See Table 1 for details of concentrations.

was used for statistics. Statistical significance was reported if $P < 0.05$ was achieved.

3. Results and discussion

OCs are well known endocrine disrupters that have been proposed as risk factors for BC due to their estrogenic properties. However, the molecular mechanisms underlying this process are still unclear. In the present study, we evaluated the effects exerted on the expression of a set of 94 genes (including 68 human kinases) by two different realistic OC mixtures that were previously described as highly prevalent in healthy women and in women diagnosed with BC (Boada et al., 2012).

3.1. Cytotoxic effect of the H- and BC-mixtures

Before testing the effect of the environmentally relevant mixtures on the selected genes, we assessed the cytotoxic response induced by these two OC mixtures in primary human breast epithelial cells (HMEC). As shown in Fig. 1, the two OC mixtures showed similar cytotoxic effects on HMEC. At concentrations of 50× and 100×, survival of cells exposed to the H-mixture was 46.2 and 15.0%, respectively, referred to the control (ANOVA test, $p = 0.024$ and $p = 0.009$, respectively). For the BC-mixture, cell survival at 50× and 100× was 39.9 and 19.1%, respectively, referred to the control (ANOVA test, $p = 0.017$ and $p = 0.010$, respectively). We did not observe statistically significant differences between concentrations at 1× and 10× compared with the control. Nevertheless, it has to be highlighted that the present results are different to those previously reported for individual OCs on HMEC (Valeron et al., 2009). In that sense, we have previously published that HMEC survived in the presence of 100× or higher concentrations of aldrin, dieldrin, p,p′-DDD, o,p′-DDE, p,p′-DDE, or p,p′-DDT taken individually or in combination. Moreover, while p,p′-DDE is not highly toxic for HMEC, the combination with other DDT metabolites makes the mixture highly cytotoxic (Valeron et al., 2009). Similarly, aldrin and dieldrin show a very different effect when they are tested alone or in combination (Valeron et al., 2009). However, endrin and lindane have not been previously evaluated. Since we have observed a dramatic decrease of cell proliferation of HMEC exposed to the two OC mixtures assayed and both mixtures included endrin and/or lindane, it is possible that these OCs (together or separately) condition the survival of HMEC. Although the biological effect exerted by a mixture of chemicals depends not only on the quantitative and qualitative composition of the mixture but also on the time of exposure and the type of cell tested, our findings point to the possibility that endrin and/or lindane exert a deleterious effect on primary human breast epithelial cell. This suggestion agrees with other authors who have published induction of micronuclei by low doses of lindane in MCF-7 cell line (Kalantzi et al., 2004), which would be enough to induce apoptosis in primary breast cancer cells. These results clearly indicated that the role played by other less known OCs, such as cyclodiene (*i.e.* aldrin, dieldrin, endrin) or hexachlorocyclohexane (*i.e.* lindane) pesticides should also be taken into account in studies regarding breast cells proliferation (Valeron et al., 2009).

3.2. Global protein kinases transcriptional regulation

The tyrosine kinase (TK) family includes many growth factor receptors, cell cycle regulators, and oncoproteins. TKs are regulatory proteins that play an important role in the cell growth and differentiation of normal cells. The TKs represent a major class of proto-oncogenes and may be involved in the progression and metastasis of cancer cells. Several TKs have been found to be induced or inhibited in breast cancer, and subsequently have been proposed to play a role in carcinogenesis (Meric et al., 2002). Thus, the gene expression profile of 94 kinases- and non-kinase genes was studied after the exposure of HMEC to the two OC mixtures (H-mixture and BC-mixture). For the global expression analyses, we included all the genes that were regulated by the OC mixtures after normalization by 18S gene. A total of 80 genes were successfully determined. We observed that the gene expression pattern was different in HMEC exposed to the H-mixture compared to HMEC exposed to BC-mixture (Fig. 2). Thus, the global profile of expression was significantly different for each mixture, suggesting a clearly differentiated biological effect of the mixtures assayed. The fold-induction was 0.397 and 0.243 for H-mixture and BC-mixture, respectively ($p < 0.0001$, Fig. 2A), supporting the hypothesis that OC mixtures could cause differential effects on normal human breast epithelial cells at environmentally relevant concentrations (Valeron et al., 2009). A total of 57 genes were down-regulated in HMEC exposed to H-mixture (20 genes were up-regulated). In the other hand, 21 genes were down-regulated in HMEC exposed to BC-mixture (56 genes were up-regulated). The six most regulated genes for each mixture are detailed in Table 2. Most down-regulated genes by the exposure to the OC mixtures were genes involved in the ATP binding process.

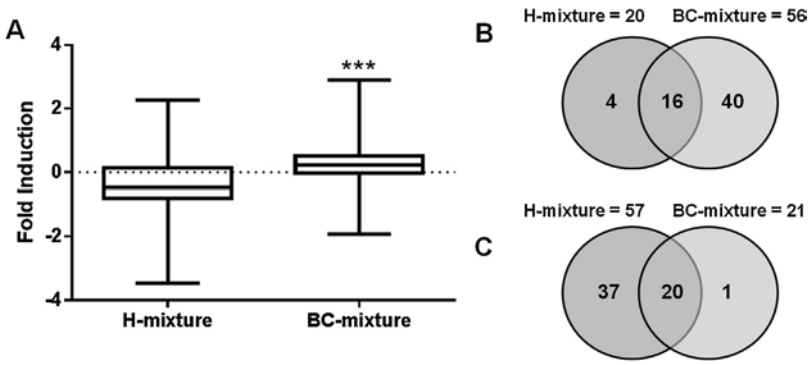

Fig. 2. Effects of healthy mixture (H-mixture) and breast cancer mixture (BC-mixture) on gene expression profiling of primary human mammary epithelial cells. **A**: Box plot showing a statistical evaluation of the differences in the mean expression changes induced by H-mixture and BC-mixture. The lines inside the boxes represent the medians, the boxes cover the 25–75th percentiles, and the minimal and maximal values are shown by the ends of the bars. Median value for H-mixture = −0.47, median value for BC mixture = 0.24. $^{***}p < 0.0001$. **B**: Venn diagram showing the number of genes up-regulated by H-mixture and BC-mixture. The overlapping area shows genes for which expression was altered by both pesticide mixtures. Value of universal (U), defined as the total number of genes represented in the diagramwas 76 genes. **C**: Venn diagram showing the number of genes down-regulated by H-mixture and BC-mixture. The overlapping area shows genes for which expression was altered by both pesticide mixtures. $U = 78$ genes.

Table 2
Genetic phenotypes and characteristics of the 3 most regulated genes by the healthy (H-mixture) and breast cancer (BC-mixture) organochlorine compound mixtures in HMEC. Fold induction ± standard deviation (SD) are included.

Gene symbol	Unigene	Location	Gene ontology (Function)[a]	Fold induction
H-mixture				
LRRC17	Hs.567412	7q22.1	Bone marrow development	2.26 ± 0.44
NTRK2	Hs.494312	9q22.1	ATP binding	1.99 ± 0.69
GFRA3	Hs.58042	5q31.1	Axon guidance receptor activity	1.28 ± 0.01
EPHA3	Hs.123642	3p11.2	ATP binding	−3.46 ± 0.07
DDR2	Hs.275757	1q23.3	ATP binding	−2.49 ± 0.07
EPHB6	Hs.380089	7q33	ATP binding	−2.47 ± 0.01
BC-mixture				
FLT4	Hs.646917	5q35.3	Growth factor binding	2.90 ± 0.01
NTRK2	Hs.494312	9q22.1	ATP binding	2.32 ± 0.55
EPHB1	Hs.116092	3q21	ATP binding	1.84 ± 0.01
ALK	Hs.654469	2p23	NF-kappaβ-inducing kinase activity	−1.92 ± 0.02
RET	Hs.350321	10q11.2	ATP binding	−1.66 ± 0.07
EPHA3	Hs.123642	3p11.2	ATP binding	−1.18 ± 0.53

[a] Information obtained from the National Library of Medicine (NLM), available at www.ncbi.nlm.nih.gov/gene.

The inhibition of the ATP binding process observed in the present study agrees with an inhibition of the ATPase activity by OCs recently reported by other authors (Bircsak et al., 2013). Such inhibition could decrease the removal of chemicals from the cell and, therefore, an alteration of the disposition of xenobiotics is possible. Furthermore, there are evidences of a close interaction between other environmental pollutants (*i.e.* polycyclic aromatic hydrocarbons) and the ATP-binding cassette (ABC) system in primary human hepatocytes (Le Vee et al., 2015), which may induce alteration of the expression of genes associated to the transport of xenobiotics, and even disruption of lipid homeostasis (Layeghkhavidaki et al., 2014). Our results need additional research to further characterize the functional regulation of ATP binding activity and determine whether OCs may alter the transporter-mediated disposition of other chemicals. In addition, other biological processes such as "bone marrow development" or "nuclear factor *kappa*-light-chain-enhancer of activated *B* cells (NF-kappaB) inducing kinase activity" were also modified through the regulation of leucine rich repeat containing 17 (*LRRC17*) and anaplastic lymphoma receptor tyrosine kinase (*ALK*) genes, respectively (Table 2). Both pathways are related to the immune system, which is often disrupted by environmental estrogens and other endocrine disruptors (Narita et al., 2007). NF-kappaB mediates the effects exerted by lindane in testicular cells. Thus, cytoplasmic levels of NF-kappaB are decreased after the exposure to lindane, driven cells to apoptosis in response to this OC (Saradha et al., 2009). We observed a similar pattern of regulation in HMEC (Table 2), especially in those cells exposed to the H-mixture, where *ALK* gene appeared more down-regulated (fold induction = −2.47, data not shown). Thus, the level of expression of *ALK* changed from −2.47 to −1.92 by the action of the H-mixture and the BC-mixture, respectively. This slight induction agrees with other authors who published that *ALK* is expressed in different subtypes of human breast cancer (Perez-Pinera et al., 2007). However, a mixture of OCs including p,p′-DDD, p,p′-DDE, o,p′-DDE, aldrin and dieldrin induced a 30-fold increase in the expression of *ALK* (Valeron et al., 2009), suggesting that not only the combination of substances but also the concentration are critical in the regulation of genes.

Three genes were only present in cells exposed to H-mixture (*NTRK1, PDGFRA*, and *GFRA3*) and 3 genes were only present in cells exposed to BC-mixture (*EPHA5, EPBH1,* and *FLT4*) (Table 3). It has to be highlighted that, despite its up-regulation with one mixture, the expression was completely avoid with the other. The biological consequences of this lack of expression are field for speculation and deserve to be investigated in detail in the future.

A total of 20 and 56 genes were up-regulated by the action of H-mixture and BC-mixture, respectively (Fig. 2B). From them, a total of 16 genes were commonly up-regulated by both mixtures. In the other hand, a total of 57 and 21 genes were down-regulated by the action of H-mixture and BC-mixture, respectively (Fig. 2C). From them, a total of 20 genes were commonly down-regulated by both mixtures. The fold induction for the 36 commonly genes that appeared inversely regulated was −0.525 and 0.250 for H-mixture and BC-mixture, respectively ($p < 0.0001$). These results indicate a clear polarization in the regulation of some genes depending on each OC mixture and support the hypothesis that the effect on genes, and by extension the effect on the cell, depends on the specific mixture of chemicals.

Table 3
Genetic phenotypes and characteristics of the subgroup of genes ($n = 6$) that were regulated only by a pesticide mixture.

Gene symbol	Unigene	Location	OMIM	Phenotype[a]	Fold induction ± SD		
					MIM number	H-mixture	BC-mixture
NTRK1	Hs.406293	1q23.1	191315	Insensitivity to pain, congenital, with anhidrosis Medullary thyroid carcinoma, familial	256800 155240	1.21 ± 0.10	Not regulated
PDGFRA	Hs.74615	4q12	173490	Gastrointestinal stromal tumor, somatic Hypereosinophilic syndrome, idiopathic, resistant to imatinib	606764 607685	1.24 ± 0.01	Not regulated
GFRA3	Hs.58042	5q31.2	605710	Form a complex with RET. No phenotype reported.	NA	1.28 ± 0.01	Not regulated
EPHA5	Hs.654492	4q13.1	600004	EPH/ELK subfamily of PTKs receptor. No phenotype reported.	NA	Not regulated	1.76 ± 0.01
EPHB1	Hs.116092	3q22.2	600600	EPH/ELK subfamily of PTKs receptor. No phenotype reported.	NA	Not regulated	1.84 ± 0.01
FLT4	Hs.646917	5q35.3	136352	Hemangioma, capillary infantile, somatic Lymphedema, hereditary, type IA	602089 153100	Not regulated	2.90 ± 0.03

Abbreviations: RET, Rearranged during Transfection Protooncogene (OMIM 164761); PTK, Receptor protein tyrosine kinases; NA, not available; SD, standard deviation.
[a] Information obtained from the Online Mendelian Inheritance in Man (OMIM), available at www.ncbi.nlm.nih.gov/omim.

3.3. Oncogenes specifically regulated by the H- and BC-mixtures

We calculated the absolute distance of expression for the differentially regulated genes, and the five most differentially regulated genes were shown in Fig. 3. Glial cell line derived neurotrophic factor family receptor alpha 1 (*GFRA1*) was down-regulated by the exposure of cells to the H-mixture but was up-regulated by the exposure to the BC-mixture (fold induction = −1.20 and 0.83, respectively; absolute distance = 2.03). *GFRA1*, together with other proteins, binds artemin (*ARTN*), which has been implicated in promoting oncogenicity, tumor growth and invasiveness in diverse human malignancies including breast cancer (Kang et al., 2009). It has been published that the expression of *GFRA1*, especially when combined with *ARTN* expression, may be useful predictors of disease progression and outcome in specific subtypes of mammary carcinoma (Esseghir et al., 2007; Wu et al., 2013). Moreover, mRNA levels of *GFRA1* are higher in patients younger than 35 years old and in advanced stages of the disease (Wu et al., 2013). The fact that the BC-mixture exerted a clear up-regulation of that gene is of interest, and offers a novel role of environment on gene regulation that deserves attention, especially in a region with high levels of mortality due to the disease (Cabanes et al., 2009). It has to be highlighted that early age of diagnosis and advanced stages (III–IV) are well known bad prognosis factors for BC.

The fold induction of v-kit Hardy–Zuckerman 4 feline sarcoma viral oncogene homolog (*KIT*) was 1.11 and −0.83 for HMEC exposed to 10× of H-mixture and BC-mixture during five days, respectively (absolute distance = 1.94) (Fig. 3). *KIT* is a proto-oncogene located at 4q12 that encodes a transmembrane receptor tyrosine kinase named *c-kit* (Bose et al., 2010). The expression and function of *c-kit* in breast cancer is a quite controversial subject, but several studies have proposed that the loss of *c-kit* expression is associated with tumor progression even for BC (Roussidis et al., 2007). As reported above, we observed a down-regulation of *KIT* in HMEC exposed to the BC-mixture, reinforcing an environmental regulation of that gene, as it was previously reported (Valeron et al., 2009).

BC-mixture caused 1.25-fold increase in the expression of basic helix-loop-helix family, member a15 (*BHLHB8*) (fold induction = −0.37 for H-mixture; absolute distance = 1.62) (Fig. 3). We report here for the first time the expression of *BHLHB8* in HMEC and its regulation by different mixtures of OCs. This gene is a tissue-restricted Class II basic helix-loop-helix (*bHLH*) transcription factor expressed in lactating mammary glands which is essential for the maintenance of the fully differentiated alveolar state (Zhao et al., 2006). It is also expressed by human neoplastic and non-neoplastic plasma cells (Yeung et al., 2012) and is down-regulated in gastric chief cells undergoing experimentally induced metaplasia (Lennerz et al., 2010). The consequences of this regulation for cell's fate are unknown and deserve deep research.

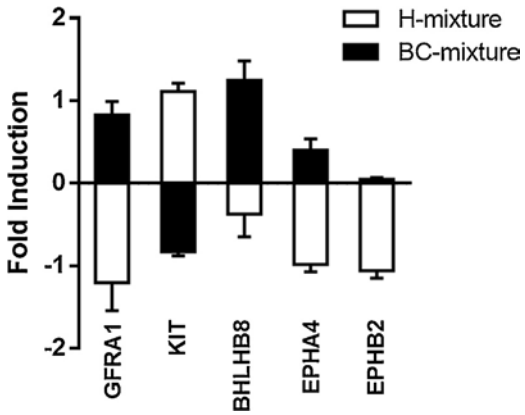

Fig. 3. Effects exerted by H-mixture and BC-mixture of OCs on the five genes that showed the most different regulation: GFRA1, KIT, BHLHB8, EPHA4 and EPHB2 mRNA levels. HMEC were exposed to the 10× OC mixtures for 5 days. 18S expression was performed for each sample as housekeeping gene. The results shown represent the fold changes of each sample relative to the reference sample.

Finally, *EPHA4* and *EPHB2* genes were down-regulated in HMEC exposed to 10× of the H-mixture during five days (fold induction = −0.98 and −1.06, respectively; absolute distance with BC-mixture = 1.38 and 1.11, respectively) (Fig. 3). The role of Eph receptors in cancer is extremely complex and remains controversial, with evidence suggesting both tumor promoting and tumor suppressive functions (Miguelena Bobadilla et al., 2015; Ruiz-Suarez et al., 2015). However, several pre-clinical and laboratory studies support the function of Eph receptor tyrosine kinases in growth, metastasis, and neovascularization of breast cancer (Miguelena Bobadilla et al., 2015). It has to be taken into account that there are 14 receptors (9 class A and 5 class B) and 8 ligands (5 class A and 3 class B) present in the human genome, with expression patterns that often overlap and promiscuous interaction between ligands and receptors that include bi-directional signaling and pleiotropic functions which makes highly difficult to discriminate the real role of specific genes of this family (Ruiz-Suarez et al., 2015). In general terms, expression of many of the Eph receptors is often elevated in a wide variety of tumors, including breast cancer (Miguelena Bobadilla et al., 2015). We have addressed the expression of these genes in HMEC, which is a non-carcinogenic primary cell line. It has been reported that ephrin-induced Eph receptor forward signaling in nontransformed mammary epithelial cells appears to transduce an inhibitory signal that may keep cells quiescent and noninvasive (Rivero et al., 2015; Boada et al., 2015). We observed that the H-mixture down-regulated the expression of some members of the family, which were slightly up-regulated by the exposure of cells to the BC-mixture (fold induction = 0.4 and 0.05 for *EPHA4* and *EPHB2*, respectively). Taken together, our results reinforce the role of *EPHA4* and *EPHB2* in the breast tissue and suggest a novel environmental regulation of these genes which could be relevant for breast carcinogenesis.

4. Conclusions

The present work attempted to evaluate the potential effects exerted by environmentally relevant OC mixtures found in healthy women and breast cancer patients on normal human epithelial breast cells. Our results confirm that the effects exerted by these two different OC mixtures on the gene expression profile of HMEC were clearly different. While BC-mixture up-regulated the expression of oncogenes associated to breast cancer (GFRA1 and BHLHB8), H-mixture down-regulated the expression of tumor suppressor genes (EPHA4 and EPHB2). The induction of oncogenes and inhibition of tumor suppressor genes are two mechanisms necessary for the initiation of oncogenesis. Our findings suggest that both OC mixtures regulate genes associated with carcinogenesis but in different ways and we pointed up an environmental regulation for a subset of kinases that would help to understand the breast carcinogenesis induced by organochlorine compounds. Different cell mechanisms seem to be compromised by the actions of these compounds, at least on a theoretical level, reason for which the present results have to be taken with caution and considered as a hypothesis generating study. Finally, further research is needed to understand the biological consequences of such regulation, and validation of gene regulation as well as evaluation of consequences at protein level is also needed.

Conflict of interest

The authors declare that there is no conflict of interests.

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ARTÍCULO 3

Simvastatin down-regulates differential genetic profiles produced by organochlorine mixtures in primary breast cell (HMEC)

La Simvastatina disminuye la regulación de perfiles génicos diferenciales producidos por mezclas de organoclorados en células de mama primarias (HMEC)

OBJETIVO 4

Evaluar los efectos de la Simvastatina en los patrones de expresión génica inducidos por la mezcla de OCs encontrada en pacientes con cáncer de mama.

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Letter to the Editor

Simvastatin down-regulates differential genetic profiles produced by organochlorine mixtures in primary breast cell (HMEC)

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ABSTRACT

Women all over the world are exposed to an unavoidable contamination by organochlorine pesticides and other chemical pollutants. Many of them are considered as xenoestrogens and have been associated with the development and progression of breast cancer. We have demonstrated that the most prevalent pesticide mixtures found in healthy women and in women diagnosed with breast cancer modulates the gene expression in human epithelial mammary cells. Statins are well-known cholesterol-depleting agents acting as inhibitors of cholesterol synthesis. Since the early 1990s, it has been known that statins could be successfully used in cancer therapy, including breast cancer, but the exact mechanism behind anti-tumor activity of the statins remains unclear. In the present study we evaluated the effect of simvastatin in the gene expression pattern induced by realistic organochlorine mixtures found in breast cancer patients. The gene expression of 94 genes related with the cell signaling pathways were assessed. Our results indicate that simvastatin exerts a global down regulating effect on successfully determined genes (78.7%), thus attenuating the effects induced by organochlorine mixtures on the gene profile of human mammary epithelial cells. This effect was more evident on genes whose function is the ATP-binding process (that also were particularly up-regulated by pesticide mixtures). We also found that MERTK (a proto-oncogene which is overexpressed in several malignancies) and PDGFRB (a member of the platelet-derived growth factor family whose expression is high in breast-cancer cells that have become resistant to endocrine therapy) were among the genes with a higher differential regulation by simvastatin. Since resistance to treatment with tyrosine kinase inhibitors is closely related to MERKT, our findings would enhance the possible utility of statins in breast cancer treatment, i.e. improving therapeutic results combining statins with tyrosine Kinase inhibitors.

1. Introduction

Breast cancer (BC) is the most common cancer diagnosed in women worldwide [1]. The etiology of BC is complex, involving genetic, epigenetic and environmental factors [2]. Among them, the best established factors contributing to BC are related to cumulative exposure of the breast tissue to estrogens [3]. However, other environmental factors have been proposed as risk factors for BC during the past decades, including smoking habits [4], alcohol intake [5], obesity, diet [6,7], and exposure to environmental pollutants [8,9]. Among environmental pollutants, exposure to organohalogenated contaminants, specially organochlorine pesticides (OCPs), has been linked to BC etiology due to their endocrine-disrupting properties (estrogenic or antiandrogenic effects) [10–12] and to their chemical characteristics (high lipophilicity and resistance to biotransformation) that result in their accumulation in the environment, food, biota, and human beings worldwide [13,14]. In this context, the ubiquity and universal presence of OCPs in the environment is a matter of public health concern [15–17]. Despite the fact that most OCPs were banned in Western countries in the late 1970's, most Western populations present a basal and unavoidable degree of contamination by OCPs [18–20].

Most OCPs are considered as xenoestrogens and may modulate steroid sex hormones homeostasis [21]. In fact, there are a number

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of studies that seem to indicate that prolonged exposure to environmental estrogens may play a critical role in the cellular and molecular changes that occur during breast carcinogenesis [22–24]. These changes may influence cellular events to facilitate cell proliferation, avoid apoptosis and achieve invasive characteristics [25,26]; and these changes may be conditioned by individual compounds or mixtures of compounds [27]. It is well known that the activation of membrane receptors and/or regulation in gene expression are the main molecular mechanisms and signaling pathways that underline the action of chemicals involved in BC development [28–30]. Nonetheless, the association between environmental pollutants and increased risk of BC is nowadays controversial and needs further research. Thus, while several earlier studies suggested a positive association [24,31–34], other studies showed no increased risk [16,35,36]. These opposite results may be explained by the fact that most studies focused in the study of isolated compounds, and they did not take into account that human beings are exposed simultaneously to multiple OCPs. The biological effects exerted by the mixture of OCPs vary considerably from those exerted by isolated chemicals [27,37]. In that sense, we recently described that the mixture of the most frequently OCPs found in women diagnosed with BC from a case-control study in Spain, up-regulated the expression of oncogenes associated to breast cancer in human mammary epithelial cells [21]. These findings indicate that environmental pollutants may modulate the expression of genes associated with carcinogenesis, thus possibly influencing the incidence of the disease in certain populations in relation to the level of environmental contamination by OCPs.

Statins, widely prescribed cholesterol-lowering drugs in the primary and secondary prevention of cardiovascular disease, act by reducing cholesterol synthesis through the inhibition of 3-hydroxy-3-methylglutayl coenzime A reductase (HMGCR), the rate-limiting enzyme of the mevalonate pathway [38,39]. Despite their traditional use in hypercholesterolemia and other cardiovascular diseases, increasing research has addressed the favorable anticancer effects exerted by statins [40–43], even for breast cancer [44]. HMGCR has been suggested to harbor oncogenic potential and deregulation of the mevalonate pathway [45] and *in vitro* studies using lipophilic statins have shown reduced tumor cell proliferation, low invasiveness, and higher survival. In addition, *in vivo* studies have confirmed statin-induced tumor growth inhibition associated with reduced tumor cell proliferation and survival [46–49].

Previous statin breast cancer trials have reported changes in single genes, mainly affecting the gene expression profile of kinases, such as Extracellular-signal-Regulated Kinase (ERK) or c-Jun N-terminal kinases (JNK) [50], but statin-mediated changes in cancer specific whole-genome expression profiles have not been reported. Gene expression profiling has been extensively used to classify tumors, to identify biologic signatures and to search for novel biomarkers [51,52]. The comparison of gene expression profiles after a given treatment enables identification of important signaling pathways and may detect transcriptional responses to a specific therapy [53].

As statins and OCPs seem to modulate kinases signaling pathways and because statins are being administered as part of breast-cancer therapy, alone or in combination with varied inhibiting compounds of tyrosine kinase-receptors [49,50], the aim of our study was to explore if statins (specifically simvastatin) may affect the gene expression pattern induced by a realistic organochlorine mixtures in primary human mammary epithelial cells; and secondarily, to evaluate the modulation exerted by simvastatin on the expression of genes potentially related with BC development to explore the possibility that the wide use of these drugs may play a role in environmentally-induced BC.

2. Materials and methods

2.1. Chemicals

Pesticides (lindane, aldrin, dieldrin, endrin, *p,p'*-DDE, *p,p'*-DDD, and *p,p'*-DDT) were purchased from Dr. Ehrenstorfer, Reference Materials (Augsburg, Germany). Simvastatin, dimethylsulfoxide (DMSO), and all other products common cited throughout this work were purchased from Sigma (Saint Louis, MI, USA) unless stated otherwise. The OCPs were dissolved in ultrapure DMSO to prepare stock solutions (1 mg/mL) and stored at −20 °C until use.

2.2. OCP mixtures

Since humans are exposed to mixtures of OCPs and not to isolated compounds, we previously assayed the effect exerted by the mixtures detected in a population-based study formed by 103 healthy women and 121 women diagnosed with BC from the Canary Islands, Spain [12]. In order to test environmentally relevant concentrations (as close as possible to the levels found in human beings), the mixture chosen for this study was the 10x-mixture observed in the upper bound (95th percentile) of prevalent OCPs found in the serum of women diagnosed with BC (BC-mixture: lindane plus aldrin plus dieldrin plus endrin plus *p,p'*-DDE plus *p,p'*-DDD plus *p,p'*-DDT) and healthy women (H-mixture: lindane plus aldrin plus dieldrin plus *p,p'*-DDE plus *p,p'*-DDD plus *p,p'*-DDT). Doses of H-mixture and BC-mixture were previously assessed and published (Table 1) [21]. Simvastatin was added to the OCP mixtures at a final concentration of 10 μM (H-SimV and BC-SimV, respectively). The OCP mixtures were dissolved in DMSO and diluted in phenol red-free Mammary Epithelial Basal Medium (MEBM) purchased from Lonza (Walkers-ville, MD, USA). Final DMSO concentration was ≤0.001% in all experiments.

2.3. Cell lines and culture conditions

The primary human breast epithelial cell line (HMEC) was purchased from Lonza (Walkersville, MD, USA). As guaranteed by the provider, this cell line has all the characteristics of the normal epithelium, including genetic characteristics. HMEC cells were cultured in phenol red-free MEBM containing the required growth supplements suggested by the manufacturer. HMEC were incubated in 5% CO_2 atmosphere at 37 °C. In each experiment, OCP mixture plus simvastatin (10 μM) was added to the fresh medium. All assays were performed at least in triplicate and each dose-group within an experiment was assayed using triplicate wells.

2.4. Protein kinases transcriptional regulation arrays

Simvastatin was added in the indicated concentrations 36 h before harvesting cell. HMEC were exposed to the 10x OCP

Table 1
H-mixture and BC-mixture 1X final concentrations (nM) calculated from the median levels of organochlorine pesticides (ng/g lipid) previously described in a population-based case-control study [21].

Compound	Healthy women (H-mixture)	Breast cancer patients (BC-mixture)
Aldrin	2.8	3.2
Dieldrin	1.2	1.9
Endrin	33.6	0.0
Lindane	3.8	7.6
p,p'-DDE	22	20
p,p'-DDD	4	34.6
p,p'-DDT	40	9.2

mixtures during five days. Thereafter, total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) in accordance with the instructions of the manufacturer. The required quantities of RNA were measured with a NanoDrop ND-1000 UV–Vis Spectrophotometer (NanoDrop, Wilmington, DE, USA). The ratio quality/integrity was verified in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). First-strand cDNAs were generated from 0.5 μg of the total RNA using the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was performed using TaqMan® Low Density Array (Applied Biosystems, Human Protein Kinase Array: Cat # 4367784). Each mix reaction containing 5 μl of reverse transcribed RNA and 50 μl of Taqman Universal PCR Master Mix (Applied Biosystems) was done on an ABI Prism7900HT (Applied Biosystems) in a final volume of 100 μl. As stated previously [21], conditions for amplification were as follows: 1 cycle of 94.5 °C for 2 min, followed by 40 cycles of 97 °C for 30 s and 59.7 °C for 1 min. The raw data were recorded with the SDS 2.2.2 software of the instrument. Differential expression was determined by delta–delta Ct method. 18S expression was performed for each sample as housekeeping gene. All assays were performed at least in quadruplicate. Gene expression was normalized against the housekeeping gene 18S and the fold induction was calculated comparing the regulation exerted by the mixtures against the control DMSO. Mean values for each probe were $\log_2$ transformed to have an escalated interpretation of the expression ratios. The TaqMan Low Density array contains assays for 68 human kinases and 26 non-kinases genes involved in signal transduction and mediate protein–protein interaction, transcriptional regulation, neural development, or cell adhesion [21].

2.5. Western blot analysis

For protein expression assay, cells from different experimental conditions were collected. The lysis was performed in a lysis buffer containing 50 mM Tris-ClH pH 7.6, 5 mM EDTA, 0.5% Triton X-100, 0.5% sodium deoxycholate, 15 mM β-glycerophosphate, 10 mM $Na_2P_2O_7$, 50 mM NaF, 200 μM orthovanadate, 20 μg/mL aprotinin, 20 μg/mL leupeptin and 1 mM phenylmethylsulfonyl fluoride. Twenty micrograms of total proteins were analyzed by SDS-electrophoresis on 10% polyacrylamide gels (SDS-PAGE). After transfer proteins to membrane (Trans-blot Turbo Mini PCDF, Bio-Rad), the blots were incubated with the corresponding specific primary antibodies against RhoA (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-p42/p44-MAPK or beta-actin (1:2000, Cell Signaling Technology, Danvers, MA). Immunocomplexes were visualized by enhanced chemiluminescence detection (Immun-Star-HRP-Chemiluminescence and ChemiDoc XRS System, Bio-Rad) with the use of a HRP-conjugated goat anti-mouse or a HRP-conjugated goat anti-rabbit secondary antibodys (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.6. Statistical analysis

The data were expressed as the means ± SD. The significance of differences between the groups was tested by ANOVA test. GraphPad Prism 6 program (GraphPad Software, San Diego, CA) was used for statistics. Statistical significance was reported if $p < 0.05$ was achieved (two tailed).

3. Results and discussion

Women all over the world suffer an inadvertent exposure to OCPs, and these environmental pollutants are well known endocrine disrupters that have been proposed as risk factors for BC. It has been described a differential gene expression pattern in human cells exposed to different OCP mixtures, but the biological relevance of this result is still unclear [21,54]. In the present study, we evaluated if such differential gene expression pattern found in HMEC exposed to environmentally relevant OCP mixtures could be affected by the intake of statins, a class of widely prescribed drugs potentially associated with the evolution of the disease.

3.1. Cytotoxic effect of the H- and BC-mixtures plus simvastatin (H-SimV and BC-SimV)

We assessed the cytotoxic response induced by these two OCP mixtures plus simvastatin in HMEC. We did not observe statistically significant differences between concentrations at 1x and 10x compared with the control. In these mixtures, we tested different concentrations of simvastatin at different times in the range of 36–48 h. The concentrations and time chosen for this trial did not produce differences in cell viability compared to controls (data not shown).

3.2. Global protein kinases transcriptional regulation

The tyrosine kinase (TK) family includes many growth factor receptors, cell cycle regulators, and oncoproteins that play an important role in the cell growth and differentiation of normal cells. The TKs represent a major class of proto-oncogenes and may be involved in the progression and metastasis of cancer cells. Thus, in this work, the gene expression profile of 94 kinases- and non-kinase genes was studied after the exposure of HMEC to two OCP mixtures (H-mixture and BC-mixture) plus simvastatin (H-SimV and BC-SimV). Of 94 genes, a total of 74 genes were successfully determined (78.7%).

For the global expression analysis, we included all the genes that were regulated by the OCP mixtures after normalization by 18S gene. We observed that the global gene expression profile was not significantly different in HMEC exposed to the H-SimV compared to HMEC exposed to BC-SimV (Fig. 1A). Thus, fold-induction was −0.312 ± 0.162 and −0.279 ± 0.118 for H-SimV and BC-SimV, respectively (mean ± standard deviation), suggesting that the presence of simvastatin attenuate the changes in genetic profiles produced by both H-mixture and BC-mixture without simvastatine. As described previously [21], the mean global profile of expression induced by H-mixture and BC-mixture were 0.397 and 0.243, respectively ($p < 0.0001$).

As showed in Fig. 1B and C, a total of 29 genes were up-regulated in HMEC exposed to H-SimV (45 genes were down-regulated). On the other hand, 20 genes were up-regulated in HMEC exposed to BC-SimV (54 genes were down-regulated). A total of 19 genes were commonly regulated but in an inverted way. However, the fold induction of these genes was −0.278 and −0.449 for H-SimV and BC-SimV, respectively (difference between means = 0.172, which was not significant). This result indicates that the polarization previously described in gene regulation associated to the specific mixture of chemicals [21] was also smoothed by the presence of simvastatin.

3.3. Effects of simvastatine in the regulation of the expression of specific genes

The most regulated genes for each mixture are detailed in Table 2. Only three of the regulated genes (NTRK2, EPHA3 and ALK) by both H-SimV and BC-SimV mixtures matched with those regulated by these mixtures in the absence of simvastatin as published by Rivero et al. (2016). Of these three genes, the most intensive change in the regulation was referred to EPHA3 which moves from −3.46 to −1.2 fold induction described for the H-mixture and

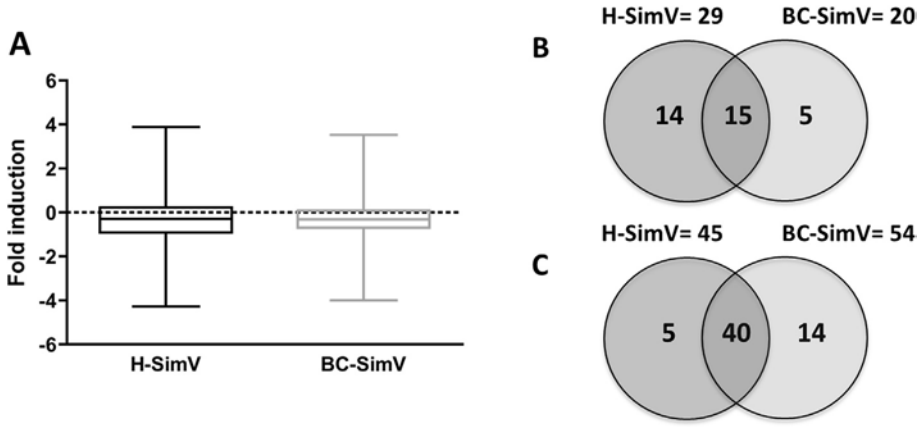

Fig. 1. Effects in presence of simvastatin of healthy mixture (H-SimV) and breast cancer mixture (BC-SimV) on gene expression profiling of breast cancer primary cell line. (A) box plot showing a statistical evaluation of the differences in the mean expression changes induced by H-SimV and BC-SimV mixtures. The lines connect the medians, the boxes cover the 25th to 75th percentiles, and the minimal and maximal values are shown by the ends of the bars. Mean value for H-SimV = −0.312, mean value for BC-SimV = −0.279, (ANOVA test; p, not significant). (B) Venn diagram showing the number of genes up-regulated by H-SimV and BC-SimV mixtures. The overlapping area shows genes for which expression was altered by both pesticide mixtures. Value of universal - U -, defined as the total number of genes represented in the diagram 74 genes. (C) Venn diagram showing the number of genes down-regulated by H-SimV and BC-SimV mixtures. The overlapping area shows genes for which expression was altered by both pesticide mixtures, U = 74 genes.

Table 2
Genetic phenotypes and characteristics of the 4 most regulated genes by the healthy (H-SimV) and breast cancer (BC-SimV) organochlorine compound mixtures in HMEC. Fold induction ± standard deviation (SD) are included.

Gene symbol	Unigene	Location	Gene Ontology (Function)[a]	Fold Induction
H-mixture plus SimV				
NTRK2	Hs.494312	9q22.1	ATP binding	3.88 ± 0.06
EPHA8	Hs.283613	1p36.12	ATP binding	3.13 ± 0.002
KIT	Hs.479754	4q12	Protein binding	3.02 ± 0.02
PDGFRB	Hs.509067	5q33.1	Platelet activating factor receptor	1.80 ± 0.14
MERTK	Hs.306178	2q14.1	ATP binding, protein binding	−4.30 ± 0.05
BHLHB8	Hs.674510	7q21.3	Transcriptional activator	−3.87 ± 0.16
LRRC2	Hs.657345	3p21.31	Tumor supressor	−3.81 ± 0.09
RET	Hs.350321	10q11.2	ATP binding	−3.71 ± 0.05
BC-mixture plus SimV				
LRRC17	Hs.646917	5q35.3	Bone marrow development	3.50 ± 0.34
NTRK2	Hs.494312	9q22.1	ATP binding	2.70 ± 0.10
NTRK1	Hs.406293	1q21-q22	ATP binding, activation MAPKK activity	1.46 ± 0.10
EPHA3	Hs.123642	3p11.2	ATP binding	1.30 ± 0.28
LRRC2	Hs.657345	3p21.31	Tumor supressor	−3.99 ± 0.46
ALK	Hs.654469	2p23	NF-kappaβ-inducing kinase activity	−2.38 ± 0.09
PLXND1	Hs.301685	3q22.1	Protein binding	−2.01 ± 0.90
GRFA1	Hs.388347	10q26.11	Ras guanyl-nucleotide exchange factor activity	−1.58 ± 0.16

[a] Information obtained from the National Library of Medicine (NLM), available at www.ncbi.nlm.nih.gov/gene. Gene expression was normalized against the housekeeping gene 18S and the fold induction was calculated comparing the regulation exerted by the mixtures against the control DMSO.

the BC-mixture [21], to −1.8 and 1.3 with these mixtures plus simvastatin, H-simV and BC-SimV, respectively. NTRK2 and EPHA3 are ATP binding process genes. We have previously reported that the most frequently regulated genes by the exposure to environmentally-relevant OCP mixtures were genes involved in that process [21]. Furthermore, there are evidences of a close interaction between other environmental pollutants (i.e. polycyclic aromatic hydrocarbons) and the ATP-binding cassette (ABC) system in primary human hepatocytes [55], which may induce alteration of the expression of genes associated to the transport of xenobiotics, and even disruption of lipid homeostasis [56]. Our results showed that simvastatin may exert a relevant regulation of genes involved in the ATP binding activity and agree with other authors who have published an association between statins and genes of the ABC system [57]. In cancer, ABC transporters are relevant to resistance to drugs, including chemotherapy and hormone therapy. It is a complex process integrated by dozens of genes. Thus, the induction or inhibition of such process depends on complex interactions established among such genes. Nevertheless, our result reinforces our previous findings suggesting that OCPs may alter the transporter-mediated disposition of other chemicals [21].

Similarly, other biological processes such as “bone marrow development” or “nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kappaB) inducing kinase activity” deserved our attention. As previously reported, these processes are modified through the regulation of leucine rich repeat containing 17 (LRRC17) and anaplastic lymphoma receptor tyrosine kinase (ALK) genes, respectively [58]. Both pathways are related to the immune system, which is often disrupted by environmental estrogens and other endocrine disruptors [58]. Furthermore, NF-kB mediates the effects exerted by other OCPs, such as lindane, in testicular cells [59]. Our findings in HMEC also showed a similar pattern of regulation, mainly in those cells exposed to the BC-SimV, where LRRC17 was the most up-regulated gene (fold induction = 3.5) (Table 2). In addition, the level of expression of ALK changed from −1.92 (BC-mixture alone) to −2.38 (by the action of the BC-SimV). This slight

variation agrees with other authors who published that ALK is expressed in different subtypes of human breast cancer [60].

With regard to the expression of other genes that were differentially regulated by the two OCP mixtures, it has drawn our attention that glial cell line derived neurotrophic factor family receptor alpha 1 (GFRA1). This gene was clearly down-regulated by the exposure of HMEC to both H-SimV and BC-SimV (Fig. 2A), but it was up-regulated by the exposure to the BC-mixture [21]. GFRA1 binds artemin (ARTN), which is implicated in promoting oncogenicity, tumor growth and invasiveness in diverse human malignancies including BC [61]. GFRA1 and ARTN expression may be useful predictors of disease progression and outcome in specific subtypes of mammary carcinoma [62,63]. The fact that the simvastatin exerts a clear down-regulation of that gene is of great interest, and deserves further studies especially in relationship to BC prevention and treatment. Similarly, our group have previously described a down-regulation of v-kit Hardy–Zuckerman 4 feline sarcoma viral oncogene homolog (KIT) in HMEC exposed to the BC-mixture, reinforcing an environmental regulation of that gene [21,27]. As previously reported, absolute distance fold induction of KIT was 1.94 for HMEC exposed to both, H-mixture and BC-mixture. However, in the present work, our results showed that the fold induction of KIT was 3.02 and 0.42 for HMEC exposed to H-SimV and BC-SimV, respectively (absolute distance = 2.6) (Fig. 2A). The expression and function of c-kit in breast cancer is a quite controversial subject, but several studies have proposed that the loss of c-kit expression is associated with tumor progression even for BC [64]. In this sense, our data may suggest that this gene could be a target in BC treatment. In relation to the basic helix-loop-helix family, member a15 (BHLHB8), our previous results showed that BC-mixture caused 1.25-fold increase in the expression of this gene (fold induction = −0.37 for H-mixture; absolute distance = 1.62) (Fig. 2A). This gene is a tissue-restricted Class II basic helix-loop-helix (bHLH) transcription factor expressed in lactating mammary glands which is essential for the maintenance of the fully differentiated alveolar state [65]. It is also expressed by human neoplastic and non-neoplastic plasma cells [66] and is down-regulated in gastric chief cells undergoing experimentally induced metaplasia [67]. As with other genes, the level of transcript of BHLHB8 decreased by the action of simvastatin (Fig. 2A). In summary, our results indicate that GRFA1, KIT and BHLHB8, followed a similar regulation profile by simvastatin (Fig. 2A).

In addition, we calculated the absolute distance of expression for the five most differentially regulated genes by simvastatin (Fig. 2B). We found that EPHA3 was down-regulated when cells were exposed to H-SimV (fold induction = −1.8, absolute distance = 3.13). In fact, the effect of simvastatin on the induction of EPHA3 by OCP mixtures was turned from −3.4 and −1.8 for H-mix and BC-mix to −1.8 and 1.3 for H-SimV and BC-SimV, respectively. Several pre-clinical and laboratory studies support the function of Eph receptor tyrosine kinases in growth, metastasis, and neovascularization of BC [68]. In general terms, expression of many of the Eph receptors is often elevated in a wide variety of tumors, including BC [69]. We have detected the expression of these genes in HMEC, which is a non-carcinogenic primary cell line. It has been reported that ephrin-induced Eph receptor forward signaling in non-transformed mammary epithelial cells appears to transduce an inhibitory signal that may keep cells quiescent and noninvasive [12]. In any case, we observed that simvastatin had a mild effect on the expression of some members of the family (EPHA4 and EPHB2), which were slightly up-regulated by the exposure of cells to the H-SimV and BC-SimV (Fig. 2A). Taken together, our results reinforce the role of eph receptors family in the breast tissue and suggest a novel environmental regulation of these genes which could be relevant for breast carcinogenesis.

Finally, mer proto-oncogene tyrosine kinase (MERTK) and platelet derived growth factor receptor beta (PDGFRB) were also among the most differentially regulated genes by OCP mixtures containing simvastatin (Fig. 2B). The fold induction of MERTK was −4.27 and −0.13 for HMEC exposed to H-SimV and BC-SimV, respectively (absolute distance = 4.13). The fold induction of PDGFRB was 1.79 and −1.38 for HMEC exposed to H-SimV and BC-SimV, respectively (absolute distance = 3.18). MERTK is a member of Tyro3, Axl, and Mer (TAM) family of RTKs [70] with oncogenic properties that is often overexpressed or activated in various malignancies [71]. PDGFRB encodes a cell surface tyrosine kinase receptor for members of the platelet-derived growth factor family. Ligand binding induces receptor dimerization and intracellular signaling pathways that regulate cell proliferation, survival, chemotaxis and differentiation [72]. In relation to BC, although less is known, the relationship between various members of this family of receptors and different subtypes of BC is supported by various authors [73–75]. Recently, it has been published that PDGF signaling is elevated in breast cancer cells that have become resistant to endocrine therapy [76,77], and a potential role of PGDFR signaling during epithelial to mesenchymal transition (EMT) of breast cancer cells has been suggested. Moreover, it has been suggested a relationship between resistance to treatment with inhibitors of growth factors and MERTK [78,79]. Resistance to treatment with TK inhibitors is closely related to MERKT. This would enhance our findings on the possible role of statins regarding BC prevention and treatment. Extensive regulation of these two genes

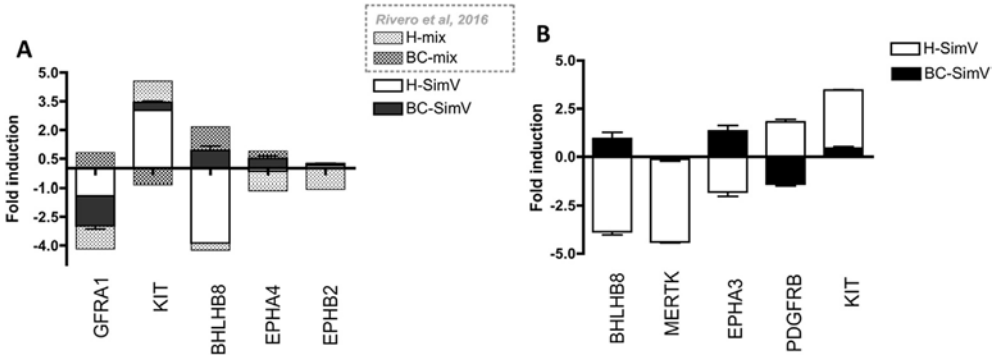

Fig. 2. (A) Comparative effects of the presence of simvastatin on the five genes that previously showed the most different regulation by H-mixture and BC-mixture (according to data previously reported: Rivero et al., 2016 [21]). (B) Effects exerted by H-SimV and BC-SimV on the five genes that showed the most different regulation: BHLHB8, MERTK, EPHA3, PDGFRB and KIT. HMEC were exposed to the 10X OC mixtures for 5 days and 10 μM of simvastatin for 36 h 18S expression was performed for each sample as housekeeping gene. The results shown represent the fold changes of each sample relative to the reference sample.

by the presence of simvastatin could shed some light in the treatment of the disease, i.e. improving therapeutic results combining statins with TK inhibitors.

Taken together, our results indicate that not only the unavoidable exposure to environmental pollutants but also medication could be critical in the regulation of genes involved in BC, and indicate, as suggested by others, that the anticancer effect of statins is a highly complex phenomenon and not only a result of their *de novo* cholesterol-lowering synthesis [50,80].

3.4. Effect of OCPs and simvastatin on the protein expression of Rho-MAPK

Since statins inhibit cholesterol synthesis at an early stage of the biosynthesis pathway, they block the production of isoprenoids, which are necessary for post-translational modifications of many proteins, including small GTPases [81]. Thus, we explored the effects of simvastatin through inhibition of prenylation of Rho proteins and extensive suppression downstream signaling pathways of these proteins, such as MAPK/ERK pathway [82]. To elucidate the effect of simvastatin on HMEC cells exposed to OCPs mixtures plus simvastatin, we focused our study on the expression of RhoA/MAPK/ERK signaling pathway.

Densitometric results suggested that RhoA and MAPK-phosphorylated expression after normalization by actin decrease slightly after exposure to OCP mixtures, in a similar way in mixtures with or without simvastatin, but such differences were not significant (Fig. 3). A similar result was obtained with combinations of other pesticide mixtures that, in the case of positive result, could helped us to clarify aspects of the regulation of these proteins (data not shown). Nonetheless, our results demonstrated that OCP mixtures induce, if any, only a subtle and non-significant effect on signaling pathways RhoGTPases-MAPK. This finding leading us to conclude that the effect of statins in gene regulation, at least in primary cells HMEC, focuses in genes that have been previously modulated by the presence of OCPs.

4. Conclusions

The present study evaluates the potential effects exerted by simvastatin in combination with environmentally relevant OCP mixtures on normal human epithelial breast cells, in an attempt to understand the molecular mechanisms behind the antioncogenic role assigned to statins. The introduction of simvastatin to the OCP mixtures attenuated the gene expression changes induced by the OCP mixtures alone. This fact might indicate a potential role for simvastatin in BC development, and give novel data about the potential utility of statins as agents for BC prevention and treatment. However, further research is needed to understand the biological consequences of such a regulation, a validation of gene regulation, as well as an evaluation of alternative pathways potentially linked to the action of simvastatin.

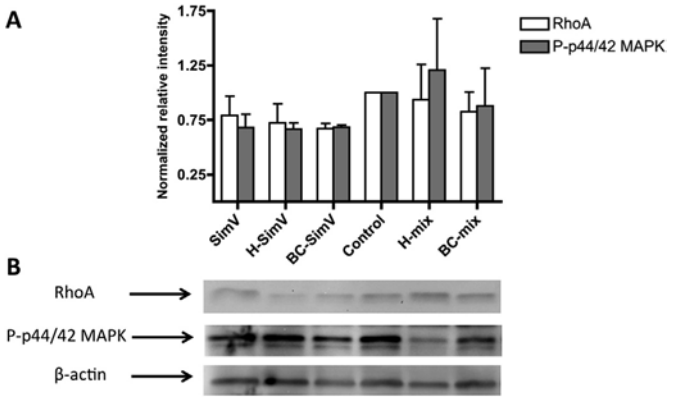

Fig. 3. Effect of organochlorine mixtures and simvastatin alone or in combination on RhoA expression and p44/42 MapK phosphorylation in HMEC cells. Cells were exposed to the 10X OC mixtures for 5 days and 10 μM of simvastatin for 36 h. (A) Bar diagram representing densitometry data (means ± SD) of three independent experiments. (B) Representative images of each protein. Equal loading is normalized with anti-β-actin in all blots.

Conflict of interest statement

The authors declare that there is no conflict of interests.

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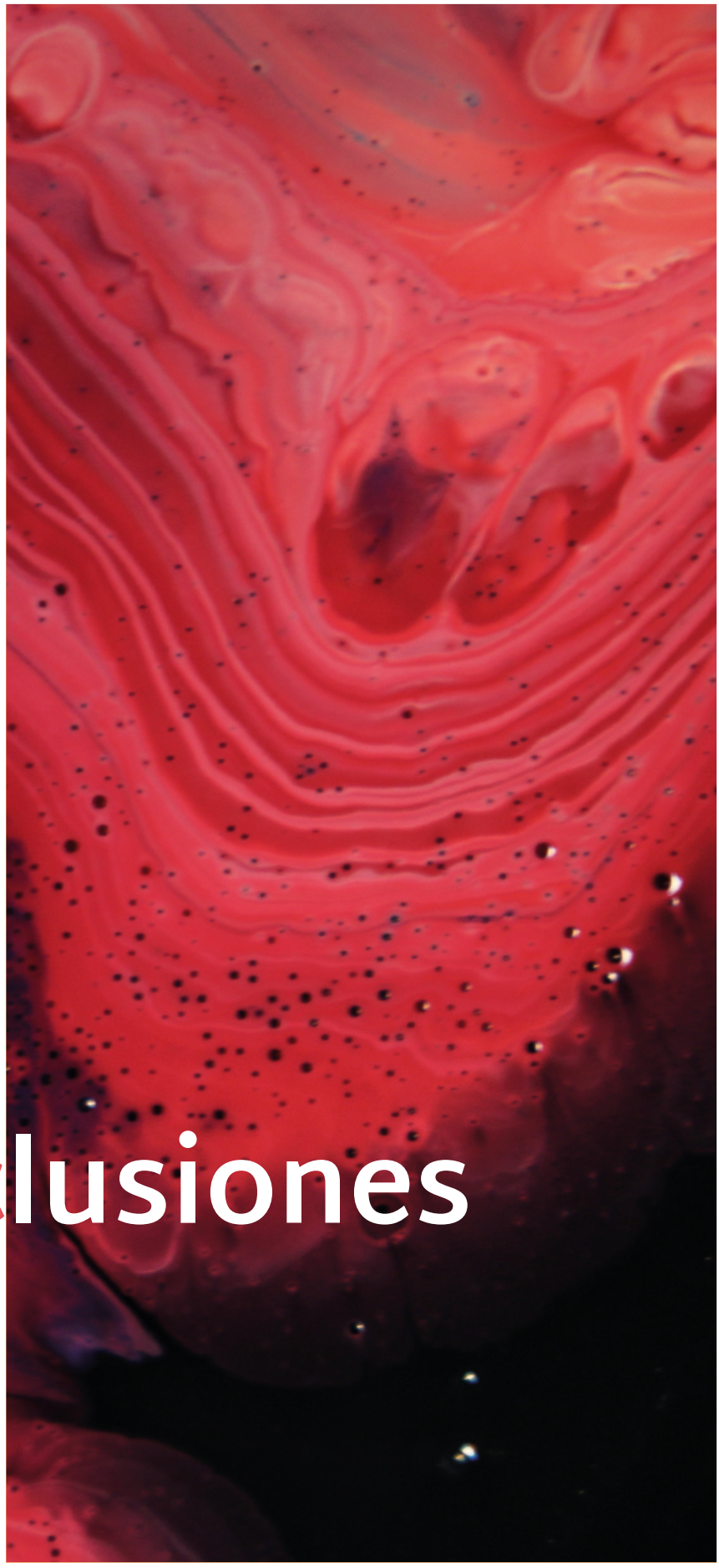


Conclusiones

1· Ambas mezclas tienen más efectos proliferativos que el estradiol a las dosis existentes en humanos (1X) y a dosis de 10X y 100X, presentando efectos citotóxicos significativos a 500X.

2· Ninguna mezcla presenta efectos estrogénicos a las dosis encontradas en humanos (1X), aunque sí exhiben la actividad estrogénica significativamente a concentraciones mayores (100X y 300X).

3· La mezcla de OCs encontrada en pacientes con cáncer de mama presenta actividad antiandrogénica incluso a bajas concentraciones (1X) lo cual, unido a la actividad estrogénica de esta mezcla a concentraciones superiores, sugiere que podría interferir en el incremento de la proliferación celular del cáncer de mama.

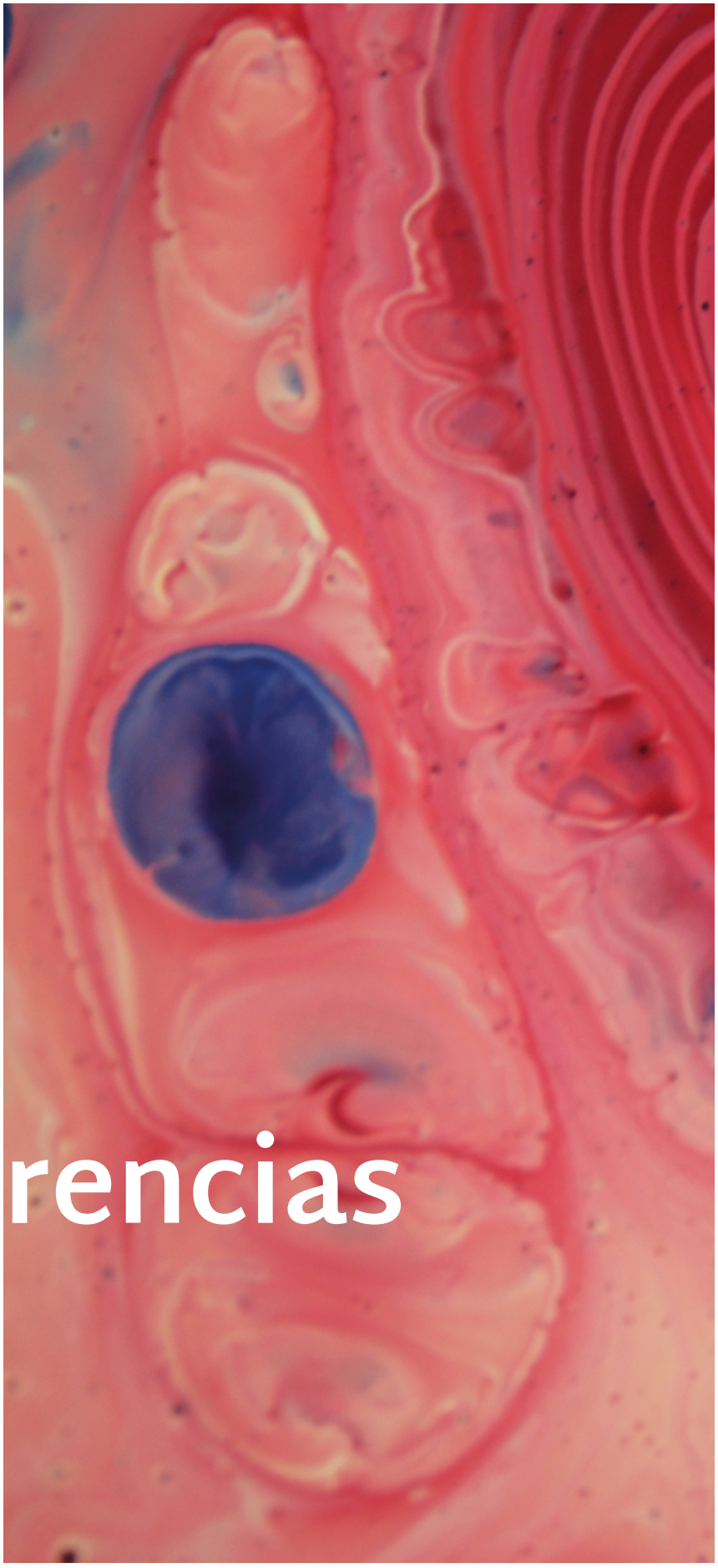
4· Ninguna de las mezclas de OCs presenta diferencias en la proliferación celular a las concentraciones reales encontradas en humanos, mostrando efectos citotóxicos a concentraciones más altas (50X y 100X).

5· El patrón de expresión génica en HMEC es significativamente diferente para ambas mezclas, lo cual sugiere que las mezclas de OCs pueden causar efectos diferenciales en células epiteliales de mama normales.

6· Mientras que la mezcla de OCs encontrada en pacientes con cáncer de mama aumenta la expresión de oncogenes asociados al cáncer de mama (GFRA1 y BHLHB8), la mezcla de OCs encontrada en pacientes sanas disminuye la regulación de genes supresores de tumores (EPHA4 y EPHB2).

7· Ninguna de las mezclas de OCs con Simvastatina produce efectos citotóxicos significativos a concentraciones bajas (1X y 10X).

8· La Simvastatina disminuye la expresión global en determinados genes, atenuando los efectos inducidos por las mezclas de OCs sobre el perfil génico de las células epiteliales de mama humana.



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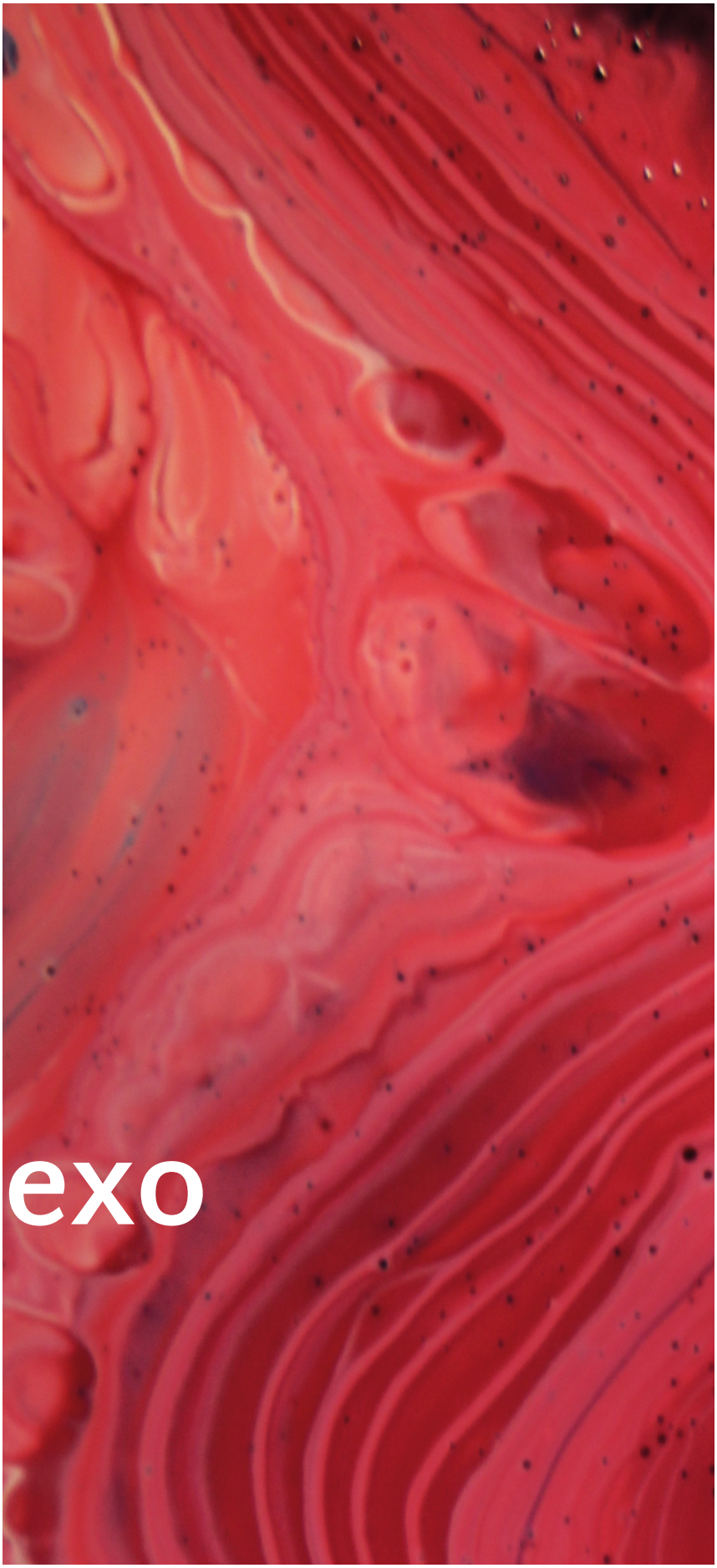

Anexo

ENCUENTRO INTERNACIONAL DEL MEDITERRÁNEO
CARTAGENA
24, 25 y 26 de junio
2015

XIII CONGRESO ESPAÑOL DE SALUD AMBIENTAL

ABRIENDO EL MEDITERRÁNEO A NUEVOS HORIZONTES

Organizan: SESA Sociedad Española de Salud Ambiental · Universidad Politécnica de Cartagena · Universidad de Murcia · CMN Campus Mare Nostrum · Región de Murcia

IX Conferencia Nacional de Disruptores Endocrinos

CERTIFICADO DE PRESENTACION DE COMUNICACIÓN CORTAS

Los siguientes autores

Henríquez-Hernández, L.A.; Boada, L.D.; Luzardo, O.P.; Rivero, J.; Zumbado, M.; Valerón, P.F

han presentado la siguiente **Comunicación Corta**

Evaluación in vitro de las mezclas de pesticidas organoclorados descritas en población femenina sana y afecta de cáncer de mama

en la Sesión de Comunicaciones Cortas, celebrada el jueves 25 de junio de 2015 de 16.00 a 17.30h, en la **"IX Conferencia Nacional de Disruptores Endocrinos"** en Cartagena

Y para que conste firmamos el presente certificado en

Cartagena, a 26 de junio de 2015.

Dra. Stella Moreno Grau
Presidenta del Comité Organizador

Dr. Alberto Torres Cantero
Presidente del Comité Científico

Reconocido de Interés Sanitario por el Ministerio de Sanidad, Servicios Sociales e Igualdad. 15/167.07 BG/mja

Felipe VI, Rey de España

y en su nombre El

Rector de la Universidad de Las Palmas de Gran Canaria

Considerando que, conforme a las disposiciones y circunstancias previstas por la legislación vigente,

Don Javier Rivero Suárez

nacido el día 7 de julio de 1987 en Las Palmas de Gran Canaria (Las Palmas), de nacionalidad española,

ha superado en junio de 2013,
los estudios conducentes al TÍTULO oficial de

Máster Universitario en Sanidad Animal y Seguridad Alimentaria
por la Universidad de Las Palmas de Gran Canaria

establecido por Acuerdo del Consejo de Ministros de 15 de abril de 2011,
expide el presente título oficial con validez en todo el territorio nacional,
que faculta al interesado para disfrutar los derechos que a este título
otorgan las disposiciones vigentes.

Dado en Las Palmas de Gran Canaria, a 23 de julio de 2018

El interesado, *El Rector,* *El/La Funcionario/a,*

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