Commentaires sur les impacts de l'exposition aux pesticides sur la santé humaine

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Biographie

Depuis septembre 2017, je suis chercheuse postdoctorale à l'École de Santé Publique de l'Université de Montréal. J'ai terminé mon doctorat en biologie en 2017 à l'INRS – Institut Armand-Frappier. Le sujet de recherche de mes travaux de recherche au doctorat portait sur les impacts de plusieurs pesticides (néonicotinoïdes et herbicides à base de glyphosate) sur l'expression et l'activité d'une enzyme indispensable à la synthèse des œstrogènes, l'enzyme aromatase.

À notre connaissance, nos travaux ont été les premiers à démontrer le potentiel de perturbateur endocrinien des néonicotinoïdes chez l'humain. Ces résultats ont été publiés dans des revues de grande qualité en santé environnementale et toxicologie (voir copies PDF en annexe). J'espère grandement que la commission utilisera l'expertise de chercheurs québécois comme moi qui se spécialisent dans l'évaluation de la toxicité humaine des pesticides les plus vendus et utilisés au Québec. Vous trouverez ci-joint un résumé en points formes des résultats obtenus lors de mon doctorat, ainsi que des copies de nos articles.

Résumé des résultats obtenus sur la toxicité des néonicotinoïdes

Nous avons mesuré la capacité de plusieurs pesticides néonicotinoïdes à perturber l'enzyme aromatase, enzyme clé de la synthèse des oestrogènes. Pour ce faire, nous avons utilisé deux modèles cellulaires mimant deux processus physiologiques où les oestrogènes jouent un rôle majeur.

1) **Modèle cellulaire du cancer du sein**

Environ 70% des cancers du sein diagnostiqués au Canada sont de type hormono-dépendant, c'est-à-dire que la surproduction d'oestrogènes dans le micro-environnement des cellules cancéreuses contribuent à leur croissance et à leur division. Il est aussi connu dans la littérature scientifique que dans ce type de cancer, l'aromatase (enzyme responsable de la dernière étape de formation des oestrogènes) est suractivée, ce qui mène à une production trop importante d'oestrogènes, et donc à la croissance des tumeurs. D'ailleurs, la plupart des traitements hormonaux donnés aux patientes atteintes d'un cancer du sein sont des inhibiteurs d'aromatase. Nous avons utilisé un modèle cellulaire mimant de façon très efficace l'activité de l'aromatase dans le cancer du sein de type hormono-dépendant. Nous avons exposé ce modèle cellulaire à des concentrations de néonicotinoïdes (thiamethoxam, thiachloprid et imidacloprid) similaires à ce qui est mesuré dans l'environnement (eau, sol) des régions agricoles au Canada et aux États-Unis, ainsi que ce qui a été mesuré dans des échantillons d'urine provenant de deux cohortes de femmes de la population générale et d'agriculteurs au Japon. **Nos résultats ont démontré que :**

- **Une exposition à des concentrations environnementale de néonicotinoïdes augmentait l'expression et l'activité de l'aromatase**
- **L'augmentation de l'expression de l'aromatase dans notre modèle cellulaire exposé aux néonicotinoïdes suivait le même profil d'expression de l'aromatase qui est normalement observé chez des patientes atteintes de cancer du sein hormono-dépendant**
- **Selon nos résultats, les pesticides néonicotinoïdes ont le potentiel d'être des perturbateurs endocriniens chez l'humain : l'utilisation du principe de précaution devrait être envisagée**

2) **Modèle interaction fœtus-placenta**

Pendant la grossesse, la production d'œstrogène augmente drastiquement. Cette augmentation d'oestrogènes est nécessaire au bon développement du placenta, ainsi qu'à l'établissement de la circulation sanguine utéro-placentaire. Dans nos travaux, nous avons utilisé un modèle de co-culture cellulaire mimant les interactions entre le fœtus et le placenta. Nous avons encore une fois exposé ce modèle cellulaire à des concentrations de pesticides néonicotinoïdes et d'herbicides à base de glyphosate similaire à ce qui est mesuré dans l'environnement. Nos résultats ont démontré que :

- L'exposition de la **co-culture fœto-placentaire** à des **néonicotinoïdes** a **augmenté** l'activité catalytique de l'**aromatase** et la production de deux oestrogènes, soit l'**œstrone** et de **17β-œstradiol**
- **Nous avons également mesuré une forte inhibition de la production d'œstriol**
- **En somme, les pesticides néonicotinoïdes perturbaient la production de 3 oestrogènes dans notre modèle de co-culture foeto-placentaire**
- L'exposition de la co-culture foeto-placentaire à plusieurs concentrations de glyphosate seul n'avait pas d'effet sur l'activité de l'aromatase ou la production d'œstrogènes, CEPENDANT
- **L'exposition de la co-culture foeto-placentaire à plusieurs concentrations d'herbicides à base de glyphosate (ex : formulation commerciale Roundup) perturbait fortement l'activité de l'aromatase et la production d'oestrogènes**
- De ce fait, alors que la réglementation et les doses journalières admissibles en situation d'exposition chronique sont basées exclusivement sur les données de toxicité de l'ingrédient actif (ex : glyphosate), il me semble que nous n'avons pas en main toutes les données toxicologiques pertinentes pour procéder à une évaluation du risque et une réglementation adéquate. Les données de toxicité concernant les formulations, qui sont ce qui est véritablement utilisées par les agriculteurs, devraient être incluses dans le processus de réglementation.
- Plusieurs autres études dans la littérature scientifique ont démontré une faible toxicité du glyphosate et une toxicité beaucoup plus importante des formulations commerciales des herbicides à base de glyphosate, ce qui challenge la présomption des ingrédients actifs vs inertes

Références :

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Effects of Neonicotinoids on Promoter-Specific Expression and Activity of Aromatase (CYP19) in Human Adrenocortical Carcinoma (H295R) and Primary Umbilical Vein Endothelial (HUVEC) Cells

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ABSTRACT

The enzyme aromatase (CYP19; cytochrome P450 19) in humans undergoes highly tissue- and promoter-specific regulation. In hormone-dependent breast cancer, aromatase is over-expressed via several normally inactive promoters (PII, I.3, I.7). Aromatase biosynthesizes estrogens, which stimulate breast cancer cell proliferation. The placenta produces estrogens required for healthy pregnancy and the major placental *CYP19* promoter is I.1. Exposure to certain pesticides, such as atrazine, is associated with increased *CYP19* expression, but little is known about the effects of neonicotinoid insecticides on *CYP19*. We developed sensitive and robust RT-qPCR methods to detect the promoter-specific expression of *CYP19* in human adrenocortical carcinoma (H295R) and primary umbilical vein endothelial (HUVEC) cells, and determined the potential promoter-specific disruption of *CYP19* expression by atrazine and the commonly used neonicotinoids imidacloprid, thiacloprid, and thiamethoxam. In H295R cells, atrazine concentration-dependently increased PII- and I.3-mediated *CYP19* expression and aromatase catalytic activity. Thiacloprid and thiamethoxam induced PII- and I.3-mediated *CYP19* expression and aromatase activity at relatively low concentrations (0.1–1.0 μM), exhibiting non-monotonic concentration–response curves with a decline in gene induction and catalytic activity at higher concentrations. In HUVEC cells, atrazine slightly induced overall (promoter-indistinct) *CYP19* expression (30 μM) and aromatase activity (≥ 3 μM), without increasing I.1 promoter activity. None of the neonicotinoids increased *CYP19* expression or aromatase activity in HUVEC cells. Considering the importance of promoter-specific (over)expression of *CYP19* in disease (breast cancer) or during sensitive developmental periods (pregnancy), our newly developed RT-qPCR methods will be helpful tools in assessing the risk that neonicotinoids and other chemicals may pose to exposed women.

Key words: neonicotinoids; aromatase; promoter-specific; CYP19; H295R; HUVEC

Neonicotinoids are members of a relatively new class of neuroactive insecticides that are used as seed coatings in large quantities to protect crops against pest (Tomizawa and Casida, 2005). Neonicotinoids are registered in 120 countries and their use is steadily increasing (Jeschke *et al.*, 2010), especially in corn, canola, soybeans, and the majority of fruits and vegetables. The insecticidal action of neonicotinoids is based on their relatively high selectivity for nicotinergic receptors in insects, where they act as an agonist of the postsynaptic acetylcholine receptor (Matsuda *et al.*, 2001). Among the most commonly used neonicotinoids are imidacloprid, thiacloprid and thiamethoxam (Jeschke *et al.*, 2010).

Neonicotinoids have been associated with *Colony Collapse Disorder* of honey bees (Girolami *et al.*, 2009; Henry *et al.*, 2012).

Neonicotinoids are systemic insecticides, which means they are soluble in water and absorbed by the tissues of the plant, and bees can be exposed to these chemicals through nectar and pollen (Rortais *et al.*, 2005). An exposure to a non-acutely lethal dose of thiamethoxam and imidacloprid causes a delayed onset of increased mortality in honey bees (Henry *et al.*, 2012) and a decrease in their foraging activity (Decourtye *et al.*, 2004). Neonicotinoids are also toxic to birds and mammals. For example, imidacloprid (2 and 8 mg/kg/day) adversely affects the reproductive system of male rats, by inducing DNA fragmentation, antioxidant imbalance and apoptosis (Bal *et al.*, 2012). Moreover, epididymal weight and sperm concentration were lower in imidacloprid exposed rats (Bal *et al.*, 2012). An exposure of female rats to imidacloprid (20 mg/kg/day) caused a decrease in ovarian weight, induced changes in granulosa cells of follicles (cytoplasmic clumping, lipofuscin accumulation) and altered levels of luteinizing and follicle stimulating hormones and progesterone (Kapoor *et al.*, 2011). Daily oral administration of the neonicotinoid clothianidin to female quails caused abnormal ovarian histology of the granulosa cells and a decrease in glutathione peroxidase 4 and manganese superoxide dismutase, two enzymes that protect against oxidative stress (Hoshi *et al.*, 2014). Furthermore, a 30-day exposure to thiacloprid (112.5 mg/kg) increased the serum levels of free thyroxine and triiodothyronine in rats (Sekeroglu *et al.*, 2014), demonstrating the endocrine disrupting potential of this class of pesticides. Given these observations and the increasing use of neonicotinoids, a much better understanding of their potential effects on human health is needed.

In North America, breast cancer represents a third of all female cancer diagnoses (WHO/UNEP, 2013) and 70 % of breast cancers are estrogen-dependent. In the majority of these cancers, the enzyme aromatase (CYP19) is over-expressed. CYP19 is responsible for the biosynthesis of estrogens, which stimulate the proliferation of estrogen-dependent breast cancer cells (Ghosh *et al.*, 2009). CYP19 is present in a variety of tissues and its gene expression is regulated by different tissue-specific promoters. In normal breast tissue, aromatase is expressed at a low level via the *CYP19* promoter I.4. However, in breast cancer, *CYP19* promoters PII, I.3 and I.7 become active, whereas they are silent in the healthy mammary gland (Bulun *et al.*, 2007). There is evidence that exposure to endocrine disruptors may increase the risk of developing hormone-dependent breast cancer via proestrogenic mechanisms (Birnbaum and Fenton, 2003), although focus has been mainly on estrogen receptor activation (Bouskine *et al.*, 2009; Lemaire *et al.*, 2006). Effects on aromatase and consequences for human health are less well understood. It has been demonstrated that exposure to atrazine, a widely used herbicide, induces aromatase expression and estrogen biosynthesis in certain human cell lines (Sanderson *et al.*, 2001, 2002; Thibeault *et al.*, 2014), but the effects of chemicals on the promoter- and tissue-specific expression of aromatase are largely unknown. In placenta, *CYP19* is expressed mainly via the placenta-specific promoter I.1, and the estrogens produced are essential for proper development of placenta and fetus (Albrecht and Pepe, 2010; Bukovsky *et al.*, 2003).

In this study, we developed quantitative real-time PCR techniques to determine the effects of neonicotinoids on *CYP19* gene expression via the breast cancer-relevant promoters PII and I.3, and the pregnancy-relevant placental promoter I.1, using two model human cell lines, adrenocortical carcinoma cells (H295R) and primary umbilical vein endothelial cells (HUVEC).

MATERIALS AND METHODS

Pesticides. All pesticides were obtained from Sigma-Aldrich (Saint-Louis, Missouri) (atrazine Pestanal 45330, purity > 99%; thiacloprid Pestanal 37905 purity > 99%; thiamethoxam Pestanal 37924, purity > 99%; imidacloprid Pestanal 37894, purity > 99%) and were dissolved in dimethylsulfoxide (DMSO) as 100 mM stock solutions.

In vitro models. The H295R cell line was selected for this study since it expresses all enzymes required for steroidogenesis *de novo* (Gazdar *et al.*, 1990; Hilscherova *et al.*, 2004; Rainey *et al.*, 1994; Sanderson, 2009; Zhang *et al.*, 2005) and the expression of *CYP19* in this cell line is regulated by 2 breast cancer-relevant promoters (PII and I.3) (Sanderson *et al.*, 2004), making it a suitable model for the study of *CYP19* expression and aromatase activity. It is also approved be the Organization for Economic Cooperation and Development as a tier 1 screening tool for chemically induced disruption of steroidogenesis (OECD, 2011). HUVEC cells were selected, because they were reported to express *CYP19* by its endothelial promoter I.7 (Alvarez-Garcia *et al.*, 2013) and have never been evaluated for additional *CYP19* promoter activities. Moreover, HUVEC are primary cells and are more likely to have a realistic regulation of promoter-specific *CYP19* expression.

Cell culture. H295R cells (ATCC no. CRL-2128) were obtained from the American Type Culture Collection. H295R cells were cultured in Dulbecco's modified Eagle medium/Ham's F-12 nutrient mix (DMEM/F12) containing 365 mg/ml of L-glutamine. Medium was completed with ITS+ Premix (Fisher Scientific, Waltham, Massachusetts) (final concentration in medium: 6.25 μg/ml insulin; 6.25 μg/ml transferrin; 6.25 ng/ml selenium; 1.25 mg/ml bovine serum albumin; 5.35 μg/ml linoleic acid); and Nu-serum (VWR International, Radnor, Pennsylvania) at a final concentration of 2.5%. Primary HUVEC (ATCC no. PCS-100-010) cells were obtained from the American Type Culture Collection. Primary HUVEC cells were cultured in vascular cell basal medium completed with the endothelial cell growth kit VEGF (ATCC no. PCS-100-041). Complete growth medium contained 5 ng/ml recombinant human (rh) VEGF, 5 ng/ml rh EGF, 5 ng/ml rh-FGF basic, 15 ng/ml rh-IGF-1, 10 mM L-glutamine, 0.75 units/ml heparin sulfate, 1 μg/ml hydrocortisone, 2% fetal bovine serum, and 50 μg/ml ascorbic acid.

Cell viability. The toxicity of atrazine and the neonicotinoids to H295R and HUVEC cells was determined using a WST-1 kit (Roche, Basel, Switzerland) which measures mitochondrial reductase activity of viable cells. H295R and primary HUVEC cells were plated in 96-well plates (5×10^3 cells/well) in their appropriate culture medium for 24 h. After this acclimatization period, cells were exposed to fresh medium containing increasing concentrations of atrazine, thiacloprid, thiamethoxam, or imidacloprid for another 24 h. Cells were then incubated with WST-1 substrate for 1.5 h and the formation of formezan was then measured using the absorbance at 440 nm with SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, California). Each experiment was conducted in triplicate and repeated twice using different cell passages.

RNA isolation and amplification by quantitative RT-PCR. Real-time quantitative PCR (qPCR) is a well-established method used to determine gene expression levels. Strong RNA quality control, primer design, and choice of reference genes (Taylor *et al.*, 2010)

are the key to achieving valid results. Therefore, careful consideration was given to the experimental design and qPCR validation. H295R and primary HUVEC cells were cultured in CellBind 6-well plates (Corning Incorporated, Corning, New York) (750 000 cells/well) containing 2 ml medium/well for 24 h. Cells were then exposed for 24 h to the various pesticides. Forskolin (Sigma-Aldrich) was used as a positive control for activation of PII and I.3 promoter-mediated *CYP19* gene expression and phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for I.1 promoter activation. DMSO vehicle (0.1%) was used as negative control. The 24-h exposure time was selected based on preliminary time-response experiments (0, 12, 24, 48, and 72 h) using forskolin, PMA, atrazine, and imidacloprid, which demonstrated that maximal (promoter-specific and promoter-indistinct) *CYP19* gene expression occurred at 24 h. RNA was isolated using an RNeasy mini-kit (Qiagen, Mississauga, Ontario) according to the enclosed instruction, and stored at −80°C. Purity of the RNA samples was determined using the 260 nm/280 nm absorbance ratio. Reverse transcription was performed using 0.5 μg of RNA with an iScript cDNA Synthesis Kit (BioRad, Hercules, California) and T3000 Thermocycler (Biometra, Göttingen, Germany); resultant cDNA was stored at −20°C.

Primer pairs were designed to amplify mRNA species containing an untranslated 5' region uniquely derived from each of the promoters (PII, I.3, I.4, I.1 and I.7) utilized for *CYP19* gene expression; a primer pair designed to recognize only the coding region (exons II–X) was used to amplify overall (promoter non-distinct) *CYP19* transcript. All the primer pairs were analyzed with Blast and Primer-Blast to ensure that the target sequences were unique to the gene or promoter region in question and that the product length was between 75 and 150 bp (Taylor *et al.*, 2010). Real-time quantitative PCR was performed using EvaGreen MasterMix (BioRad) with CFX96 real-time PCR Detection System (BioRad) (95°C for 5 min; 40 cycles of 95°C for 5 s and 60°C for 15 s). Standard curves for amplification by each primer pair to ensure an efficiency between 90% and 110% and r^2 value greater than 0.95 (Table 1; only PII, I.3 and I.1 promoter activity was detected in our cell models). Each experiment was conducted in triplicate and repeated twice using different cell passages.

For each pesticide and cell line, two suitable reference genes were included for normalization of *CYP19* gene expression (Table 2). Widely used reference genes such as glyceraldehyde 3-phosphate dehydrogenase (GADPH), 18S or β-actin are not always suitable, since their expression can be highly variable dependent on experimental conditions (Aerts *et al.*, 2004; Radonić *et al.*, 2004). In our case, it is crucial to evaluate the stability of the expression of different potential reference genes in each cell type and for each treatment. We determined suitable reference genes using the geNorm algorithm method (Biogazelle qbase Plus software, Zwijnaarde, Belgium) to calculate the target stability for each given condition (Taylor *et al.*, 2010). All reference genes met the criterion for gene expression stability of having an (*M*) value below 0.5.

Aromatase catalytic activity. Aromatase activity was measured using a tritiated water-release assay as described previously (Lephart and Simpson, 1991; Sanderson *et al.*, 2000). Briefly, H295R and primary HUVEC cells were cultured in 24-well plates (250 000 or 400 000 cells/well, respectively) containing 1 ml of the appropriate culture medium. After 24 h, cells were exposed to various concentrations of pesticides and incubated for 24 h. The treated medium was then removed and cells were washed twice with 500 μl PBS 1×. A volume of 250 μl of culture medium

Table 1. Primer Pair Sequences Used for Amplification of Promoter-Specific *CYP19* Expression and Their Amplification Characteristics in H295R and Primary HUVEC Cells

CYP19 Promoter	Primer Pairs (5'–3')	Amplification Characteristics	Tissue-Specific Expression	Reference and Accession Number
CYP19-coding region	Fw: TGTCTCTTTGTTCTTCATGCTATTTCTC Rv: TCACCAATAACAGTCTGGATTTCC	H295R cells: Standard curve: $r^2 = 0.980$ Efficiency: 101.0% HUVEC cells: Standard curve: $r^2 = 0.995$ Efficiency: 110.0%	Detects all aromatase transcripts regardless of promoter utilized.	Sanderson *et al.* (2000) M22246
CYP19-I.1	Fw: GGATCTTCCAGACGTCGCGA Rv: CATGGCTTCAGGCACGATGC	HUVEC cells: $r^2 = 0.966$ Efficiency: 94.3%	Placenta-specific aromatase transcript.	Klempan *et al.* (2011) NM_000103
CYP19-PII	Fw: TCTGTCCCTTTGATTTCCACAG Rv: GCACGATGCTGGTGATGTTATA	H295R cells: Standard curve: $r^2 = 0.977$ Efficiency: 108.5%	Expressed in ovaries, testes, and stroma of breast cancer patients	Heneweer *et al.* (2004) S52794
CYP19-I.3	Fw: GGGCTTCCTTGTTTTGACTTGTAA Rv: AGAGGGGGCAAT TTAGAGTCTGTT	H295R cells Standard curve: $r^2 = 0.960$ Efficiency: 102.7%	Expressed in ovaries, testes, and stroma of breast cancer patients	Wang *et al.* (2008) D30796

Table 2. Primer Pair Sequences of Reference Genes Used to Normalize *CYP19* Gene Expression for Each Pesticide Treatment in H295R and Primary HUVEC Cells

Cell Type	Reference Genes	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
Atrazine			
H295R	UBC	ATTTGGGTCGCGGTTCTTG	TGCCTTGACATTCTCGATGGT
	PBGD	GGCAATGCGGCTGCAA	GGGTACCCACGCGAATCAC
HUVEC	RPII	GCACCACGTCCAATGACAT	GTGCGGCTGCTTCCATAA
	PBGD	GGCAATGCGGCTGCAA	GGGTACCCACGCGAATCAC
Imidacloprid, Thiacloprid, Thiamethoxam			
H295R	RPII	GCACCACGTCCAATGACAT	GTGCGGCTGCTTCCATAA
	RPLP0	GGCGACCTGGAAGTCCAACT	CCATCAGCACCACAGCCTTC
HUVEC	UBC	ATTTGGGTCGCGGTTCTTG	TGCCTTGACATTCTCGATGGT
	RPLP0	GGCGACCTGGAAGTCCAACT	CCATCAGCACCACAGCCTTC

Abbreviations: PBGD, porphobilinogen deaminase; RPII, RNA polymerase II; RPLP0, 60 S acidic ribosomal protein P0; UBC, ubiquitin C.

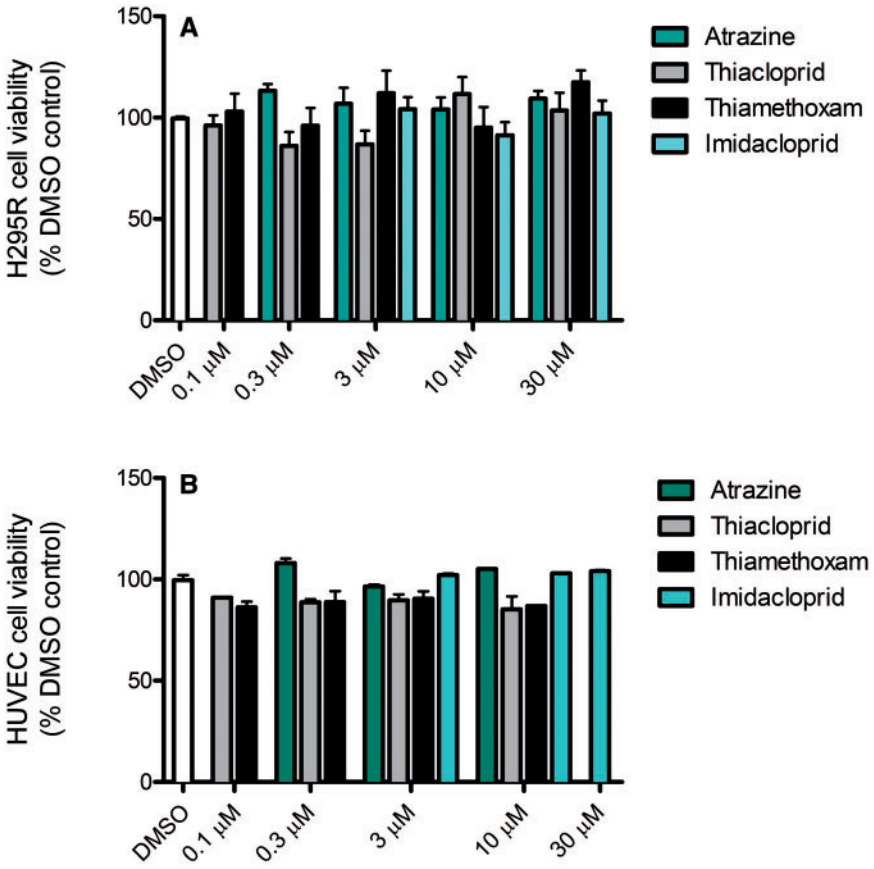

FIG. 1. Viability of H295R (A) and HUVEC (B) cells exposed for 24 h to various concentrations of atrazine, thiacloprid, thiamethoxam, or imidacloprid as a percentage of DMSO control. No statistically significant difference between pesticide treatments and DMSO control were detected (Kruskal–Wallis test; $P > .05$). Each experiment was performed twice using a different cell passage; per experiment each concentration was tested in triplicate.

(without phenol red) containing 54 nM 1β-^{3}H-androstenedione was added to each well, and cells were incubated for 90 (H295R) or 150 (HUVEC) minutes at 37°C (5% CO_2). Further steps were as described previously (Sanderson *et al.*, 2000). Tritiated water was counted in 24-well plates containing liquid scintillation cocktail using a Microbeta Trilux (PerkinElmer, Waltham, Massachusetts). Counts per minute produced by each sample were corrected for quenching to determine disintegrations per minute, which were then converted into aromatase activity (fg/h/100 000 cells) and then expressed as a percent of control activity (DMSO). Formestane (1 μM), a selective and irreversible aromatase inhibitor, was used to ensure specificity for the aromatase reaction.

Statistical analysis. Results are presented as means with standard errors of three independent experiments using different cell passages; per experiment, each concentration was tested in triplicate. The normal distribution of the residuals and the homoscedasticity of the data were verified for each analysis using JMP Software (SAS, Cary, North Carolina). Statistically significant differences ($^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$) from control were determined by one-way ANOVA followed by a Dunnett

post hoc test to correct for multiple comparisons to control using GraphPad Prism v5.04 (GraphPad Software, San Diego, California).

RESULTS

Effects of Atrazine and Neonicotinoids on Viability of H295R and Primary HUVEC Cells

A 24-h exposure to increasing concentrations of atrazine (0.3, 3, 10, 30 μM), imidacloprid (3, 10, 30 μM), thiacloprid, or thiamethoxam (0.1, 0.3, 3, 10, 30 μM) did not statistically affect the viability of H295R (Fig. 1A) or primary HUVEC cells (Fig. 1B) based on mitochondrial reductase activity.

Effects of Atrazine and Neonicotinoids on Promoter-Specific CYP19 Expression in H295R Cells

We determined the effects of a 24-h exposure to atrazine and three neonicotinoids on the PII and I.3 promoter-specific expression of *CYP19* in H295R cells. Our positive control forskolin (10 μM) increased PII- and 1.3-derived *CYP19* mRNA levels by 11.6 ± 2.6 and 13.0 ± 3.0-fold, respectively, and promoter-indistinct *CYP19* mRNA levels by 3.8 ± 0.6 μM. No evidence of I.4 or I.1 promoter activity was found in H295R cells (not shown). Atrazine increased levels of PII and I.3 promoter-derived *CYP19* transcript levels in a concentration-dependent manner, resulting in a 6-fold induction at 30 μM (Fig. 2A), as has been described previously (Fan *et al.*, 2007; Heneweer *et al.*, 2004). A 24-h exposure of H295R cells to thiacloprid resulted in a statistically significant increase in relative levels of promoter-indistinct *CYP19* mRNA at 0.3 and 10 μM (12.1- and 2.7-fold, respectively) as well as statistically significant increases in relative levels of PII- and I.3-derived *CYP19* transcript (4.6- and 3.0-fold, respectively) at 0.3 μM (Fig. 2B). Thiamethoxam at 0.1 μM strongly increased promoter-indistinct *CYP19* expression and expression via promoters PII and I.3 (Fig. 2C) by almost equal extents (between 12.2- and 15.7-fold). PII-derived mRNA levels were also significantly elevated (2.6-fold) at 0.3 μM (Fig. 2C). A 24-h exposure to 3 μM imidacloprid caused a statistically significant decrease of about 60 % in the expression of promoter-indistinct *CYP19* mRNA levels as well as the levels of PII- and I.3-derived *CYP19* transcript (Fig. 2D).

Effects of Atrazine and Neonicotinoids on Promoter-Specific CYP19 Expression in HUVEC Cells

HUVEC cells, which are primary cells of endothelial origin derived from human umbilical cord, were found to express aromatase via the major placental *CYP19* promoter I.1. Neither PII, I.3, I.4 nor I.7 *CYP19* promoter activity was detected in HUVEC

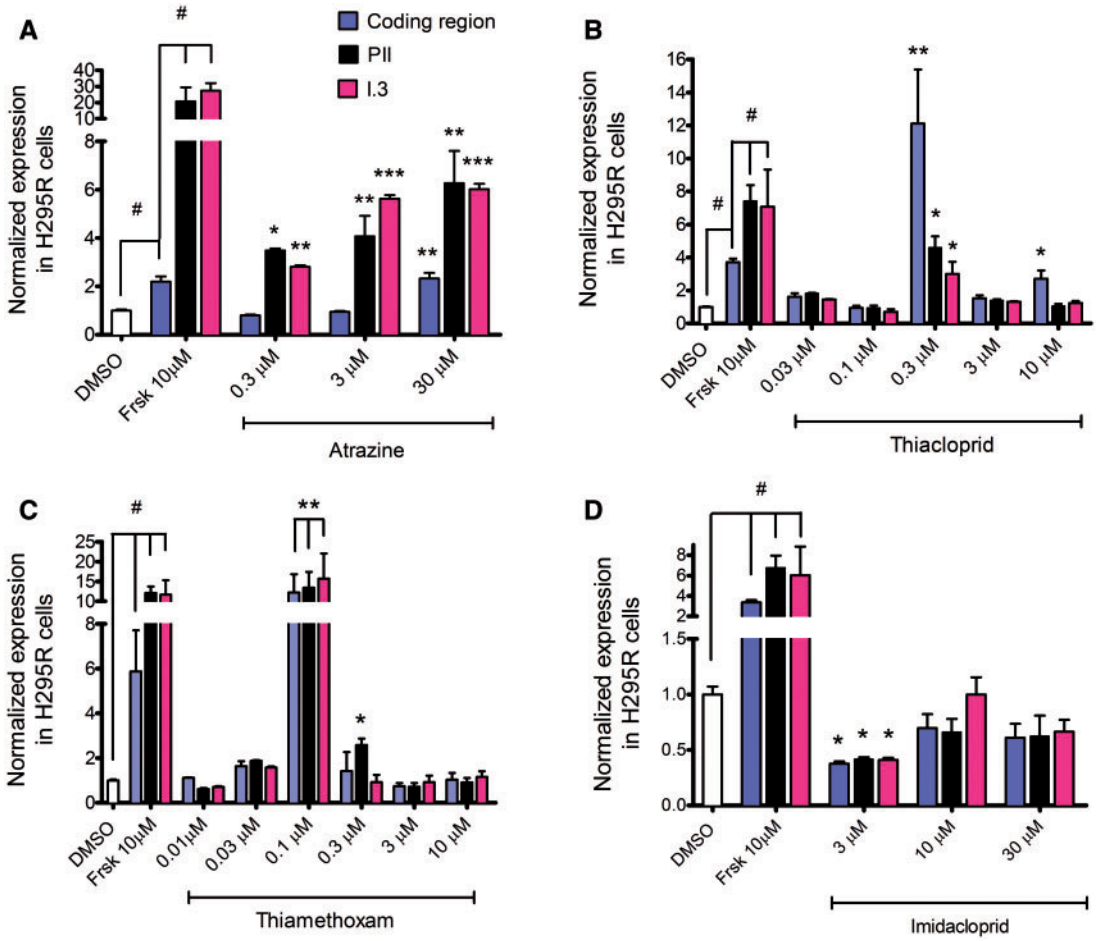

FIG. 2. Relative expression of *CYP19* (coding region) or PII and I.3 promoter-derived *CYP19* transcripts in H295R cells exposed for 24 h to various concentrations of atrazine (A), thiacloprid (B), thiamethoxam (C), or imidacloprid (D). Forskolin (Frsk) was used as a positive control for PII/I.3-mediated induction of *CYP19* expression. (#) A statistically significant difference between Frsk and DMSO control (Student t-test; $^{\#}P < 0.05$). (*, **, ***) A statistically significant difference between pesticide treatment and DMSO control (one-way ANOVA and Dunnett *post hoc* test; $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$). Experiments were performed in triplicate using different cell passages; per experiment each concentration was tested in triplicate.

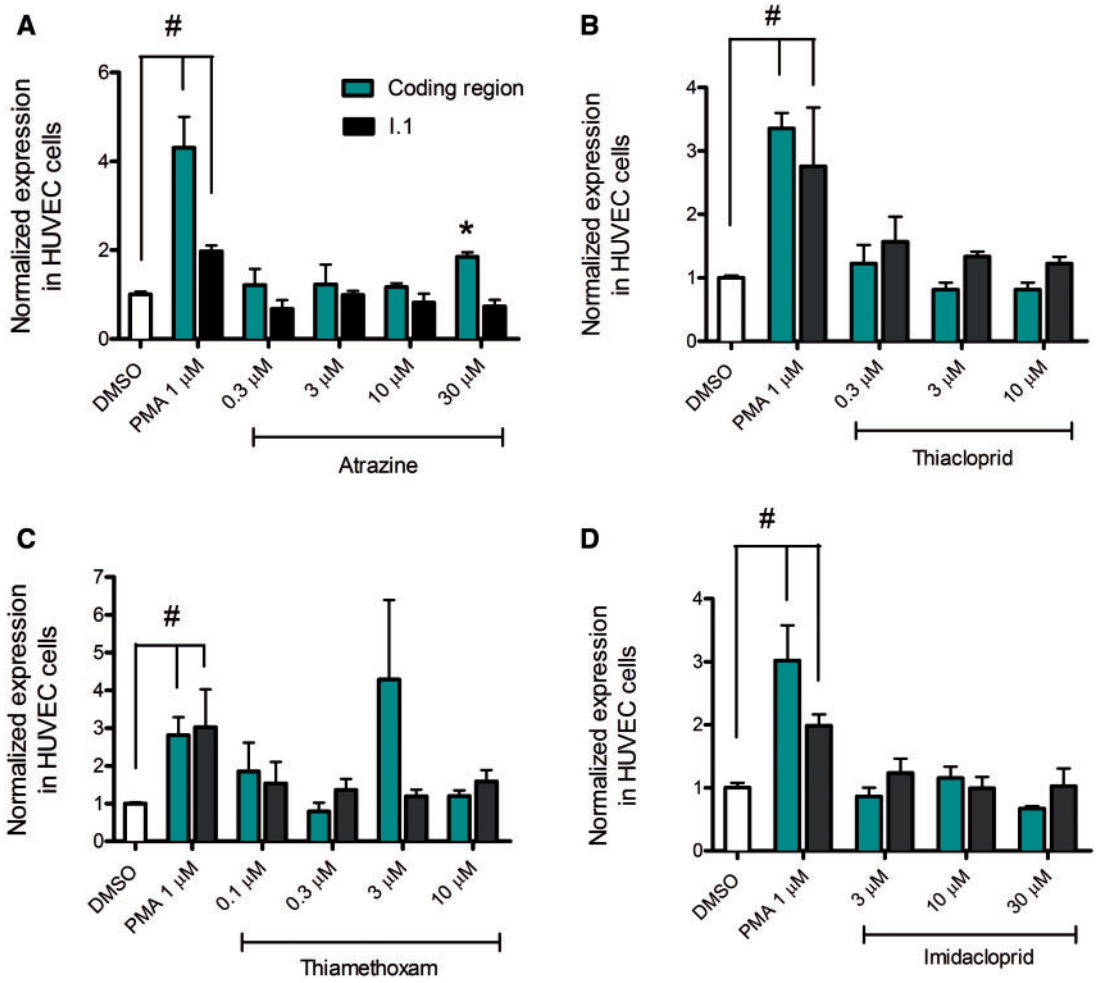

FIG. 3. Relative expression of *CYP19* (coding region) or I.1 promoter-derived *CYP19* transcripts in primary HUVEC cells exposed for 24 h to various concentrations of atrazine (A), thiacloprid (B), thiamethoxam (C), or imidacloprid (D). PMA was used as a positive control for induction of I.1-mediated induction of *CYP19* expression. (#) A statistically significant difference between PMA treatment and DMSO control (Student *t*-test; $^{\#}P < .05$). (*, **, ***) A statistically significant difference between pesticide treatment and DMSO control (one-way ANOVA and Dunnett *post hoc* test; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Experiments were performed in triplicate using different cell passages; per experiment each concentration was tested in triplicate.

cells. We also did not detect endothelial I.7 *CYP19* promoter activity in primary HUVEC cells using our qPCR method, neither with published primer pairs that were reported to detect I.7 promoter activity in these cells (Alvarez-Garcia *et al.*, 2013), nor with our own primer designs. On average, PMA induced levels of promoter-indistinct (coding region) *CYP19* mRNA by 3.3 ± 0.3-fold and levels of I.1 promoter-derived mRNA by 2.5 ± 0.3-fold (Fig. 3A–D). Atrazine slightly induced the expression of promoter non-distinct *CYP19* transcript, with a statistically significant increase of 1.9 ± 0.2-fold at 30 μM, but had had no statistically significant effects on I.1 promoter activity (Fig. 3A). The neonicotinoids did not affect overall or I.1 promoter-mediated *CYP19* expression in HUVEC cells at any tested concentrations (Fig. 3B–D).

Effects of Atrazine and Neonicotinoids on CYP19 Catalytic Activity in H295R and HUVEC Cells

To confirm whether the pesticide-induced changes in promoter-specific *CYP19* gene expression observed in H295R and primary HUVEC cells resulted in similar changes in catalytic activity of the final enzyme product, we measured the concentration-dependent effects of each pesticide on aromatase activity in each cell type. In H295R cells, forskolin (10 μM) was used as a positive control for induction of CYP19 and increased aromatase activity by 5.3 ± 2.2-fold compared to DMSO control, whereas formestane (1 μM), a positive control for inhibition of aromatase activity, reduced activity by about 77%–90% (not shown). In H295R cells, atrazine concentration-dependently induced aromatase activity, increasing it by about 2-fold at 30 μM (Fig. 4A); this is consistent with the observed increase in the promoter-specific expression of *CYP19* transcript in these cells (Fig. 2A). Thiacloprid and thiamethoxam induced aromatase activity statistically significantly at concentrations between 0.1 and 3 μM, producing biphasic concentration–response curves, with each pesticide having maximal effect at about 1 μM (Fig. 4A). Imidacloprid had no effect on aromatase activity in H295R cells within the tested concentration range (Fig. 4A).

In primary HUVEC cells, PMA (1 μM), a positive control for promoter I.1-controlled induction of *CYP19* gene expression, induced aromatase activity, on average, by 2.4 ± 0.3-fold compared to DMSO control, whereas formestane (1 μM) inhibited aromatase activity by 80%–100% (not shown). In primary HUVEC cells, atrazine induced aromatase activity concentration-dependently

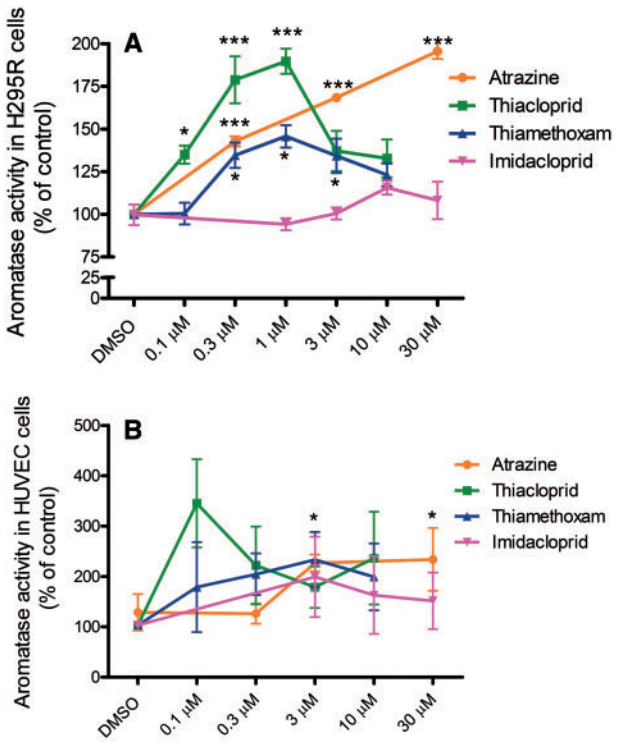

FIG. 4. Effect of atrazine, thiacloprid, thiamethoxam and imidacloprid on aromatase activity in H295R (A) and primary HUVEC (B) cells. (*, ***) A statistically significant difference between pesticide treatment and DMSO control (one-way ANOVA and Dunnett *post hoc* test; *$P < 0.05$; ***$P < .001$). Note: the asterisks (*) in B apply to atrazine only. Experiments were performed in triplicate using different cell passages; per experiment each concentration was tested in triplicate.

with statistically significant increases at 3 and 30 µM (Fig. 4B). Neither imidacloprid, thiacloprid nor thiamethoxam induced aromatase activity in a statistically significant manner (Fig. 4B).

DISCUSSION

Effects of Atrazine and Neonicotinoids on the Promoter-Specific Regulation of CYP19 in H295R Cells

Using atrazine as a positive control, our results confirm that this widely used herbicide is an effective inducer of aromatase via the promoters PII and I.3 in H295R cells. These effects are well documented and consistent with other studies (Fan *et al.*, 2007; Sanderson *et al.*, 2000, 2002). Atrazine, at concentrations higher than those currently found in the environment, also disrupted ovarian and hypothalamic function in rats, causing altered LH and prolactin synthesis (Cooper *et al.*, 2000), and induced aromatase expression via a steroidogenic factor-1 (SF-1) dependent pathway in H295R cells (Fan *et al.*, 2007). At environmentally relevant levels, atrazine exposure reduced growth rates of red drum larvae (*Sciaenops ocellatus*) exposed to 40 and 80 µg/l for 96 h (del Carmen Alvarez and Fuiman, 2005). Moreover, a study conducted in Texas coast demonstrated that atrazine levels in runoff water can reach 40–65 µg/l (Pennington *et al.*, 2001). Therefore, the observed induction of *CYP19* expression and activity by atrazine at a concentration as low as 300 nM (H295R cells), lies within an environmentally relevant range.

To our knowledge, we are the first to document the *in vitro* effects of neonicotinoids on the promoter-specific expression of *CYP19* and the catalytic activity of aromatase. In our study, thiacloprid and thiamethoxam strongly induced the expression of PII and I.3 promoter-derived *CYP19* mRNA in H295R cells, which is a well-established *in vitro* model for the study of steroidogenesis (OECD, 2011). The strong increase in expression of total (coding region) *CYP19* transcript in H295R cells exposed to 0.3 µM thiacloprid relative to the weaker increase in PII- and I.3-derived transcript levels appears to suggest the presence of other, possibly unknown, aromatase promoters or promoter-independent mechanisms of transactivation in these cells. (We confirmed that neither I.1, I.4 nor I.7 promoter activity was present in H295R cells under our experimental conditions.) Among the neonicotinoids, thiacloprid, and thiamethoxam had the greatest effects on PII and I.3 promoter-specific *CYP19* expression in H295R cells. These effects occurred at relatively low concentrations, producing biphasic or non-monotonic concentration–response curves. Although an alteration in mRNA expression does not necessarily result in a change in protein expression or enzyme activity, we show that thiacloprid and thiamethoxam altered promoter-specific CYP19 mRNA expression as well as catalytic activity (which we consider a more relevant endpoint in an *in vivo* context) in a non-monotonic manner. In toxicologically studies of endocrine disrupting chemicals, this type of biphasic response is not uncommon (Calabrese and Baldwin, 2001a, 2001b; Kennedy *et al.*, 1993; Giesy *et al.*, 2000; Jobling *et al.*, 2003; Rivest *et al.*, 2011; Sanderson *et al.*, 1998; Vandenberg *et al.*, 2012). For example, bisphenol A binds the estrogen receptor at low concentrations, but also acts as an antiandrogen at higher ones (Sohoni and Sumpter, 1998) and we have recently shown that androgen receptors are expressed in H295R cells (Robitaille *et al.*, 2015). This concentration-dependent selectivity of bisphenol A for several hormone receptors was suggested to explain the non-monotonic shape of the resultant concentration–estrogenic response curve (Vandenberg *et al.*, 2012). Also, exposure of rats to phtalates leads to a biphasic effect on aromatase activity. This was partially explained by a change in testosterone availability and by the inhibitory action of phtalate metabolites on *CYP19* transcript levels (Andrade *et al.*, 2006). Moreover, Jobling *et al.* (2003) demonstrated that a 3-week exposure to ethinylestradiol and bisphenol A in prosobrach mollusc (*Potamopyrgus antipodarum*) stimulated embryo production in an inverted U-shaped dose–response manner, where lower concentrations had a greater effect. Another study conducted on fathead minnow (*Pimophales promelas*) exposed to 4-nonylphenol (NP) demonstrated an effect of this chemical on egg production and plasma vitellogenin levels, resulting in an inverted U-shape dose-response curve (Giesy *et al.*, 2000). It remains unclear how thiacloprid and thiamethoxam produced a non-monotonic response on CYP19 expression and aromatase activity in H295R cells, but it may activate (or deactivate) different intracellular signaling factors that affect *CYP19* promoter activity at different concentrations. The action of atrazine on PII/I.3-mediated CYP19 expression occurs by increasing intracellular levels of cAMP (Sanderson *et al.*, 2002) and possibly by interacting with SF-1 (Fan *et al.*, 2007). It is further known that cAMP-mediated phosphorylation of GATA-4 is involved in SF-1 activation and subsequent stimulation of the CYP19 PII promoter (Tremblay and Viger, 2003). It is not known whether atrazine activates GATA-4 and it remains to be studied whether thiacloprid and thiamethoxam act as potent inducers via these signaling pathways or by other yet to be delineated mechanisms. Further insight into the mechanisms of induction of CYP19 by neonicotinoids is important in understanding their promoter-specificity and explaining the non-monotonicity of their concentration–response curves. This will be addressed in further studies.

Evidence of other adverse or endocrine disruptive effects of neonicotinoids has been observed in recent studies, with imidacloprid inducing apoptosis and fragmentation of seminal DNA in rats (Bal *et al.*, 2012), and causing oxidative stress and hormone disruption in female rats (Kapoor *et al.*, 2011). Moreover, a few studies demonstrated that half-lives of certain neonicotinoids, such as imidacloprid, may exceed 1000 days and that around 90% of the active ingredient in neonicotinoid formulations enters the soil (Goulson, 2013). These characteristics indicate that bioaccumulation of these pesticides may occur and eventually cause subchronic toxicities, which re-enforce the need for toxicological studies of neonicotinoids.

Effects of Atrazine and Neonicotinoids on the Promoter-Specific Regulation of CYP19 in Primary HUVEC Cells

We are the first to evaluate the effects of atrazine and neonicotinoids on *CYP19* expression and aromatase activity in primary endothelial HUVEC cells. RT-qPCR analyses showed that HUVEC cells express *CYP19* (Mukherjee *et al.*, 2002) via the placental PKC-driven I.1 promoter, which is stimulated by the phorbol ester PMA. Atrazine increased promoter non-distinct *CYP19* mRNA expression statistically significantly, but this increase in transcript was not derived from the I.1 promoter as its activity remained unchanged (Fig. 3A). A possible explanation could be the presence of another *CYP19* promoter that drives the observed changes in *CYP19* expression levels, possibly the endothelium-specific I.7 promoter (Bulun *et al.*, 2003) or an unknown one, although we could not detect any transcript using the currently published sequence for the 5'-untranslated region of I.7 promoter-derived mRNA that was discovered in breast cancer tissues (Sebastian *et al.*, 2002). Other mechanisms such as inhibition of mRNA degradation could also play a role. The absence of statistically significant effects of the neonicotinoids on aromatase activity in HUVEC cells was consistent with the lack of effects on *CYP19* expression at the mRNA level (Figs. 3B–D and 4B). We note that basal aromatase activity in HUVEC cells (2 fmole/h/10^5 cells) is considerably lower than in H295R cells (118 fmole/h/10^5 cells) and less inducible by its positive control PMA (2.4-fold) than by forskolin in H295R cells (5.3-fold). However, given the induction of *CYP19* expression and aromatase activity observed with atrazine, endothelial cells (such as HUVEC) should not be ignored as a potential target for endocrine disrupting pesticides and other environmental contaminants. For example, HUVEC cells exposed to environmentally relevant doses of bisphenol A have higher expression of proangiogenic genes such as VEGFR (Andersson and Brittebo, 2012) and exhibit mitotic abnormalities during cell division (Ribeiro-Varandas *et al.*, 2013).

Relevance of Disruption of Promoter-Specific CYP19 Expression in Health and Disease

The H295R cell line is a useful *in vitro* model for the study of the effects of chemicals on the PII and I.3 promoter-specific expression of *CYP19*. The activity of the PII and I.3 promoters of *CYP19* are associated with the over-expression of aromatase in the adipose stromal cells surrounding hormone-dependent breast tumors (Bulun *et al.*, 2007; Chen *et al.*, 2009). The consequence of induction of *CYP19* via these two promoters would be an increased local production of estrogens in close proximity to the tumor, increasing the likelihood of cancer cell proliferation. The involvement of the PII promoter in *CYP19* over-expression has also been established in ovarian cancer (Bulun *et al.*, 2007) and endometriosis, where the adipose-specific I.4 promoter also appears to be involved (Zeitoun *et al.*, 1999). Moreover, obesity is associated with higher expression of aromatase in breast fibroblasts via promoters PII/I.3, and women suffering from obesity have greater risk of developing breast cancer (Bulun *et al.*, 2012). One of the suggested pathways linking obesity with breast cancer and aromatase over-expression is the increased production of prostaglandin E_2, known to induce aromatase expression via promoters PII and I.3 (Chen *et al.*, 2007). Considering the importance of the promoter-specific character of *CYP19* expression in various tissues and disease states as well as during pregnancy, our finding that atrazine and certain neonicotinoid pesticides can modulate *CYP19* in a promoter-specific manner emphasizes the need for further study of the potential disruptive effects of environmental contaminants on steroidogenesis.

Whether human exposures to environmental contaminants that disrupt *CYP19* expression and aromatase activity are sufficiently high to cause or contribute to human disease is an important question that remains to be answered. Atrazine is found in surface waters (Smalling *et al.*, 2015) and in agricultural products (Viden *et al.*, 1987) at concentrations that have been linked to reproductive abnormalities in amphibians (Hayes *et al.*, 2003) and effects on *CYP19* expression in the brains of tadpoles of the bullfrog (*Rana catesbeiana*) (Gunderson *et al.*, 2011). Furthermore, atrazine is detected in breast milk (Balduini *et al.*, 2003) confirming that infant exposures may occur during a critical developmental period. With regards to the neonicotinoids, levels of clothianidin and thiamethoxam have been steadily increasing since 2012 in water samples from wetlands and agricultural areas in the United States and Canada (concentrations ranging from 0.002 to 3.6 μg/l) (Anderson *et al.*, 2013; Main *et al.*, 2014; Smalling *et al.*, 2015). Since a large number of studies demonstrate a link between exposures to environmental contaminants and increased risk of breast cancer (Birnbaum and Fenton, 2003; Bonefeld-Jorgensen *et al.*, 2011; Dewailly *et al.*, 1994; Fenton, 2006) or adverse birth outcomes (Rinsky *et al.*, 2012; Vafeiadi *et al.*, 2014), the increasing use and reliance on chemicals such as pesticides remains a cause of concern.

CONCLUSIONS

We have successfully developed RT-qPCR methods for the quantitative determination of the promoter-specific expression of *CYP19*. We have shown that the H295R cell line is a useful *in vitro* model for the study of chemicals that may interfere with the PII and I.3 promoter-specific expression of *CYP19* and that primary HUVEC cells express *CYP19* via the placenta-type I.1 promoter. To fully understand the promoter-specific regulation of *CYP19* in HUVEC or other endothelial cells, further studies would be helpful to assess the activation pathway(s) of the endothelial I.7 promoter. Finally, we are the first to show that neonicotinoid insecticides have the potential to increase the expression of *CYP19* in a promoter-specific manner. Given the importance of the promoter-specific expression of *CYP19* in breast cancer or during pregnancy, it is important to study how endocrine disrupting chemicals may affect the activity of individual *CYP19* promoters in order to better understand and predict the potential risk to exposed women.

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Effects of Neonicotinoid Pesticides on Promoter-Specific Aromatase (CYP19) Expression in Hs578t Breast Cancer Cells and the Role of the VEGF Pathway

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Background: Aromatase (CYP19) is a key enzyme in estrogens biosynthesis. In the mammary gland, *CYP19* gene is expressed at low levels under the regulation of its I.4 promoter. In hormone-dependent breast cancer, fibroblast cells surrounding the tumor express increased levels of *CYP19* mRNA due to a decrease of I.4 promoter activity and an increase of PII, I.3, and I.7 promoter activity. Little is known about the effects of environmental chemicals on the promoter-specific *CYP19* expression.

Objective: We aimed to determine the effects of two neonicotinoids (thiacloprid and imidacloprid) on promoter-specific *CYP19* expression in Hs578t breast cancer cells and understand the signaling pathways involved.

Methods: Hs578t cells were exposed to various signaling pathway stimulants or neonicotinoids for 24 h. Promoter-specific expression of *CYP19* was determined by real-time quantitative polymerase chain reaction and catalytic activity of aromatase by tritiated water release assay.

Results: To our knowledge, we are the first to demonstrate that the normal I.4 promoter and the breast cancer-relevant PII, I.3, and I.7 promoters of *CYP19* are active in these cells. We found that the expression of *CYP19* via promoters PII, I.3, and I.7 in Hs578t cells was, in part, dependent on the activation of two VEGF signaling pathways: mitogen-activated protein kinase (MAPK) 1/3 and phospholipase C (PLC). Exposure of Hs578t cells to environmental concentrations of imidacloprid and thiacloprid resulted in a switch in *CYP19* promoter usage, involving inhibition of I.4 promoter activity and an increase of PII, I.3, and I.7 promoter-mediated *CYP19* expression and aromatase catalytic activity. Greater effects were seen at lower concentrations. Our results suggest that thiacloprid and imidacloprid exert their effects at least partially by inducing the MAPK 1/3 and/or PLC pathways.

Conclusions: We demonstrated *in vitro* that neonicotinoids may stimulate a change in *CYP19* promoter usage similar to that observed in patients with hormone-dependent breast cancer. https://doi.org/10.1289/EHP2698

Introduction

Background

In 2017, 26,300 women were diagnosed with breast cancer in Canada (Canadian Cancer Society's Advisory Committee on Cancer Statistics 2017). In the United States, it was expected that 252,710 new cases of breast cancer would be diagnosed in 2017 (American Cancer Society 2017). Of these cases, 83% were estrogen-receptor and/or progesterone-receptor positive (American Cancer Society 2017). In this type of cancer, increased local estrogen is produced, resulting in greater concentrations in the tumor microenvironment, which stimulates the proliferation of breast cancer epithelial cells (Ghosh et al. 2009; Yamaguchi and Hayashi 2009). Aromatase (CYP19) is a key enzyme in the biosynthesis of estrogens, as it is responsible of the final conversion of androstenedione to estrone, and testosterone to estradiol (Bulun et al. 2003). The *CYP19* gene is expressed in a tissue-specific manner by the activation of various promoters located in the noncoding region of the gene. In the normal breast, *CYP19* is expressed at low levels in fibroblast cells (stromal preadipocytes) and driven by the I.4 promoter (Simpson and Davis 2001).

In breast cancer, a series of events leads to the inhibition of I.4 promoter activity (Agarwal et al. 1996; Harada et al. 1993) and the activation of several promoters that are normally inactive in the stromal cells of the mammary gland, namely PII, I.3, and I.7 (Irahara et al. 2006; Subbaramaiah et al. 2012; Zhou et al. 1997). This unique switch in promoter usage results in an increase of overall *CYP19* gene expression, aromatase catalytic activity, and subsequent estrogen biosynthesis. Moreover, malignant epithelial cells synthesize prostaglandin E_2 (PGE_2), which binds to its G-protein-coupled PGE_2 receptor to stimulate the production of cyclic AMP (cAMP), which results in increased *CYP19* expression through activation of promoters PII and I.3 (Chen et al. 2007; Subbaramaiah et al. 2012). PGE_2 can also activate the orphan nuclear receptor homologue-1 (LRH-1), known to induce *CYP19* expression in breast tissue (Zhou et al. 2005).

Increased levels of PGE_2, and other inflammatory factors such as TNFα and IL-11 in the tumor microenvironment only partially explain the promoter-switch in regulation of *CYP19* expression that occurs in hormone-dependent breast cancer patients. Another potential contributor to the promoter-switch in *CYP19* expression is the vascular endothelial growth factor (VEGF) receptor signaling pathway. The VEGF receptor (VEGFR) signaling pathway plays a central role in angiogenesis. More precisely, secretion of VEGF is associated with proliferation of vascular endothelial cells (Schneider and Sledge 2007). It has been demonstrated that VEGF and its receptors are overexpressed in breast cancer (Adams et al. 2000; Konecny et al. 2004). Furthermore, we know that VEGF promotes angiogenesis and endothelial cell permeability by activating ERK 1/2 (MEK/MAPK1/3) (Breslin et al. 2003; Pai et al. 2001; Xu et al. 2008) and PLC/PKC (Cross and Claesson-Welsh 2001; Jiang et al. 2016).

Given the importance of aromatase in hormone-dependent breast cancer, understanding the regulation of the promoter-specific expression of *CYP19* is paramount to assessing potential impacts of environmental contaminants on the development of this disease. Indeed, there is growing evidence that exposure to contaminants, such as pesticides, is a risk factor for hormone-dependent breast cancer (Cohn et al. 2007; Ibarluzea et al. 2004; Mathur et al. 2002; Xu

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et al. 2010). A lot of research has focused on effects of endocrine disruptors on the estrogen receptor (Bouskine et al. 2009; Roy et al. 2009; Rubin et al. 2001). The enzyme aromatase has been identified as a target for endocrine disrupting chemicals, including environmental pesticides (Sanderson 2006). However, we have little information on the roles that environmentally relevant levels of chemicals may play in the disruption of aromatase expression or activity. It has been demonstrated that the widely used herbicide atrazine induces estradiol synthesis in human cell lines by the activation of PII/I.3-mediated *CYP19* expression (Caron-Beaudoin et al. 2016; Sanderson et al. 2002). Furthermore, our laboratory recently demonstrated that the neonicotinoids thiacloprid and thiamethoxam induced PII/I.3-mediated *CYP19* expression as well as aromatase catalytic activity in a nonmonotonic manner in H295R adrenocortical carcinoma cells, at relatively low concentrations (Caron-Beaudoin et al. 2016). We also demonstrated that three neonicotinoids (thiacloprid, thiamethoxam, and imidacloprid) increased the production of estrone and estradiol, yet strongly inhibited the production of estriol in a fetoplacental coculture model of steroidogenesis during pregnancy (Caron-Beaudoin et al. 2017). To the best of our knowledge, the impacts of neonicotinoid insecticides on human health have not been studied in any detail, but an increasing body of evidence suggests they have the potential to disrupt endocrine functions (Bal et al. 2012; Hoshi et al. 2014; Kapoor et al. 2011; Şekeroğlu et al. 2014). For example, female rats exposed to imidacloprid through diet (20 mg/kg per day) showed decreased ovarian weights and alterations in progesterone and follicle-stimulating hormone levels (Kapoor et al. 2011).

Neonicotinoids are widely used pesticides that have been linked to Honey Bee Colony Collapse Disorder (Goulson 2013; Henry et al. 2012). In 2012, 216,000 kg of active neonicotinoids were applied on 11 million hectares of land in Canada (Main et al. 2014). These insecticides exert their effects by binding to nicotinic acetylcholine receptors (Matsuda et al. 2001), and they are used as a seed coating in a variety of crops, fruits, and vegetables (Elbert et al. 2008). Neonicotinoid half-lives can reach 1,250 days for imidacloprid (Main et al. 2014), and these insecticides are detected in surface water and soil (Schaafsma et al. 2015; Starner and Goh 2012; Stokstad 2013). Due to their relative persistence in the environment, and because neonicotinoids are used as seed treatments and repeatedly applied, concerns regarding human exposure have been raised. Imidacloprid has been detected in 89% of water samples in California, and concentrations exceeded the U.S. Environmental Protection Agency's aquatic life benchmark dose in 19% of samples (Starner and Goh 2012). In wetlands in Saskatchewan, Canada, concentrations of clothianidin and thiamethoxam were found to be as high as 3,110 ng/L (Main et al. 2014). Furthermore, it was recently demonstrated that residues of at least one neonicotinoid were detected in vegetables and fruits purchased from grocery stores in Boston, Massachusetts, with concentrations reaching 100.7 ng/g. In this study, at least two different neonicotinoids were detected in 72% of fruits and 45% of vegetables (Chen et al. 2014). Finally, a study conducted in Japan analyzed neonicotinoid metabolites in urine samples of farmers. 3-Furoic acid, the major metabolite of the neonicotinoid dinotefuran, was detected in all urine samples, with concentrations reaching 0.13 μM (Nomura et al. 2013). Urinary neonicotinoid levels were also measured in females from the general Japanese population, and thiacloprid and imidacloprid were detected at concentrations up to 0.01 μM (Ueyama et al. (2015). The human exposure to neonicotinoid insecticides highlights the need to investigate their potential endocrine disrupting effects, especially at environmentally relevant concentrations.

Objectives

Using Hs578t cells as a breast cancer-relevant *in vitro* model, we aimed to understand the signaling pathways implicated in the expression of *CYP19* via the activity of promoters I.4, I.7, I.3, and PII and whether neonicotinoids can induce a promoter-switch in *CYP19* expression, as has been described in breast cancer patients (Irahara et al. 2006).

Methods

Reagents

Thiacloprid (Pestanal®; cat. no. 37905, purity >99%) and imidacloprid (Pestanal®; cat. no. 37894, purity >99%) were obtained from Sigma-Aldrich (Saint-Louis, MO) and dissolved in sterile-filtered dimethylsulfoxide (DMSO; cat. no. 67-68-5, Sigma-Aldrich) as 100 mM stock solutions. The MAPK 1/3 pathway inhibitor PD98059 was purchased from Fisher Scientific and dissolved in DMSO as a 50 mM stock solution. The phospholipase C (PLC) inhibitor U73122 (Calbiochem) was dissolved in DMSO as a 2 mM stock solution. Forskolin and dexamethasone were obtained from Sigma-Aldrich and dissolved in DMSO as 10 mM and 100 μM stock solutions, respectively. VEGF was purchased from the American Type Culture Collection (ATCC) at a concentration of 5.0 μg/mL.

Cell Culture and Experimental Design

Hs578t cells (ATCC, cat. no. HTB-126) are triple-negative breast cancer epithelial cells derived from a 74-y-old patient with mammary carcinoma. Cells from low passages (below 9) were cultured in Dulbecco's modified Eagle medium (DMEM, cat. no. 30-2002, Sigma-Aldrich) containing 4 mM L-glutamine, 4,500 mg/L glucose, 1 mM sodium pyruvate, and 1,500 mg/L sodium bicarbonate. Medium was completed with 10% fetal bovine serum (FBS) and 0.01 mg/mL of bovine insulin (Sigma-Aldrich). Hs578t cells were exposed to various concentrations of each compound in culture medium at a final DMSO concentration of 0.1%.

Real-Time Quantitative Polymerase Chain Reaction

For the Real-Time Quantitative Polymerase Chain Reaction experiments, Hs578t cells were cultured for 24 h in 6-well plates (CellBind, Corning Incorporated) (7.5×10^5 cells/well) containing 2 mL medium/well. For subsequent exposures, medium was removed, Hs578t cells were washed with 500 μL PBS (1X) and 2 mL of treated medium was added. To determine which *CYP19* promoters are active, Hs578t cells were exposed for 24 h to 10 μM forskolin, 100 nM dexamethasone, or 2.5 ng/mL VEGF. Dexamethasone (100 nM) was used as a known inducer of I.4-mediated *CYP19* expression, whereas forskolin (10 μM) was used to induce PII/I.3-mediated *CYP19* expression. VEGF (2.5 ng/mL) was used as a potential inducer of I.7-mediated *CYP19* expression (Kalluri and Zeisberg 2006). Control cells were exposed to 0.1% DMSO. To determine which VEGF signaling pathways are implicated in the expression of *CYP19* via promoters PII, I.3 or I.7, Hs578t cells were pretreated with a PLC inhibitor (2 μM U73122) or a MAPK 1/3 inhibitor (50 μM PD88059) 4 h prior to the addition of forskolin or VEGF for 24 h. Forskolin is known to increase calcium release (Schmidt et al. 2001), which can activate the PLC pathway. Furthermore, it is known that the MAPK 1/3 signaling pathway is activated by VEGF (Breslin et al. 2003; Cross and Claesson-Welsh 2001; Lee et al. 1998). Therefore, we tested the potential involvement of the PLC pathway in PII and I.3 promoter-specific *CYP19* expression by pretreating Hs578t cells with U73122 4 h prior to the addition of forskolin, and the potential involvement of

the MAPK 1/3 pathway in I.7 promoter-specific *CYP19* expression by pretreating with PD98059 4 h prior to addition of VEGF.

Conditions for Neonicotinoid Exposures for Real-Time Quantitative PCR Experiments

Hs578t cells were exposed to thiacloprid (0.03, 0.1, 0.3, 3, and 10 μM) or imidacloprid (0.03, 0.1, 0.3, and 3 μM) for 24 h. These concentrations are similar to those found in urine samples of farmers and women from the general population in Japan (Nomura et al. 2013; Ueyama et al. 2015). Finally, to determine if neonicotinoids exert their effects on *CYP19* expression via the PLC and/or MAPK1/3 pathways, Hs578t cells were pretreated with the selective inhibitors (2 μM U73122 or 50 μM PD88059) 4 h prior to a 24-h exposure to 0.1 μM thiacloprid or imidacloprid. After treatment, medium was removed and Hs578t cells were washed twice with 500 μL PBS (1X) prior to RNA isolation (see the section "RNA isolation and amplification by quantitative RT-PCR" below).

Exposure Conditions for the Aromatase Catalytic Activity Assay

Hs578t cells were cultured in 24-well plates (400,000 cells/well) containing 1 mL of culture medium. After 24 h, medium was removed and Hs578t cells were washed with 500 μL PBS (1X) before 1 mL of treated medium was added. To determine the impact of changes in promoter-specific *CYP19* expression on aromatase activity, Hs578t cells were exposed to 10 μM forskolin, 100 nM dexamethasone, or 2.5 ng/mL VEGF for 24 h. To determine the effects of neonicotinoids on aromatase activity, Hs578t cells were exposed to thiacloprid (0.03, 0.1, 0.3, 3, and 10 μM) or imidacloprid (0.03, 0.1, 0.3, and 3 μM) for 24 h. Control cells were exposed to 0.1% DMSO. Formestane (1 μM), a selective and irreversible aromatase inhibitor, was used to verify the specificity of the assay for the aromatization reaction. Prior to the aromatase assay, the treated medium was removed and the cells were washed twice with 500 μL PBS (1X).

Cell Viability

The cytotoxicity of thiacloprid and imidacloprid was determined using a WST-1 kit (Roche), which measures mitochondrial reductase activity in viable cells. Hs578t cells were incubated for 24 h in 96-well plates (5×10^3 cells/well) in culture medium. After this period, cells were exposed for 24 h to fresh medium containing various concentrations of thiacloprid (0.03, 0.1, 0.3, 3, and 10 μM) or imidacloprid (0.03, 0.1, 0.3, and 3 μM). Cells were then incubated with WST-1 substrate for 1.5 h, after which the formation of formezan was measured at an absorbance wavelength of 440 nm using a SpectraMax M5 spectrophotometer (Molecular Devices).

RNA Isolation, Reverse Transcription, and Amplification by Quantitative PCR

Real-time quantitative PCR was designed and performed following recommendations from Taylor et al. (2010). RNA was isolated using an RNeasy mini-kit (Qiagen) according to the manufacturer's instructions, and stored at −80°C. The absorbance ratio at 260 nm/280 nm was used to determine purity of the RNA samples. Reverse transcription was performed with an iScript cDNA Synthesis kit (BioRad) and T3000 Thermocycler (Biometra) using 1 μg of RNA. Resultant cDNA was preamplified using SsoAdvanced PreAmp SuperMix (BioRad) and T3000 Thermocycler following the manufacturer's instructions. cDNA and preamplified cDNA were stored at −20°C. Primer pairs were designed to selectively amplify mRNA species containing an untranslated 5′ region uniquely derived from the promoters I.4, PII, I.3, or I.7 of *CYP19*; a primer pair designed to recognize only the coding region (exons II–X) was used to amplify overall (promoter nondistinct) *CYP19* transcript (Table 1). All the primer pairs were analyzed with Blast and Primer-Blast (National Center for Biotechnology Information) to ensure their selectivity. Real-time quantitative PCR was performed with EvaGreen MasterMix (BioRad) using a CFX96 Real-Time PCR Detection System (BioRad) (95°C for 5 min; 40 cycles of 95°C for 5 s, and 60°C for 15 s) (Table 1). Suitable reference genes were selected using the geNorm algorithm method (version 1.5; Biogazelle qbase Plus software). For treatments with thiacloprid, UBC (forward primer 5′–3′: ATTTGGGTCGCGGTTCTTG; reverse primer 5′–3′: TGCCTTGACATTCTCGATGGT) and RPLP0 (forward primer 5′–3′: GGCGACCTGGAAGTCCAACT; reverse primer 5′–3′: CCATCAGCACCACAGCCTTC) reference genes were used; for imidacloprid, we used UBC and PBGD (forward primer 5′–3′: GGCAATGCGGCTGCAA; reverse primer 5′–3′: GGGTACCCACGCGAATCAC). For forskolin, dexamethasone and VEGF treatments alone, UBC and RPLP0 were used as reference genes.

Table 1. Primer pair sequences and amplification characteristics for the amplification of promoter-specific *CYP19* expression in Hs578t cells.

CYP19 promoter	Primer pairs (5′–3′)	Amplification characteristics in Hs578t	Tissue-specific expression	Reference and NCBI accession number
CYP19-coding region	Fw: TGTCTCTTTGTTCTTCATGCTATTTCTC	Standard curve: $r^2 = 0.991$	Detects all aromatase transcripts regardless of promoter utilized	Sanderson et al. 2000)
	Rv: TCACCAATAACAGTCTGGATTTCC	Efficiency: 92.8%		M22246
CYP19-I.4	Fw: GGCTCCAAGTAGAACGTGACCAACTG	Standard curve: $r^2 = 0.941$	Expressed in fibroblasts in the normal mammary gland	Heneweer et al. 2004)
	Rv: CAGCCCAAGTTTGCTGCCGAA	Efficiency: 101.9%		S52794
CYP19-PII	Fw: TCTGTCCCTTTGATTTCCACAG	Standard curve: $r^2 = 0.937$	Expressed in ovaries, testes and stroma of breast cancer patients	Heneweer et al. 2004)
	Rv: GCACGATGCTGGTGATGTTATA	Efficiency: 108.9%		S52794
CYP19-I.3	Fw: GGGCTTCCTTGTTTTGACTTGTAA	Standard curve: $r^2 = 0.969$	Expressed in ovaries, testes and stroma of breast cancer patients	Wang et al. 2008)
	Rv: AGAGGGGGCAATTTAGAGTCTGTT	Efficiency: 95.7%		D30796
CYP19-I.7	Fw: ACACTCAGCTTTTTCCCAACA	Standard curve: $r^2 = 0.983$	Expressed in endothelial cells and stroma of breast cancer patients	NM_001347251
	Rv: TTTCACCCCTTTCTCCGGTC	Efficiency: 90.7%		

Note: Fw, forward; NCBI, National Center Biotechnology Information; Rv, reverse.

Aromatase Catalytic Activity

Aromatase activity was measured using the tritiated water-release assay as described previously (Caron-Beaudoin et al. 2016; Sanderson et al. 2000). A volume of 250 µL of culture medium (without phenol red) containing 54 nM 1β-^{3}H-androstenedione was added to each well, and cells were incubated for 150 min at 37°C (5% CO_2). As described previously (Lephart and Simpson 1991; Sanderson et al. 2000), 200 µL of culture medium underwent chloroform extraction to separate the substrate 1β-^{3}H-androstenedione from tritiated water ($^{3}H_2O$), a product of the aromatization reaction. To remove any remaining substrate, the aqueous fraction treated with dextran-coated charcoal. The amount of tritiated water released was counted in 24-well plates containing liquid scintillation cocktail using a Microbeta Trilux (PerkinElmer, Waltham, MA). Counts per minute emitted from each sample were corrected for quenching to determine disintegrations per minute, which were then converted into aromatase activity (fg/h per 100,000 cells) and ultimately expressed as a percent of control activity (cells treated with 0.1% DMSO).

Statistical Analysis

Results are presented as means with standard errors of three independent experiments using different cell passages; per experiment, each treatment was tested in triplicate. The normal distribution of the residuals and the homoscedasticity of the variance were verified for each analysis using JMP Pro 13 Software (SAS Institute Inc.). Statistically significant differences (* = equals $p < 0.05$; ** = equals $p < 0.01$; *** = equals $p < 0.001$) from control were determined by Student t-test or one-way analysis of variance (ANOVA) followed by a Dunnett post hoc test to correct for multiple comparisons to control using GraphPad Prism (version 5.04; GraphPad Software).

Results

Promoter-Specific Expression of CYP19 in Hs578t Cells

None of the tested neonicotinoid insecticides was cytotoxic at the tested concentrations of 0.03, 0.1, 0.3, 3, and 10 µM (see Figure S1). We determined the effects of a 24-h exposure to various pharmacological compounds on the promoter-specific induction of *CYP19* gene expression in Hs578t cells (Figure 1A). In Hs578t cells exposed to vehicle control (0.1% DMSO), basal *CYP19* expression was driven by the I.4 promoter (Cq = 31; quantification cycle; the amplification cycle at which accurate quantification of expression levels can be made), the PII (Cq = 32), I.7 (Cq = 33.5), and I.3 (Cq = 36) promoters of aromatase. In Hs578t cells exposed to 100 nM dexamethasone, I.4 promoter-derived *CYP19* mRNA levels were induced 264 ± 85 fold compared with DMSO control, whereas no significant differences were observed in PII, I.3, and I.7 promoter-derived *CYP19* mRNA levels. Cells treated with forskolin (10 µM) exhibited 4.6 ± 0.4 and 2.3 ± 0.3 fold higher PII and I.3 promoter-mediated *CYP19* expression, respectively, than did DMSO controls. By contrast, no significant effects were observed on transcripts derived from the I.7 promoter (Figure 1A). Finally, Hs578t cells treated with VEGF exhibited significantly higher I.7 and PII-mediated *CYP19* expression (13.7 ± 1.2 and 6.6 ± 0.4 fold, respectively) compared with DMSO control (Figure 1A).

Aromatase Catalytic Activity in Hs578t Cells

Formestane inhibited aromatase catalytic activity in Hs578t cells. Dexamethasone (100 nM), forskolin (10 µM), and VEGF (2.5 ng/mL) induced aromatase catalytic activity in Hs578t cells by 1,973 ± 673, 166 ± 23, and 169 ± 25 fold, respectively, compared with DMSO controls (Figure 1B).

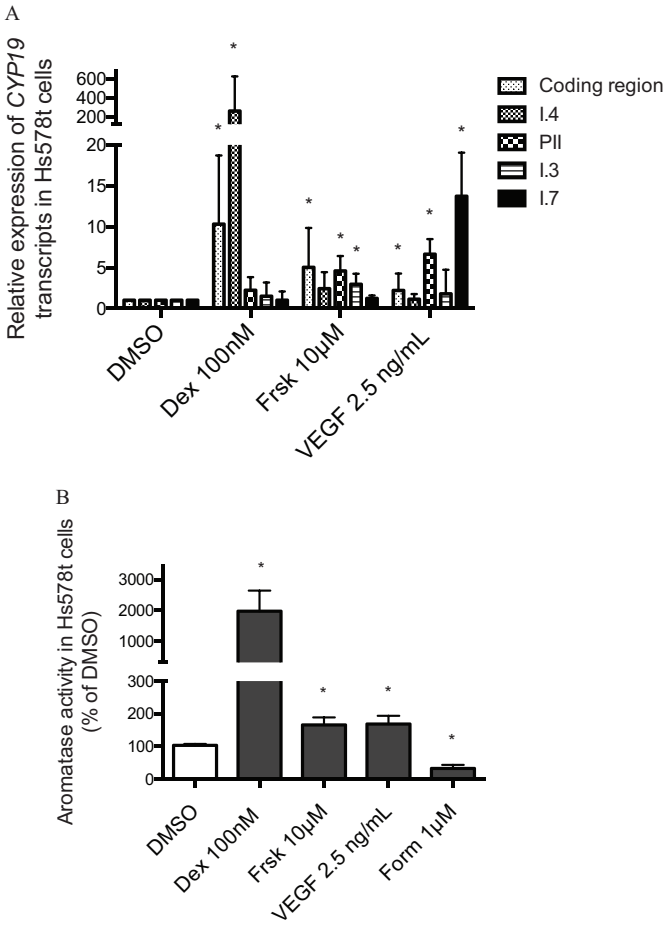

Figure 1. (*A*) Relative expression of *CYP19* coding region (nonpromoter-specific or total), and I.4, PII, I.3, and I.7 promoter-derived *CYP19* transcripts in Hs578t cells (fold DMSO control). (*B*) Aromatase catalytic activity in Hs578t cells exposed to dexamethasone (DEX) 100 nM, forskolin (Frsk) 10 µM, vascular endothelial growth factor (VEGF) 2.5 ng/mL, or formestane (Form) 1 µM. Experiments were performed in triplicate with three different cell passages; per experiment, each treatment was tested in triplicate. Cells were exposed to treatments for 24 h. *, $p < 0.05$. Statistically significant difference between treatments compared with DMSO (Student *t*-test).

Effects of Inhibition of the PLC and MAPK 1/3 Pathways on Promoter-Specific Expression of CYP19 in Hs578t Cells

To assess the involvement of two VEGF signaling pathways (PLC and MAPK 1/3) in the promoter-specific expression of *CYP19*, Hs578t cells were pretreated with pathway-selective inhibitors 4 h prior to addition of VEGF or forskolin. Pretreatment of Hs578t cells with the PLC inhibitor U73122 (2 µM) prior to forskolin (10 µM) treatment resulted in significantly lower relative (to DMSO control) expression of *CYP19* coding region than treatment with forskolin alone (1.4 ± 0.2 vs. 5.7 ± 0.83 fold; Figure 2A) this was also true for PII-mediated *CYP19* expression (2.7 ± 1.1 vs. 17.7 ± 9.5 fold; Figure 2B), and I.3-mediated *CYP19* expression (2.0 ± 0.8 vs. 17.7 ± 9.5 fold; Figure 2C). Furthermore, pretreatment of Hs578t cells with the MEK/MAPK 1/3 inhibitor PD98059 (50 µM) prior to VEGF (2.5 ng/mL) treatment also resulted in a significantly lower relative expression of *CYP19* coding region than treatment with VEGF alone (0.59 ± 0.25 vs. 4.3 ± 1.3 fold; Figure 2A); this was also the case

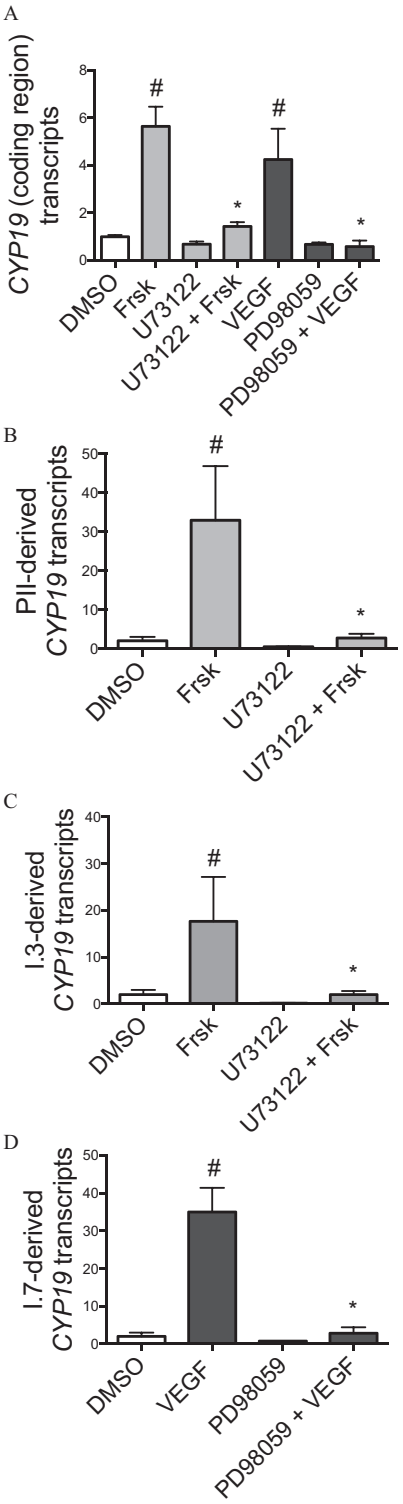

Figure 2. Relative expression of (*A*) *CYP19* coding region (nonpromoter-specific or total), and *CYP19* transcripts derived from promoters (*B*) PII, (*C*) I.3, and (*D*) I.7 in Hs578t cells (fold DMSO control). Cells were exposed for 24 h to 10 μM forskolin (Frsk) or 2.5 ng/mL VEGF, inducers of PII/I.3 or I.7 promoter-mediated *CYP19* expression, in the presence or absence of selective inhibitors of the PLC (U73122; 2 μM) or MEK/MAPK 1/3 (PD98059; 50 μM) signaling pathways. Experiments were performed in triplicate with three different cell passages; per experiment, each treatment was tested in triplicate. *, $p < 0.05$. Statistically significant difference between Hs578t cells pretreated with U73122 compared with those treated with Frsk alone, or between Hs578t cells pretreated with PD98059 compared with those treated with VEGF alone (Student *t*-test). #, $p < 0.05$. Significantly different from DMSO control (Student *t*-test).

for I.7-mediated *CYP19* expression (2.9 ± 1.6 vs. 35.0 ± 6.4 fold; Figure 2D).

Effects of Neonicotinoids on Promoter-Specific Expression of CYP19 in Hs578t Cells

Generally, in Hs578t cells treated for 24 h with thiacloprid, at all concentrations above 0.03 μM (0.1–10 μM), I.4 promoter-mediated *CYP19* expression was lower than in control cells, whereas levels of PII, I.3, and I.7 promoter-derived *CYP19* transcripts as well as overall (promoter-nonspecific coding region) *CYP19* transcript were increased (Figure 3A). Above 0.03 μM, relatively lower concentrations appeared to have greater effects. Hs578t cells exposed to 0.1 μM thiacloprid had lower *CYP19* expression via the I.4 promoter (0.046 ± 0.041 fold of DMSO controls), whereas PII (34.49 ± 12.07 fold), I.7-mediated *CYP19* (3.54 ± 0.80) and overall coding region expression (57.37 ± 37.22 fold) were significantly higher than DMSO controls (Figure 3A). I.3-mediated *CYP19* expression was higher (2.00 ± 0.05 fold) than DMSO controls, although this was not statistically significant. We observed an increase of the catalytic activity of aromatase at 0.1, 0.3, and 10 μM thiacloprid, with greater increases at relatively lower concentrations (Figure 3B).

In Hs578t cells treated for 24 h with the neonicotinoid imidacloprid, differences in promoter-specific *CYP19* expression were observed in concentrations of 0.1–3 μM (Figure 4A), compared with DMSO controls. Following a 24-h exposure to 0.1 μM imidacloprid, the relative levels of I.4 promoter-derived *CYP19* transcripts were significantly lower (0.61 ± 0.10 fold) whereas PII, I.3, and I.7 promoters-derived CYP19 transcripts was higher (11.0 ± 1.1, 1.8 ± 0.3, and 7.3 ± 0.3 fold) than the DMSO control, with an overall higher coding region expression (2.7 ± 0.4 fold) relative to DMSO controls (Figure 4A). Compared with DMSO controls, the catalytic activity of aromatase was increased significantly after treatment with 0.1 μM imidacloprid (Figure 4B).

Effects of Inhibition of the PLC and MAPK 1/3 Pathways on Neonicotinoid-Mediated Changes in CYP19 Expression in Hs578t Cells

To investigate whether the effects of neonicotinoids on promoter-specific *CYP19* expression were due to an action on the PLC and/or MEK/MAPK 1/3 pathways, we determined the promoter-specific expression of *CYP19* in Hs578t cells treated with either thiacloprid or imidacloprid (0.1 μM) in the presence of a selective inhibitor of either the PLC (U73122, 2 μM) or MEK/MAPK 1/3 (PD98059, 50 μM) pathway. Hs578t cells pretreated with 2 μM U73122 prior to 0.1 μM thiacloprid had significantly lower expression of promoter-nonspecific *CYP19* (coding region; 32.9 ± 17.8%) than did Hs578t cells exposed to thiacloprid alone. When Hs478t cells were pretreated with 50 μM PD98059, expression of promoter-nonspecific *CYP19* transcripts were lower (56.0 ± 19.2%) than expression in cells exposed to thiacloprid alone, although this inhibition was not statistically significant (Figure 5A). Pretreatment of Hs578t cells with 2 μM U73122 prior to 0.1 μM thiacloprid resulted in significantly lower PII and I.3 promoter-mediated *CYP19* expression (17.0 ± 15.3% and 33.1 ± 17.9%, respectively) than that measured in Hs578t cells exposed to thiacloprid alone (Figure 5B,C). Furthermore, pretreatment of Hs578t cells with 50 μM PD98059 prior to 0.1 μM thiacloprid resulted in lower I.7 promoter-mediated *CYP19* expression (39.0 ± 4.1%) than that in Hs578t cells exposed to thiacloprid alone (Figure 5D).

We observed a similar trend for imidacloprid. In Hs578t cells pretreated with 2 μM U73122 prior to 0.1 μM imidacloprid, expression of promoter-nonspecific *CYP19* (coding region) transcripts was lower (33.2 ± 19.3%) than expression in Hs578t cells

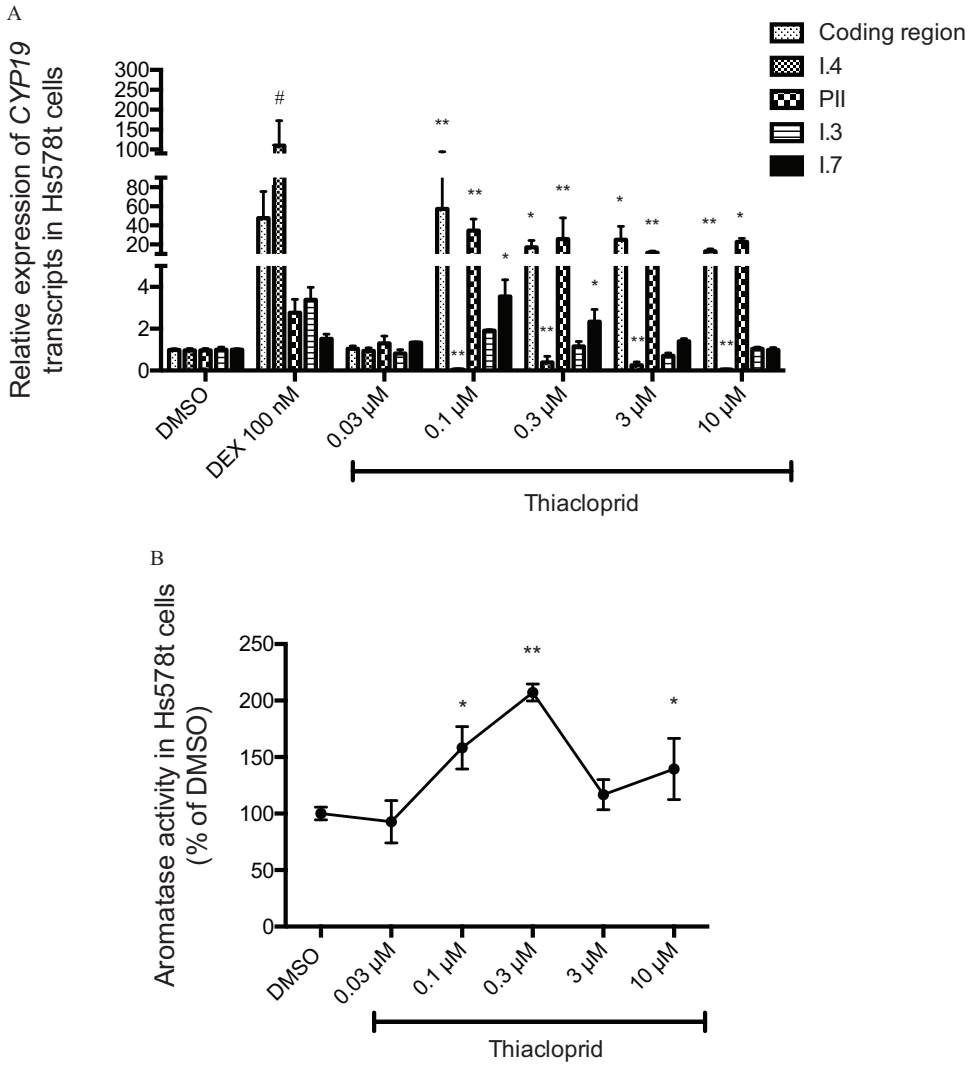

Figure 3. (*A*) Relative expression of *CYP19* coding region (nonpromoter-specific or total), and I.4, PII, I.3, and I.7 promoter-derived *CYP19* transcripts in Hs578t cells (fold DMSO control). (*B*) Aromatase catalytic activity in Hs578t cells exposed to thiacloprid (0.03, 0.1, 0.3, 3, and 10 µM). DEX (100 nM) was used as a positive control for I.4 promoter-mediated *CYP19* expression. Experiments were performed in triplicate with three different cell passages; per experiment, each treatment was tested in triplicate. DEX, dexamethasone. *, $p < 0.05$; **, $p < 0.01$). Statistically significant difference between thiacloprid and DMSO control (one-way ANOVA and Dunnett post hoc test). #, $p < 0.05$. Statistically significant difference between DEX treatment and DMSO control (Student t-test).

exposed to imidacloprid alone (Figure 5A); PII and I.3-mediated *CYP19* expression was lower ($29.7 \pm 1.8\%$ and $26.8 \pm 13.9\%$, respectively) than expression in cells exposed to imidacloprid alone (Figure 5B,C). In Hs578t cells pretreated with 50 µM PD98059 prior to 0.1 µM imidacloprid, we observed a nonsignificant lower expression of *CYP19* coding region ($37 \pm 25\%$) than expression when treated with imidacloprid alone (Figure 5A). However, the same pretreatment did not result in lower I.7 promoter-mediated *CYP19* expression (Figure 5D).

Discussion

Hs578t Cells as a Suitable Model to Study the Promoter-Specific Expression of CYP19 in Hormone-Dependent Breast Cancer

In this study, we successfully developed robust and sensitive real-time quantitative PCR methods to evaluate the expression of *CYP19* via four specific promoters, namely the normally active I.4 promoter and the breast cancer-associated promoters PII, I.3, and I.7, using Hs578t cells as a representative model of the aromatase-expressing and estrogen-producing cells typically found in the hormone-dependent breast tumor environment. Triple-negative cells do not express estrogen or progesterone receptors and do not display amplification of the human epidermal growth factor receptor 2 (HER2) (Chavez et al. 2010). Epithelial cells normally do not express aromatase, but it has been previously demonstrated that triple-negative breast cancer cells (MDA-MB-231) express *CYP19* by the activation of the adipose I.4 promoter and breast cancer-associated proximal PII/I.3 promoters, and that aromatase is catalytically active in this cell line (Knower et al. 2010; Su et al. 2008).

In breast cancer, increased *CYP19* expression and estrogen synthesis is driven by a promoter-switch involving the activation of the PII, I.3, and I.7 promoters, and inhibition of normal I.4 promoter activity (Irahara et al. 2006; Sebastian and Bulun 2001). To our knowledge, we are the first to demonstrate that Hs578t cells express *CYP19* through these four breast cancer-relevant promoters, which is key to the relevance of this cell line as an *in vitro* model of the estrogen-producing cells present in the hormone-

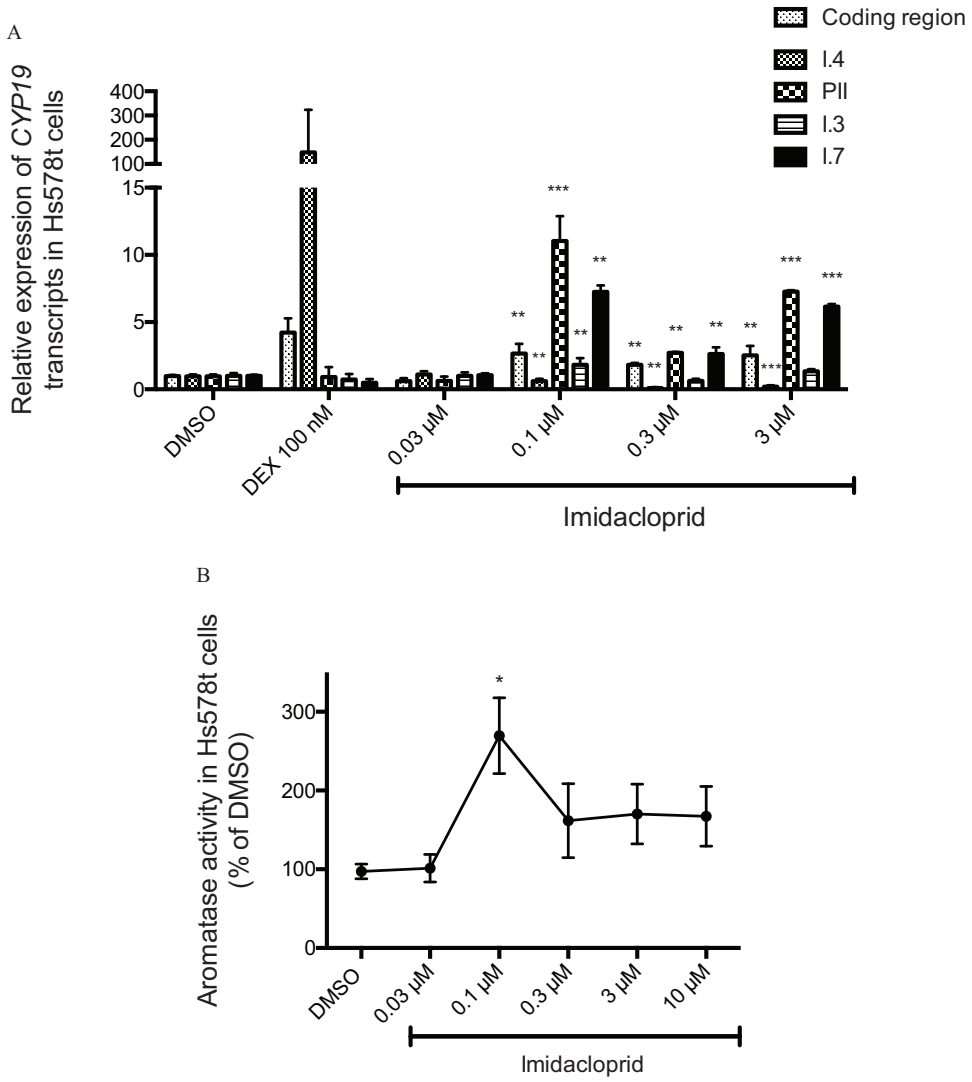

Figure 4. (*A*) Relative expression of *CYP19* coding region (nonpromoter-specific or total), and I.4, PII, I.3, and I.7 promoter-derived *CYP19* transcripts in Hs578t cells (fold DMSO control). (*B*) Aromatase catalytic activity in Hs578t cells exposed to imidacloprid (0.03, 0.1, 0.3, and 3 µM). DEX (100 nM) was used as a positive control for I.4 promoter-mediated *CYP19* expression. DEX, dexamethasone. Experiments were performed in triplicate with three different cell passages; per experiment, each treatment was tested in triplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Statistically significant difference between imidacloprid compared with DMSO control (one-way ANOVA and Dunnett post hoc test). #, $p < 0.05$. Statistically significant difference between DEX treatment and DMSO control (Student *t*-test).

dependent breast tumor environment. The mechanisms leading to this switch in *CYP19* promoter usage are not fully understood. Breast cancer epithelial cells synthesize prostaglandin E_2 (PGE_2), a G-protein–coupled receptor that stimulates the production of cAMP. Cyclic AMP then activates the protein kinase A (PKA) pathway, leading to the phosphorylation of cAMP responsive element binding protein 1 (CREB1). CREB1 then translocates to the nucleus and binds to CRE-like sequences in the PII/I.3 promoter region to stimulate promoter activity, which leads to increased expression of *CYP19* (Sofi et al. 2003; To et al. 2015; Zhao et al. 1996). PKA can also phosphorylate the transcription factor GATA-4, which recruits coactivators such as the CREB-binding protein (CBP). The resulting complex then binds to the PII promoter region of *CYP19* (Tremblay and Viger 2003).

We know less about the endothelial I.7 promoter of *CYP19*. This promoter, originally characterized by Sebastian et al. (2002), may have a role in regulating the effects of estrogens on blood vessels through its main regulator, the transcription factor GATA-2. However, it has also been demonstrated that the I.7 promoter is overactive in breast cancer (Sebastian et al. 2002). As VEGF is involved in angiogenesis in breast cancer and has a role in increasing endothelial permeability (Breslin et al. 2003), we hypothesized that I.7 promoter activation is regulated by the VEGF/MEK/MAPK 1/3 signaling pathway in Hs578t cells. It is known that MEK/MAPK 1/3 is activated by the binding of VEGF to its receptors (VEGFR-1 and VEGFR-2) (Breslin et al. 2003; Cross and Claesson-Welsh 2001; Lee et al. 1998). We also know that the activation of VEGFR-2 in endothelial cells stimulates the PLC/PKC pathway (Cario et al. 2004; Cross and Claesson-Welsh 2001; Jiang et al. 2016), thus explaining the overexpression of PII-derived *CYP19* in Hs578t cells exposed to VEGF (Figure 1A).

Angiogenesis is associated with tumor growth and metastasis in breast cancer (Adams et al. 2000) and increased expression of VEGF and its receptors has been denoted in invasive breast carcinomas (Yoshiji et al. 1996). In our study, VEGF stimulated I.7- and PII-mediated *CYP19* expression, resulting in an increase in overall (nonpromoter-specific) expression of *CYP19* (Figure 1A) and aromatase catalytic activity (Figure 1B). Using an inhibitor of the MEK/MAPK 1/3 pathway, we also demonstrated that the VEGF-mediated overexpression of I.7 promoter-derived *CYP19*

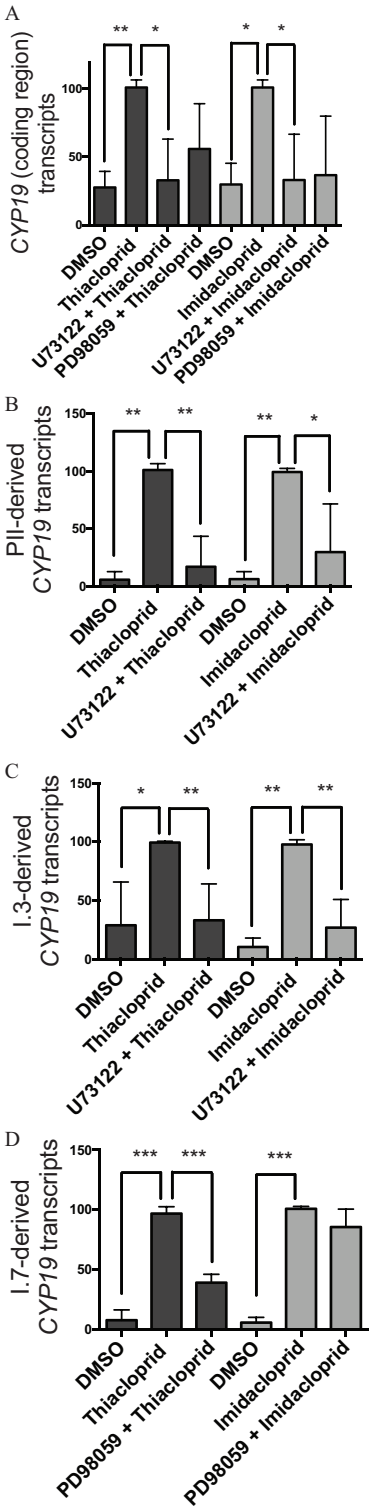

Figure 5. Relative expression of (*A*) *CYP19* coding region (nonpromoter-specific or total), and *CYP19* transcripts derived from promoters (*B*) PII, (*C*) I.3, and (*D*) I.7 in Hs578t cells exposed to thiacloprid (0.1 μM) or imidacloprid (0.1 μM) in the presence or absence of selective inhibitors of the PLC (U73122, 2 μM) or MEK/MAPK 1/3 (PD98059, 50 μM) signaling pathways. Relative transcript levels are expressed as a percentage (%) of the response of Hs578t cells exposed to 0.1 μM thiacloprid or imidacloprid (100%). Experiments were performed in triplicate with three different cell passages; per experiment, each treatment was tested in triplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Statistically significant difference between inhibitor pretreatment and neonicotinoid treatment alone (Student *t*-test).

was at least in part mediated through the MEK/MAPK 1/3 pathway (Figure 2A,D). This finding supports our hypothesis and is consistent with the literature. Indeed, it has been demonstrated that VEGF increases human endothelial permeability via the MEK pathway (Breslin et al. 2003), that incubation of endothelial cells with PD98059 reduces MAPK 1 activity (Pai et al. 2001), and that VEGF induces the phosphorylation of MAPK 1/2 (Xu et al. 2008). We have also demonstrated that treatment with forskolin stimulates PII/I.3-mediated *CYP19* expression and induces aromatase activity (Figure 1A,B), and that this effect is mediated at least in part through the PLC pathway (Figure 2A–C). This result is also supported by a study showing that in HEK-293 cells, forskolin induces calcium release (Schmidt et al. 2001), an important component of the PLC pathway. These results suggest that VEGF signaling pathways, and more specifically the PLC and MEK/MAPK 1/3 pathways, are involved in PII/I.3 and I.7-mediated *CYP19* expression in Hs578t breast cancer cells.

Effects of Neonicotinoids on the Promoter-Specific Expression of CYP19

Certain contaminants such as atrazine exert estrogenic activity by increasing *CYP19* expression and aromatase activity (Fan et al. 2007; Sanderson et al. 2002), which would result in increased biosynthesis of estrogens (Caron-Beaudoin et al. 2017; Eldridge et al. 1994). Moreover, we have previously shown that atrazine, and recently, that several neonicotinoid insecticides induce the promoter-specific expression of *CYP19* and/or its catalytic activity in various *in vitro* cell systems (Caron-Beaudoin et al. 2017; Caron-Beaudoin et al. 2016).

In the present study, we have found that treatment of Hs578t breast cancer cells with the neonicotinoids thiacloprid and imidacloprid results in an overall increase in *CYP19* expression and catalytic activity of aromatase compared with control (Figures 3 and 4), an observation consistent with a neonicotinoid-induced switch in *CYP19* promoter usage. Results from pretreatment of cells with VEGF pathway inhibitors suggest that thiacloprid increases PII/I.3 and I.7 promoter-mediated *CYP19* expression through activation of the PLC and MEK/MAPK 1/3 pathways (Figures 5 and 6). We observed a similar promoter-specific response in Hs578t cells exposed to imidacloprid, although inhibition of the MEK/MAPK 1/3 pathway did not statistically significantly alter the response of the cells to this neonicotinoid (Figures 5 and 6). Exposure of Hs578t cells to thiacloprid and imidacloprid resulted in an increase of predominantly PII promoter-derived *CYP19* transcripts and a more modest increase in I.3 promoter-derived transcripts compared with control. This differential effect on the two promoters is not unusual because similar expression patterns have been observed in primary adipose stromal cells exposed to phorbol 12-myristate 13-acetate (PMA), PGE_2, or forskolin (Heneweer et al. 2004; Zhao et al. 1996). We also previously observed an increase in PII/I.3 promoter-mediated *CYP19* expression in H295R cells exposed to the neonicotinoids thiacloprid, imidacloprid, and thiamethoxam (Caron-Beaudoin et al. 2016).

Limitations and Perspectives

Cell–cell communication during hormone-dependent breast cancer progression has been widely studied. For instance, communication between epithelial cancer cells and fibroblastic cells surrounding the tumor leads to a desmoplastic reaction associated with the accumulation of fibroblasts (preadipocytes) due to inhibition of their differentiation into stromal adipocytes. Preadipocytes have greater *CYP19* expression than differentiated stromal cells and are key actors in the overproduction of estrogens in the tumor microenvironment, leading to proliferation of cancer cells (Kalluri and

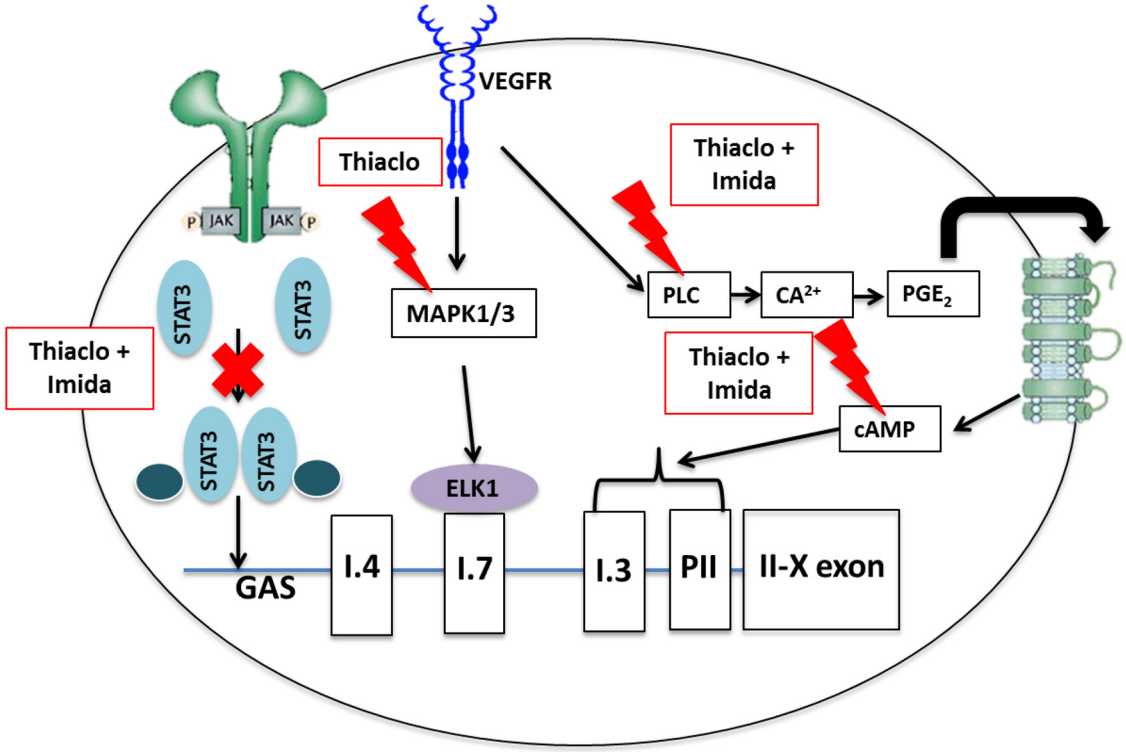

Figure 6. Proposed signaling pathways implicated in the promoter-specific expression of *CYP19* in Hs578t cells. It is proposed that thiacloprid and imidacloprid block the JAK/STAT3 pathway, which regulates I.4 promoter activity via a yet unknown mechanism. In addition, based on the effects of pathway inhibitors, the neonicotinoids are proposed to increase PII/I.3-mediated *CYP19* expression in Hs578t cells via stimulation of the PLC pathway. Thiacloprid may also induce I.7-mediated *CYP19* expression by stimulation of the MEK/MAPK 1/3 pathway. $CA2^{2+}$, calcium ion; cAMP, cyclic adenosine monophosphate; X, inhibition; ELK, electron transport system transcription factor; GAS, gamma interferon activation site; Imida, imidacloprid; JAK, Janus kinase; lightning bolt, activation/stimulation; MAPK, mitogen-activated protein kinases; PGE_2, prostaglandin E_2; PLC, phospholipase C; STAT, signal transducer and activator of transcription protein; Thiaclo, thiacloprid; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

Zeisberg 2006; Meng et al. 2001; Zhao et al. 1996). Therefore, using a single-cell bioassay has its limitations, given that we are not able to adequately mimic the cellular interactions during breast cancer progression. However, the present study in Hs578t cells provides crucial information for a better understanding of the mechanisms underlying the expression of *CYP19* by breast cancer-relevant promoters, as well as the impacts of neonicotinoids on these processes. We are currently developing a cellular coculture model in which Hs578t cells together with estrogen-responsive epithelial breast cancer cells will produce a more representative model of the tumor microenvironment. This coculture model will provide a more physiologically and toxicologically relevant study tool to better understand impacts of environmental contaminants on hormone-dependent breast cancer.

Conclusions

To the best of our knowledge, the present study is the first to describe the promoter-specific expression of *CYP19* via the normal mammary promoter I.4 and the breast cancer-relevant promoters PII, I.3, and I.7 in Hs578t cells. We have further shown that exposure of these cells to concentrations of the neonicotinoid insecticides thiacloprid and imidacloprid similar to what is found in urine of farmers and women from the general population in Japan increase *CYP19* expression, associated with a decrease in I.4 promoter activity and an increase in the activities of promoters PII, I.3, and I.7. The observed promoter-switch appears to involve the VEGF-mediated PLC and MAPK 1/3 signaling pathways (Figure 6). This unique switch in promoter usage induced by thiacloprid and imidacloprid is a process usually observed in patients with progressive hormone-dependent breast cancer. However, the molecular targets of thiacloprid and imidacloprid involved in this promoter-switch remain unknown. Future work should also focus on investigating the signaling pathways implicated in the decrease of I.4-mediated *CYP19* expression in Hs578t cells in response to the promoter-switch induced by exposed to neonicotinoids. Our findings highlight the need for further research to assess the potential impacts of low-dose and chronic exposure to neonicotinoids on endocrine processes affecting women's health.

Acknowledgments

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The use of a unique co-culture model of fetoplacental steroidogenesis as a screening tool for endocrine disruptors: The effects of neonicotinoids on aromatase activity and hormone production

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ABSTRACT

Estrogen biosynthesis during pregnancy is dependent on the collaboration between the fetus producing the androgen precursors, and the placenta expressing the enzyme aromatase (*CYP19*). Disruption of estrogen production by contaminants may result in serious pregnancy outcomes. We used our recently developed *in vitro* co-culture model of fetoplacental steroidogenesis to screen the effects of three neonicotinoid insecticides on the catalytic activity of aromatase and the production of steroid hormones. A co-culture of H295R human adrenocortical carcinoma cells with fetal characteristics and BeWo human choriocarcinoma cells which display characteristics of the villous cytotrophoblast was exposed for 24 h to various concentrations of three neonicotinoids: thiacloprid, thiamethoxam and imidacloprid. Aromatase catalytic activity was determined in both cell lines using the tritiated water-release assay. Hormone production was measured by *ELISA*. The three neonicotinoids induced aromatase activity in our fetoplacental co-culture and concordingly, estradiol and estrone production were increased. In contrast, estriol production was strongly inhibited by the neonicotinoids. All three pesticides induced the expression of *CYP3A7* in H295R cells, and this induction was reversed by co-treatment of H295R cells with exogenous estriol. *CYP3A7* is normally expressed in fetal liver and is a key enzyme involved in estriol synthesis. We suggest that neonicotinoids are metabolized by *CYP3A7*, thus impeding the 16α-hydroxylation of fetal DHEA(-sulfate), which is normally converted to estriol by placental aromatase. We successfully used the fetoplacental co-culture as a physiologically relevant tool to highlight the potential effects of neonicotinoids on estrogen production, aromatase activity and *CYP3A7* expression during pregnancy.

1. Introduction

The use of *in vitro* models in toxicology has significantly enhanced our understanding of the mechanisms by which chemicals cause adverse effects in humans and wildlife. However, to mimic the interactions that occur *in vivo* is a challenge when whole animal or human studies are not possible. Well thought-out *in vitro* models, such as the use of co-culture models, are promising approaches to study the communication between different cell types in a more complex context. As example, a co-culture using primary human mammary fibroblasts and MCF-7 (epithelial breast cancer cells) was developed by (Heneweer et al., 2005) to study intercellular interactions in breast cancer. More recently, we developed a co-culture model that reproduces the steroidogenic fetoplacental unit and can be used to evaluate the impacts of endocrine disruptors on this delicate aspect of fetoplacental communication (Hudon Thibeault et al., 2014; Hudon Thibeault et al., 2017).

During pregnancy, the fetoplacental unit plays an important endocrine role, ensuring, amongst others, estrogen (estrone, estradiol and estriol) biosynthesis. Estrogens are required in several physiological processes during pregnancy, such as the formation of the syncytiotrophoblast and regulation of uteroplacental blood flow (Yashwanth et al., 2006). Maternal cholesterol is converted to androgen precursors in the fetus by the action of several enzymes, such as cytochrome P450 17 (*CYP17*), sulfotransferase 2A1 (*SULT2A1*) and steroid 16α-hydroxylase (*CYP3A7*). In the placenta, *CYP19* (aromatase) is responsible for the final step in estrone, estradiol and estriol biosynthesis (Leeder et al., 2005; Rainey et al., 2002). Alone, the placenta cannot produce estrogens *de novo*, as it needs the steroid precursors synthesized

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by the fetus (for complete steroidogenesis pathway, see Fig. 7 in Results section). Estriol is uniquely produced during pregnancy, and its synthesis requires correct functioning of the fetoplacental unit (Mucci et al., 2003). Thus, a disruption in biosynthesis of estrogens such as that of estriol may adversely alter development and influence important indicators of fetal health like birth weight and head circumference (Kaijser et al., 2000; Troisi et al., 2003). Moreover, decreased free estriol in maternal serum has been associated with growth retardation, reduced Apgar scores and postnatal complications in a control study of 869 women (Gerhard et al., 1986).

Exposure during pregnancy to contaminants such as heavy metals, pesticides, polychlorinated biphenyls (PCBs) and phthalates have been linked to fetal growth retardation (Siddiqui et al., 2003), spontaneous abortions, learning disabilities (Hu, 1991; Abadin et al., 1997), reduced birth weight, preterm birth (Jacobson et al., 1990) and disruption of reproductive development (Mylchreest et al., 2000; Honma et al., 2002; Foster, 2006). It is not surprising that exposure to certain chemicals during pregnancy can lead to adverse pregnancy and birth outcomes, since *in utero* development is a critical window of vulnerability of the embryo (Bellinger, 2013). The fetoplacental co-culture model of steroidogenesis developed in our laboratory (Hudon Thibeault et al., 2014) allows us to study chemicals that may impair estrogen biosynthesis or that of other key placental hormones such as β-human chorionic gonadotropin (β-hCG), potentially leading to serious pregnancy complications (Albrecht and Pepe, 1999; Albrecht et al., 2000; Svedas et al., 2002). We have reported earlier that prochloraz, a widely-used fungicide, and norfluoxetine, a selective serotonin-reuptake inhibitor, strongly inhibited aromatase activity and estrogen production in our fetoplacental co-culture model (Hudon Thibeault et al., 2014; Hudon Thibeault et al., 2017).

Neonicotinoids are some of the most widely used insecticides in the world. For example, thiamethoxam and clothianidin were both in the top 10 most sold insecticides in Canada in 2010 (Health Canada, 2014). By 2012, neonicotinoids were applied to 11 million hectares in Canada, representing >216,000 kg of active neonicotinoid (Main et al., 2014). Neonicotinoids are mostly used as seed coatings on the vast majority of crops, fruits and vegetables. Moreover, their physicochemical characteristics (K_{ow} and pK_a) explain their systemic properties and their distribution throughout the entire plant (Bonmatin et al., 2015; Simon-Delso et al., 2015). For this reason, neonicotinoid insecticides also target pollinators, mammals and humans. The scientific community is increasingly accepting that exposure to these insecticides partially explains the worldwide decline in honeybees populations (Decourtye et al., 2004; Girolami et al., 2009; Henry et al., 2012; Goulson, 2013). Neonicotinoid insecticides are also persistent in the environment. Half-lives in soil vary and can reach 1250 days for imidacloprid (Main et al., 2014). Because of their persistence and repeated application, it is expected that neonicotinoids will continue to accumulate in soil (Stokstad, 2013). A recent study analyzed neonicotinoid levels in surface waters from 136 wetlands across Saskatchewan, Canada. Clothianidin and thiamethoxam concentrations were detected in the majority of water samples, reaching concentrations as high as 3110 ng/L (Main et al., 2014). Moreover, human populations are also exposed to neonicotinoids through diet. A study conducted in Boston, Massachusetts, analyzed neonicotinoid residues in honey, fruits and vegetables purchased in local grocery stores. Imidacloprid was the most frequently detected neonicotinoid in the samples. At least one neonicotinoid was detected in all the tested fruits and vegetables. Also, in 72% of fruits and 45% of the tested vegetables, two or more neonicotinoids were detected, with concentrations reaching 100.7 ng/g (Chen et al., 2014). Furthermore, a study conducted with a cohort of 147 farm workers from northeastern Japan evaluated the presence of neonicotinoid metabolites in urine. A metabolite of the neonicotinoid dinotefuran, 3-furoic acid, was detected in 100% of the samples at concentrations as high as 0.13 μM (Nomura et al., 2013). Moreover, the concentrations of 6-chloronicotinic acid, a metabolite of imidacloprid and thiacloprid, reached concentrations of 0.05 μM (Nomura et al., 2013).

In recent years, a growing number of studies have evaluated the endocrine disrupting potential of neonicotinoid insecticides. We demonstrated that two neonicotinoids (thiacloprid and thiamethoxam) induce aromatase expression in a promoter-specific manner *in vitro*, targeting promoters known to be overexpressed in breast cancer (Caron-Beaudoin et al., 2016). Moreover, Bal et al. (2012) found that male rats exposed to imidacloprid (2 mg/kg/day) through diet showed increased apoptosis and fragmentation of seminal DNA. In female rats exposed to the same neonicotinoid (20 mg/kg/day), Kapoor et al. (2011) noted decreased ovarian weight and altered levels of follicle stimulating hormone and progesterone.

In this study, we used our recently developed fetoplacental co-culture model as a screening tool to determine the effects of three widely used neonicotinoid insecticides on steroidogenesis in the human fetoplacental unit, and more precisely, on aromatase activity, estrogen production and *CYP3A7* expression (key enzyme in the fetal production of the estriol precursor 16α-hydroxyDHEA(-sulfate). Our previous work showed that two neonicotinoid insecticides, thiacloprid and thiamethoxam, induced *CYP19* expression and aromatase activity at environmentally relevant concentrations in human H295R adrenocortical carcinoma cells displaying characteristics of the fetal adrenal cortex and which represents the fetal compartment of our fetoplacental co-culture (Caron-Beaudoin et al., 2016). Therefore, we hypothesized that neonicotinoids may also disrupt the production of estrogens within the fetoplacental unit.

2. Materials and methods

2.1. Chemicals

All pesticides were purchased from Sigma-Aldrich (St-Louis, MO) (thiacloprid, Pestanal 37905, purity > 99%; thiamethoxam, Pestanal 37924, purity > 99%; imidacloprid, Pestanal 37894, purity > 99%). All neonicotinoids were dissolved in dimethylsulfoxide (DMSO) as 30 or 100 mM stock solutions.

2.2. Feto-placental co-culture

The feto-placental co-culture (Hudon Thibeault et al., 2014) consists of H295R adrenocortical carcinoma and BeWo choriocarcinoma cells. H295R cells have the characteristics of the fetal adrenocortex (Gazdar et al., 1990; Staels et al., 1993) as well as that of fetal liver (Hudon Thibeault et al., 2014) and reflect the steroidogenesis that would occur in the fetal compartment. BeWo cells are a well documented model of the placental trophoblast (Ellis et al., 1990; Nampoothiri et al., 2007). This co-culture model of the fetoplacental unit is capable of *de novo* production of estrogens, including the unique pregnancy estrogen estriol, under our experimental conditions (Hudon Thibeault et al., 2014). Briefly, BeWo (ATCC no. CCL-98) and H295R (ATCC no. CRL-2128) cells were cultured separately in their respective recommended media. BeWo cells were cultured in DMEM/F-12 without phenol red (Catalog no. 11039021, Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS; Hyclone, Tempe, AZ). H295R were cultured in DMEM/F-12 (Catalog no. 11039021, Thermo Fisher Scientific, Waltham, MA), supplemented with 2.5% Nu Serum (BD Biosciences, Mississauga, ON, Canada) and 1% ITS + Premix (BD Biosciences, Mississauga, ON, Canada). Once confluent, BeWo and H295R cells were trypsinized, and H295R cells were seeded in 24-well plates at a concentration of 2.5×10^4 cells/well, whereas BeWo cells were seeded in transwell inserts (Corning Life Sciences, Corning, NY) at a concentration of 1.25×10^4 cells/insert, After 24 h, the co-culture was assembled by adding the inserts containing BeWo cells to the 24-well plates containing H295R cells. The original culture media were removed and replaced with co-culture media which contained DMEM/F-12 without phenol red, supplemented with 2.5% stripped Nu Serum, 1% ITS + Premix and 1% stripped FBS, and the various concentrations of the neonicotinoids or

vehicle control (DMSO at a final concentration of 0.1%). Neonicotinoid concentrations were chosen based on our previous study that determined their effects on *CYP19* expression in H295R cells and an absence of cytotoxicity (Caron-Beaudoin et al., 2016). Moreover, the selected concentrations are environmentally relevant as our lower range is similar to what is measured in urine samples from farm workers (Nomura et al., 2013). Exposures were for 24 h in an incubator at 37°C with a humidified atmosphere containing 5% CO_2.

2.3. CYP19 catalytic activity

Aromatase activity was determined as previously described (Sanderson et al., 2000; Hudon Thibeault et al., 2014; Caron-Beaudoin et al., 2016). Briefly, after the 24-h exposure period, the treated media in the insert and well were combined and removed (and stored at −80°C for hormone quantification), and H295R and BeWo cells were washed twice with 1× PBS. The inserts containing BeWo cells were removed from the wells containing H295R cells and placed in 12-well plates for further steps. A volume of 250 μL of serum and additive-free culture medium containing 54 nM 1β-^{3}H-androstenedione (Perkin Elmer, Wellesley, MA) was added to H295R cells. This volume was 50 μL for BeWo cells. Cells were incubated for 90 min at 37°C, during which there was a linear conversion of 1β-^{3}H-androstenedione (and release of tritiated water). Tritiated water was extracted from the reaction medium of each of the cell types, and counted in plates containing liquid scintillation cocktail using a Microbeta Trilux (Perkin Elmer). Formestane (1 μM), an irreversible *CYP19* inhibitor, was used to ensure the specificity of the aromatase reaction. Forskolin (10 μM) was used as a positive control for the induction of aromatase activity.

2.4. Hormone quantification

The following hormones, dehydroepiandrosterone (DHEA), androstenedione, β-human chorionic ganodotropin (β-HCG), estradiol, estrone and estriol) were quantified in the co-culture media (well and insert pooled) using *ELISA* kits from DRG Diagnostics (Marburg, Germany).

2.5. RNA isolation and quantitative RT-PCR

RNA isolation and quantitative RT-PCR were performed as describe in Caron-Beaudoin et al. (2016). Briefly, H295R cells were cultured in CellBind 6-well plates (Corning Inc., Corning, New York) at a concentration of 750,000 cells/well in 2 mL medium/well for 24 h. Cells were subsequently exposed for 24 h to thiacloprid, thiamethoxam or imidacloprid at 3 and 10 μM, which were the concentrations with the greatest effects on aromatase catalytic activity in the co-culture. To investigate whether the inhibitory effects of neonicotinoids on estriol production in the co-culture were responsible for altering *CYP3A7* enzyme expression, H295R cells were pretreated for 4 h with estriol (5 ng/mL), prior to a 24-h exposure to thiacloprid, thiamethoxam or imidacloprid (3 μM). DMSO (0.1% or 0.2% in the case of co-treatments) was used as a vehicle control. RNA was extracted using the RNeasy mini-kit (Qiagen, Mississauga, Ontario) according to enclosed instructions and stored at −80 °C. The 260/280 nm absorbance ratio was used to verify RNA purity. Reverse transcription was subsequently performed with 1 μg of RNA using the iScript cDNA Synthesis Kit (BioRad, Hercules, CA) and T3000 Thermocycler (Biometra, Göttingen, Germany): cDNA was stored at −20°C for quantitative PCR.

Primer pair sequences and standard curves characteristics for the reference genes (*UBC* and *RPII*), as well as *CYP3A7* and *SULT2A1*, are described in Table 1. Real-time quantitative PCR was performed using EvaGreen MasterMix (BioRad) with CFX96 real-time PCR Detection System (BioRad). Housekeeping genes were selected based on their stability for each pesticide treatment (gene expression stability (M) value below 0.5) using the geNorm algorithm method (Biogazelle qbase Plus Software, Zwijnaarde, Belgium).

2.6. Statistical analysis

Experiments were performed 3 or 4 times using different cell passages, and per experiment each treatment was conducted in triplicate. Results are presented as means with standard errors. One-way analysis of variance (ANOVA) followed by a Dunnett *post-hoc* test, or a Student *t*-test was performed (JMP Software, SAS, Cary, NC), depending on the experimental design. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of neonicotinoids on CYP19 catalytic activity

In this study, we determined the effects of three widely used neonicotinoids (thiacloprid, thiamethoxam and imidacloprid) on the catalytic activity of *CYP19* in a co-culture of H295R and BeWo cells. Forskolin increased aromatase activity by 3.9 and 12-fold in BeWo and H295R cells, respectively (Fig. 1). Formestane (1 μM) decreased its activity by 80–90% in both cell lines (not shown).

A 24-h exposure to increasing concentrations of thiacloprid induced aromatase activity in both cell types in co-culture (Fig. 1A). In BeWo cells, thiacloprid induced aromatase activity by 1.3-fold at 10 μM, which was statistically significant. In H295R cells, thiacloprid increased aromatase activity by 1.9- and 2.0-fold above control at a concentration of 0.1 and 0.3 μM, respectively. At greater concentrations this increase was no longer apparent.

A 24-h exposure of the co-culture to various concentrations of thiamethoxam induced aromatase activity in H295R cells by 1.8- and 2.2-fold above control, at 3 and 10 μM, respectively (Fig. 1B). Thiamethoxam did not affect the catalytic activity of *CYP19* in the BeWo cell compartment of the co-culture (Fig. 1B). Finally, a 24-h exposure of the co-culture to 3 and 10 μM imidacloprid induced the aromatase activity in H295R cells by 2.2- and 2.4-fold, respectively (Fig. 1C), whereas in BeWo cells it was induced significantly by 2.3-fold at 10 μM only (Fig. 1C).

3.2. Effects of neonicotinoids on hormone production

Basal production of dehydroepiandrosterone (DHEA) (1962 ± 166 pg/mL), androstenedione (38.8 ± 1.4 pg/mL), estriol (486.2 ± 131.8 pg/mL), estradiol (21.9 ± 3.7 pg/mL) and estrone (363.1 ± 13.3 pg/mL) by the co-culture was similar to that found in our previous study (Hudon Thibeault et al., 2014) which initially characterized the model, although estrone and β-HCG production in our study was slightly higher than previously reported.

Thiacloprid increased DHEA production statistically significantly at 3 μM (3506 ± 450 pg/mL) and 10 μM (3912 ± 402 pg/mL), compared to vehicle control (1961 ± 166 pg/mL) (Fig. 2A), although it had no effect on androstenedione production (Fig. 2B). Thiacloprid increased β-hCG production to a statistically significant extent at 0.1 μM (140.8 ± 24.6 mIU/mL) compared to DMSO (74.5 ± 12.3 mIU/mL), but not at greater concentrations (Fig. 2C). Thiacloprid significantly increased estradiol and estrone production by the co-culture at 0.1 and 0.3 μM, resulting in estradiol concentrations of 108.7 ± 32.6 and 55.4 ± 7.1 pg/mL, and estrone concentrations of 617.2 ± 48.6 and 499.7 ± 79.4 pg/mL, respectively, compared to vehicle control (estradiol: 21.9 ± 3.7 pg/mL; estrone: 363.1 ± 13.3 pg/mL) (Fig. 2D–E). Strikingly, estriol production in the co-culture was strongly inhibited by 0.3 and 3 μM thiacloprid (146.5 ± 76.0 and 148.8 ± 78.3 pg/mL), decreasing production to about 30% of DMSO control (486.2 ± 131.8 pg/mL) (Fig. 2F).

In the co-culture exposed to thiamethoxam, DHEA production was increased concentration-dependently and was significantly greater

Table 1
Sequences of the primer pairs and their amplification characteristics for *CYP3A7* and *SULT2A1* transcripts and that of two reference genes (*UBC* and *RPII*), in H295R cells.

Gene	Primer pairs (5′-3′)	Amplification characteristics	Reference
CYP3A7	Fw: CTCTTTAAGAAAGCTGTGCCCC Rv: GGGTGGTGGAGATAGTCCTA	Standard curve: $r^2 = 0.97$ Efficiency: 117.6%	Kondoh et al. (1999)
SULT2A1	Fw: TCGTCATAAGGGATGAAGATGTAATAA Rv: TGCATCAGGCAGAGAATCTCA	r^2: 0.982 Efficiency: 118.6%	Shiraki et al. (2011)
UB	Fw: ATTTGGGTCGCGGTTCTTG Rv: TGCCTTGACATTCTCGATGGT	Standard curve: $r^2 = 0.981$ Efficiency: 109.0%	Vandesompele et al. (2002)
RPII	Fw: GCACCACGTCCAATGACAT Rv: GTGCGGCTGCTTCCATAA	Standard curve: $r^2 = 0.986$ Efficiency: 100.1%	Radonić et al. (2004)

than control (1790 ± 29.5 pg/mL) by 1.67-fold at 0.3 μM (2992 ± 166 pg/mL), by 1.9-fold at 3 μM (3464 ± 550 pg/mL) as well as at 10 μM (3436 ± 466 pg/mL) (Fig. 3A). The production of androstenedione and β-hCG remained unchanged (Fig. 3B–C). Thiamethoxam concentration-dependently increased estradiol production with a 5.0-fold increase at 10 μM (108.6 pg/mL) compared to DMSO control (21.9 ± 3.7 pg/mL) (Fig. 3D). Estrone production was increased by 2-fold (735.2 pg/mL) at 10 μM thiamethoxam compared to control production (363.1 ± 13.3 pg/mL) (Fig. 3E). Estriol production was inhibited by about 80% at all tested concentrations of thiamethoxam compared to DMSO control (Fig. 3F).

In the co-culture exposed to imidacloprid, DHEA production was significantly increased at all concentrations by about 1.7-fold (3088 ± 325 to 3381 ± 475 pg/mL) compared to control (1962 ± 166 pg/mL) (Fig. 4A), whereas androstenedione production was not affected (Fig. 4B–E). The production of β-hCG was increased statistically significantly at an imidacloprid concentration of 10 μM (181.1 ± 19.0 mIU/mL) by about 2.8-fold above control (64.5 ± 18.9 mIU/mL) (Fig. 4C). Estradiol production was strongly elevated at 10 μM imidacloprid (158.4 ± 51.9 pg/mL) and was about 7.2-fold greater than control (Fig. 4D). Imidacloprid at all concentrations inhibited estriol production by about 80% compared to DMSO control (Fig. 4F).

3.3. Effects of neonicotinoids on mRNA levels of CYP3A7 and SULT2A

In the co-culture exposed to each of the three neonicotinoids, a significant decrease in estriol production was observed. *SULT2A1*, expressed in the fetal adrenal, and *CYP3A7*, expressed in the fetal liver, are key enzymes in the biosynthesis of the precursor for estriol, which is produced predominantly in placenta. Therefore, we wished to confirm the presence of *SULT2A1* and *CYP3A7* in H295R cells by RT-qPCR and then determine the potential effects of neonicotinoids on their expression. As a novel finding, we were able to detect *SULT2A1* and *CYP3A7* expression in H295R cells, confirming our previous suggestion that H295R cells act as the fetal adrenocortical as well as liver compartment of the co-culture model. *SULT2A1* expression was not affected by exposure to thiacloprid, thiamethoxam or imidacloprid at 3 or 10 μM (data not shown). A 24-h exposure of H295R cells to thiacloprid at 3 and 10 μM resulted in a statistically significant increase in levels of *CYP3A7* expression by 6.29 and 6.83-fold, respectively, compared to DMSO control (Fig. 5). Thiamethoxam at 3 μM induced *CYP3A7* mRNA levels statistically significantly by 5.88-fold compared to DMSO control. A 24-h exposure to 3 μM imidacloprid increased *CYP3A7* expression in H295R cells by 3.75-fold compared to DMSO control, although this induction was not statistically significant (Fig. 5).

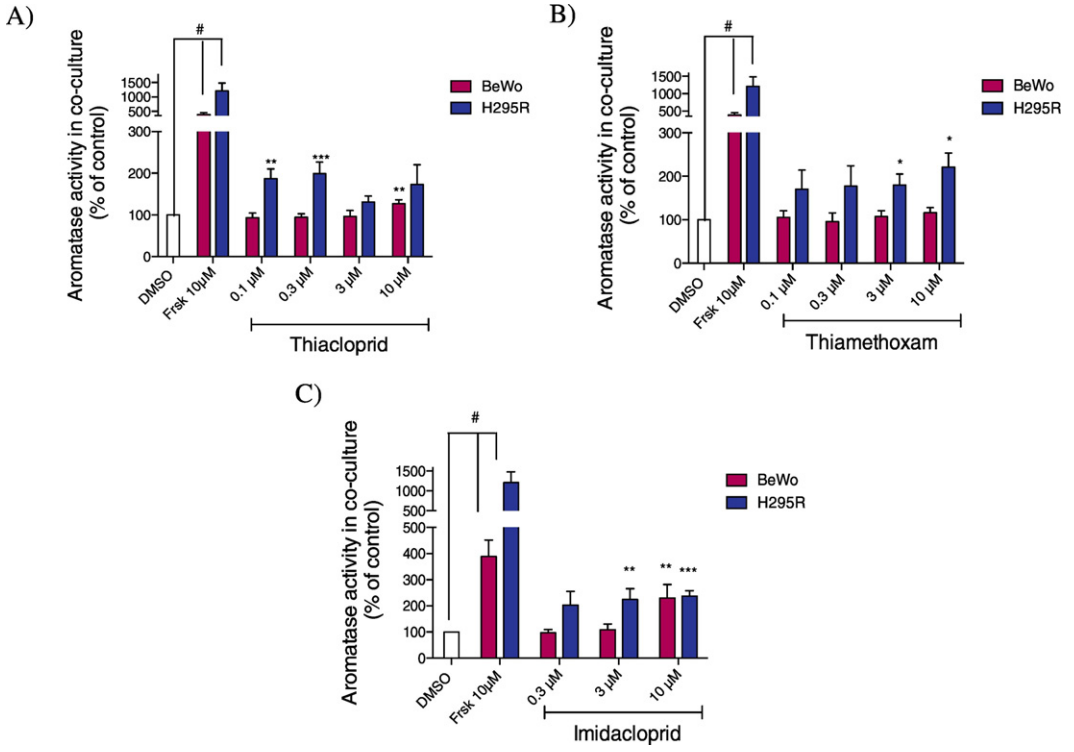

Fig. 1. Concentration-dependent effects of thiacloprid (A), thiamethoxam (B) and imidacloprid (C) on aromatase activity in H295R and BeWo cells in co-culture. Forskolin (Frsk) was used as a positive control for induction of aromatase activity. (#) A statistically significant difference between Frsk treatment and DMSO control (Student *t*-test; $P < 0.05$). (*, **, ***) A statistically significant difference between neonicotinoid treatment and DMSO control (one-way ANOVA and Dunnett *post hoc* test; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Experiments were performed 4 times using different cell passages; per experiment each concentration was tested in triplicate.

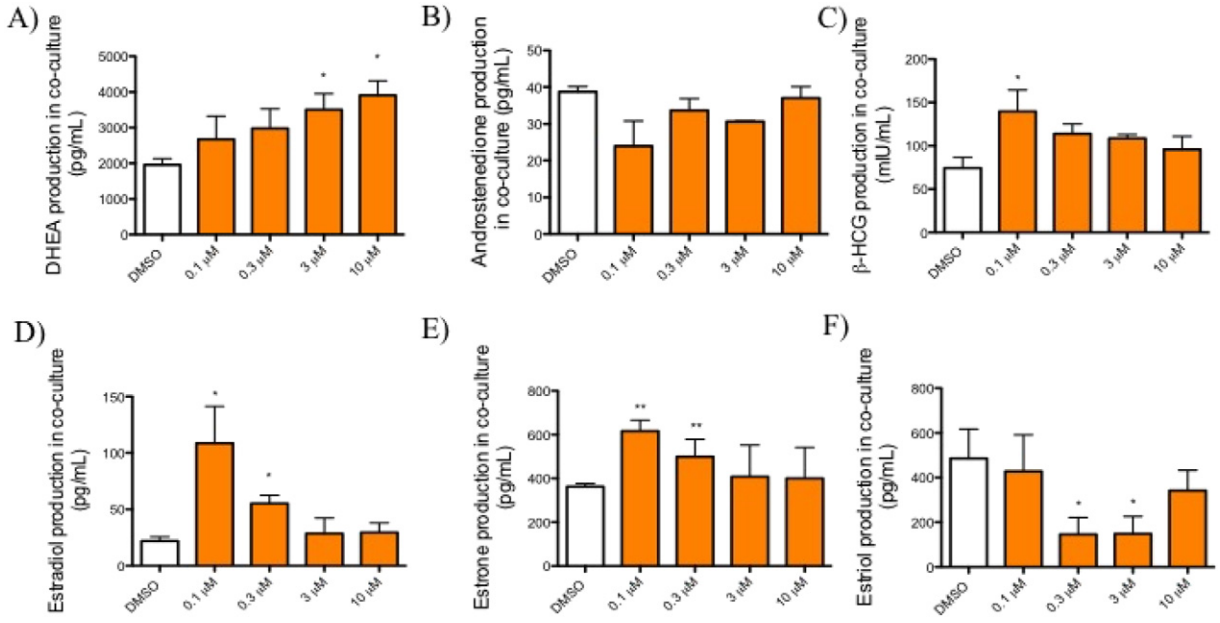

Fig. 2. Concentration-dependent effects of thiacloprid on DHEA (A), androstenedione (B), β-hCG (C), estradiol (D), estrone (E) and estriol (F) production by a co-culture of H295R and BeWo cells. (*, **, ***) A statistically significant difference between neonicotinoid treatment and DMSO control (one-way ANOVA and Dunnett *post hoc* test; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Experiments were performed 4 times using different cell passages; per experiment each concentration was tested in triplicate.

To determine whether the increased *CYP3A7* gene expression was related to the decreased estriol levels in response to the neonicotinoids, we determined the effect of estriol supplementation (using a concentration normally found in the co-culture under basal conditions) on the expression of *CYP3A7* in response to thiacloprid, thiamethoxam or imidacloprid. Co-treatment of H295R cells with estriol (5 ng/mL) prior to a 24-h exposure to thiacloprid, thiamethoxam or imidacloprid at 3 μM resulted in significantly reduced levels of *CYP3A7* expression compared to the neonicotinoid treatments alone, which all increased *CYP3A7* levels significantly above DMSO control. In the case of imidacloprid, co-treatment with estriol reduced imidacloprid-induced *CYP3A7* to levels not significantly different from DMSO control.

4. Discussion

4.1. Fetoplacental co-culture as a screening tool for endocrine disrupting chemicals

We have successfully applied our previously developed fetoplacental co-culture model as a unique screening tool to evaluate the potential endocrine disrupting effects of a series of neonicotinoids on steroidogenesis during pregnancy. Naturally, our co-culture system has some limitations –it uses cancer cells, not primary cells and cannot fully describe the complex interactions that occur *in vivo*, but as a model of fetoplacental steroidogenesis it is capable of producing a variety of hormones important

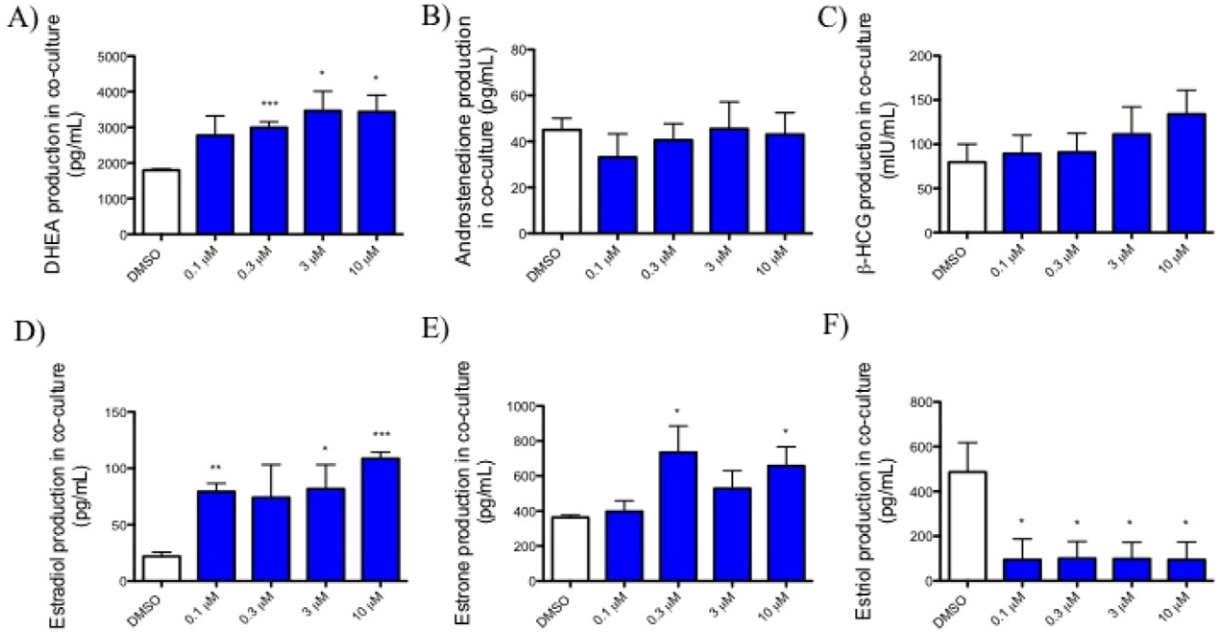

Fig. 3. Concentration-dependent effects of thiamethoxam on DHEA (A), androstenedione (B), β-hCG (C), estradiol (D), estrone (E) and estriol (F) production by a co-culture of H295R and BeWo cells. (*, **, ***) A statistically significant difference between neonicotinoid treatment and DMSO control (one-way ANOVA and Dunnett *post hoc* test; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Experiments were performed 4 times using different cell passages; per experiment each concentration was tested in triplicate.

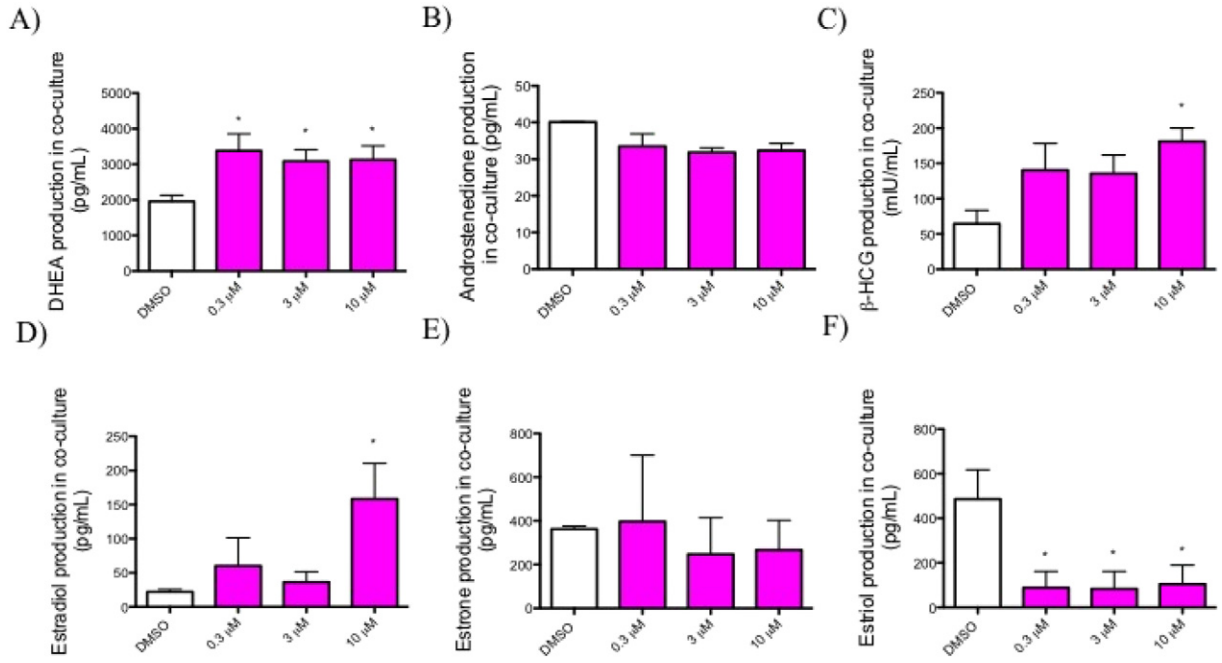

Fig. 4. Concentration-dependent effects of imidacloprid (on DHEA (A), androstenedione (B), β-hCG (C), Estradiol (D), Estrone (E) and Estriol (F) production by H295R and BeWo cells in co-culture). (*, **, ***) A statistically significant difference between neonicotinoid treatment and DMSO control (one-way ANOVA and Dunnett *post hoc* test; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Experiments were performed 4 times using different cell passages; per experiment each concentration was tested in triplicate.

during pregnancy. We measured aromatase activity in both the fetal (H295R) and placental (BeWo) compartments of the co-culture model with basal activity being at least 15 times higher in BeWo cells than in H295R cells (Fig. 1A–C), as previously demonstrated (Hudon Thibeault et al., 2014). This corresponds well with the *in vivo* situation where the placenta is responsible for the majority of *CYP19* expression and estrogen biosynthesis (Mesiano and Jaffe, 1997; Hanley et al., 2001; Rainey et al., 2002; Pezzi et al., 2003).

It is well understood that estrogen production in the fetoplacental unit is dependent upon a close collaboration between the fetus and the placenta. As the human placenta does not express significant levels of *CYP17* or its associated catalytic activities, it is, despite high levels of aromatase activity, incapable of producing significant amounts of estrogens *de novo* and is dependent on androgen precursors produced by the fetal adrenal and liver. During pregnancy, up to 90% of the unique pregnancy estrogen estriol is derived from fetal precursors (Gerhard et al., 1986). As previously shown, our fetoplacental co-culture model produces significant quantities of estriol (Figs. 2F, 3F, 4F), whereas each cell type in monoculture produces negligible amounts (Hudon Thibeault et al., 2014), further supporting the physiological relevance of our co-culture as a representative model of the fetoplacental steroidogenic unit and justifying its use as a meaningful screening tool for the effects of potential endocrine disrupting chemicals during pregnancy (Hudon Thibeault et al., 2017).

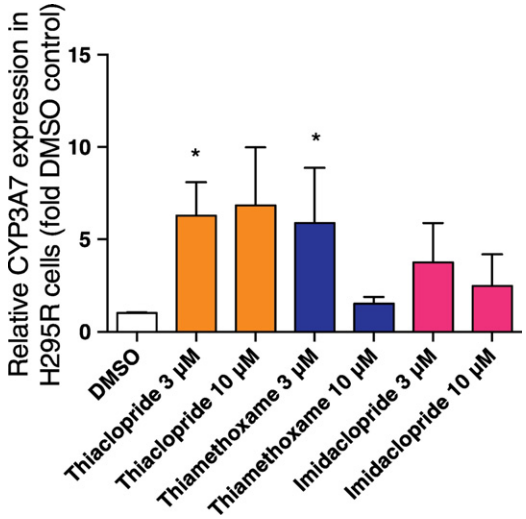

Fig. 5. Relative expression levels of CYP3A7 in H295R cells exposed for 24-h to 3 or 10 μM of thiacloprid, thiamethoxam or imidacloprid. DMSO was used as a negative control. (*, **, ***) A statistically significant difference between neonicotinoid treatment and DMSO control (one-way ANOVA and Dunnett *post hoc* test; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Experiments were performed 3 times using different cell passages; per experiment each concentration was tested in triplicate.

4.2. Disruption of fetoplacental steroidogenesis by neonicotinoid insecticides

It is widely accepted that an important number of environmental contaminants have estrogen-like activity. The most commonly studied mechanism for estrogenic effects is the binding of chemicals to the estrogen receptor, whereby they act as (partial) agonists. This mechanism has been demonstrated for several contaminants to which pregnant women may be exposed, such as bisphenol A (Diel et al., 2002; Vivacqua et al., 2003; Heneweer et al., 2005; Wetherill et al., 2007; Chou et al., 2011). However, estrogen receptor activation is not the only possible mechanism by which environmental contaminants may exert pro- or antiestrogenic activity. For example, atrazine, a herbicide widely used in North America, induces aromatase activity and expression in various human cell lines (Sanderson et al., 2000, 2001, 2002; Sanderson, 2006; Fan et al., 2007; Caron-Beaudoin et al., 2016). A growing number of studies have determined that neonicotinoid insecticides are potential endocrine disruptors. A recent study showed that clothianidin, a neonicotinoid and metabolite of thiamethoxam, caused DNA fragmentation in germ cells of male quails (Hoshi et al., 2014). Kapoor et al. (2011) showed that female rats exposed to neonicotinoids had lower ovarian weight, as well as LH and progesterone levels. We also previously demonstrated that two neonicotinoids, thiacloprid and thiamethoxam, induced *CYP19* expression and aromatase activity in H295R cells at environmentally relevant concentrations (Caron-Beaudoin et al., 2016). In the present study, the neonicotinoids thiacloprid, thiamethoxam and

imidacloprid induced aromatase activity in H295R and BeWo cells in co-culture (Fig. 1), highlighting the tissue- and promoter-specific differences in the effects of neonicotinoids on *CYP19* expression, aromatase activity and estrogen biosynthesis. Beside the potential direct effects of neonicotinoids on *CYP19* expression, the tested pesticides also increased the synthesis of DHEA and β-hCG (the latter an important early indicator of pregnancy), resulting in the increased production of estradiol and estrone that we observed in the co-culture. It is known that hCG induces cAMP intracellular levels (Massicotte et al., 1981), which we have shown can increase aromatase expression and activity (Sanderson et al., 2002). Estrogen biosynthesis is, therefore, complex and cannot be explained simply by the production of precursors like DHEA or androstenedione.

4.3. Possible metabolism of neonicotinoids by fetal CYP3A7

In this study, a 24-h exposure of our fetoplacental co-culture to each of the three neonicotinoids resulted in a significant decrease of estriol production (Figs. 2F, 3F, 4F). SULT2A1 and CYP3A7 are key enzymes in the fetal production of the estriol precursor 16α-hydroxyDHEA(-sulfate). We are the first to report the presence of *CYP3A7* transcript in H295R cells, the fetal compartment of our co-culture model (Figs. 5–6), and we confirmed a previous report that SULT2A1 is expressed in H295R cells (Oskarsson et al., 2006). We further found that the neonicotinoids only affected the expression of *CYP3A7*, which was increased. It is known that *CYP3A4* is involved in the metabolism of neonicotinoids in human adult liver (Schulz-Jander and Casida, 2002; Dorne et al., 2005; Shi et al., 2009; Casida, 2011). Adult hepatic *CYP3A4* is structurally closely related to fetal hepatic *CYP3A7*, sharing an amino acid sequence that is 88% similar (Komori et al., 1989; Schuetz et al., 1989; Komori et al., 1990). As fetal *CYP3A7* is considered to have the same catalytic function as adult *CYP3A4* (Lacroix et al., 1997), we hypothesize that neonicotinoid insecticides are metabolized by *CYP3A7* in the fetoplacental unit, thus competing with the ability of this enzyme to produce 16α-hydroxylated DHEA (and its sulfate conjugate) which are the precursors for estriol synthesis (Fig. 7).

This would explain the decreased estriol biosynthesis and the accumulation of DHEA in our co-culture when exposed to neonicotinoids.

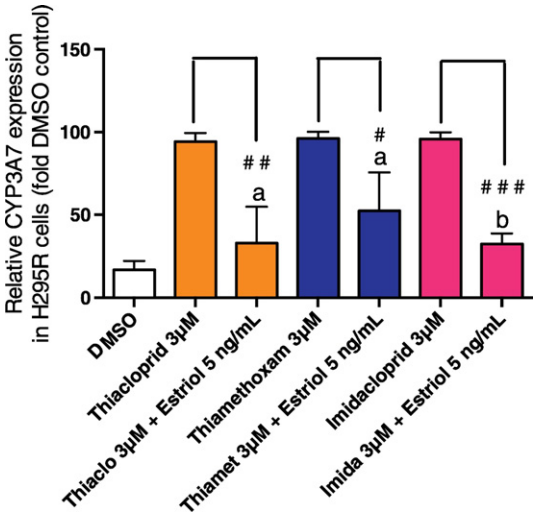

Fig. 6. Relative expression of CYP3A7 in H295R cells exposed for 24-h to 3 μM of thiacloprid, thiamethoxam or imidacloprid ('inducers'), with or without co-treatment with estriol (5 ng/mL). CYP3A7 expression levels in cells co-treated with estriol were expressed as a % of those in cells treated with each neonicotinoid alone (100%). (#, ##, ###) A statistically significant difference between neonicotinoid- and co-treated cells; Student *t*-test; #$P < 0.05$. ##$P < 0.01$; ###$P < 0.001$. (a) No statistical difference between co-treatment and DMSO control, (b) A statistically significant difference between co-treatment and DMSO control; Student *t*-test; $P < 0.05$. Experiments were performed 3 times using different cell passages; per experiment each treatment was tested in triplicate.

The excess androgen precursors would result in the overproduction of estrone and estradiol by the placental compartment of the co-culture, resulting in the increased levels of these two estrogens we observed (Figs. 2D, E, 3D, E, 4D, E).

We further suggest that the strong decrease in estriol levels triggers a positive feedback mechanism that increases *CYP3A7* gene expression, thus explaining the *CYP3A7* overexpression in H295R cells (the fetal compartment of the co-culture). To provide evidence for this hypothesis, we determined the effects of thiacloprid, thiamethoxam and imidacloprid on *CYP3A7* expression in H295R cells supplemented with a normalizing concentration of estriol to overcome the strong suppression of estriol synthesis by the neonicotinoids (Fig. 6). Estriol reversed the neonicotinoid-mediated induction of *CYP3A7* mRNA expression, suggesting such a positive feedback mechanism may exist. During pregnancy, levels of circulating estrone and estradiol increase by 100-fold. This induction reaches 1000-fold for estriol, bringing its concentration up to similar levels as the other estrogens, compared to non-pregnant women (Blackburn, 2007). To reach these levels, placental estrogens use a positive feedback mechanism, consisting of upregulating DHEA(-S) production by the fetal adrenal zone. The final result of this positive feedback is the induction of placental estrogen, including estriol (Albrecht and Pepe, 1999; Kaludjerovic and Ward, 2012). The decrease in estriol levels in the fetoplacental co-culture model exposed to neonicotinoids may activate this positive feedback mechanism, leading to overexpression of *CYP3A7* in H295R cells (Fig. 5). The control of placental estrogen production by this positive feedback mechanism would also explain why neonicotinoid-induced *CYP3A7* expression is reversed by a co-treatment with estriol (Fig. 6).

4.4. Implications for human health

During pregnancy, estrogens regulate uteroplacental blood flow, trophoblast invasion and cellular differentiation (Yashwanth et al., 2006). Disruption of estrogen biosynthesis during this critical period may impact the development of the fetus and placenta, as well as affect the mother's health (Kaijser et al., 2000). For example, disruption of estrogen production has been associated with negative birth outcomes (Gerhard et al., 1986; Kaijser et al., 2000; Troisi et al., 2003). It remains unclear if environmental concentrations of neonicotinoids are sufficient to disrupt aromatase activity or hormone production (specifically estriol) in pregnant women. Nevertheless, neonicotinoids are frequently detected in vegetables and fruits (Chen et al., 2014) and their concentrations are steadily increasing in water samples in agricultural areas in North America, with concentrations up to 3.6 μg/L (about 0.012 μM for thiamethoxam) having been measured (Anderson et al., 2013; Main et al., 2014; Smalling et al., 2015). These widely used insecticides' metabolites are also detected in the urine of farm workers at concentrations similar to those used in our experiments (up to 0.05 μM for metabolite of thiacloprid and imidacloprid) (Nomura et al., 2013). Importantly, it is suspected that ingested neonicotinoids can pass through the placental barrier (Taira, 2014), since neurobehavioral deficits were measured in rat offspring exposed *in utero* to these insecticides (Abou-Donia et al., 2008). Therefore, the steadily increasing use of neonicotinoid pesticides is a cause of concern for the health of pregnant women.

5. Conclusions

We applied a fetoplacental co-culture model to screen for potential endocrine disrupting effects of neonicotinoid insecticides. We found thiacloprid, thiamethoxam and imidacloprid to induce aromatase activity in this *in vitro* model of fetoplacental steroidogenesis. The neonicotinoids increased estrone and estradiol production, while strongly inhibiting estriol production. We also have in direct *in vitro* evidence that neonicotinoids may be competing with DHEA(-S) as a substrate for *CYP3A7*, thus explaining the decrease in estriol production in the co-culture model. This study contributes to growing evidence of the

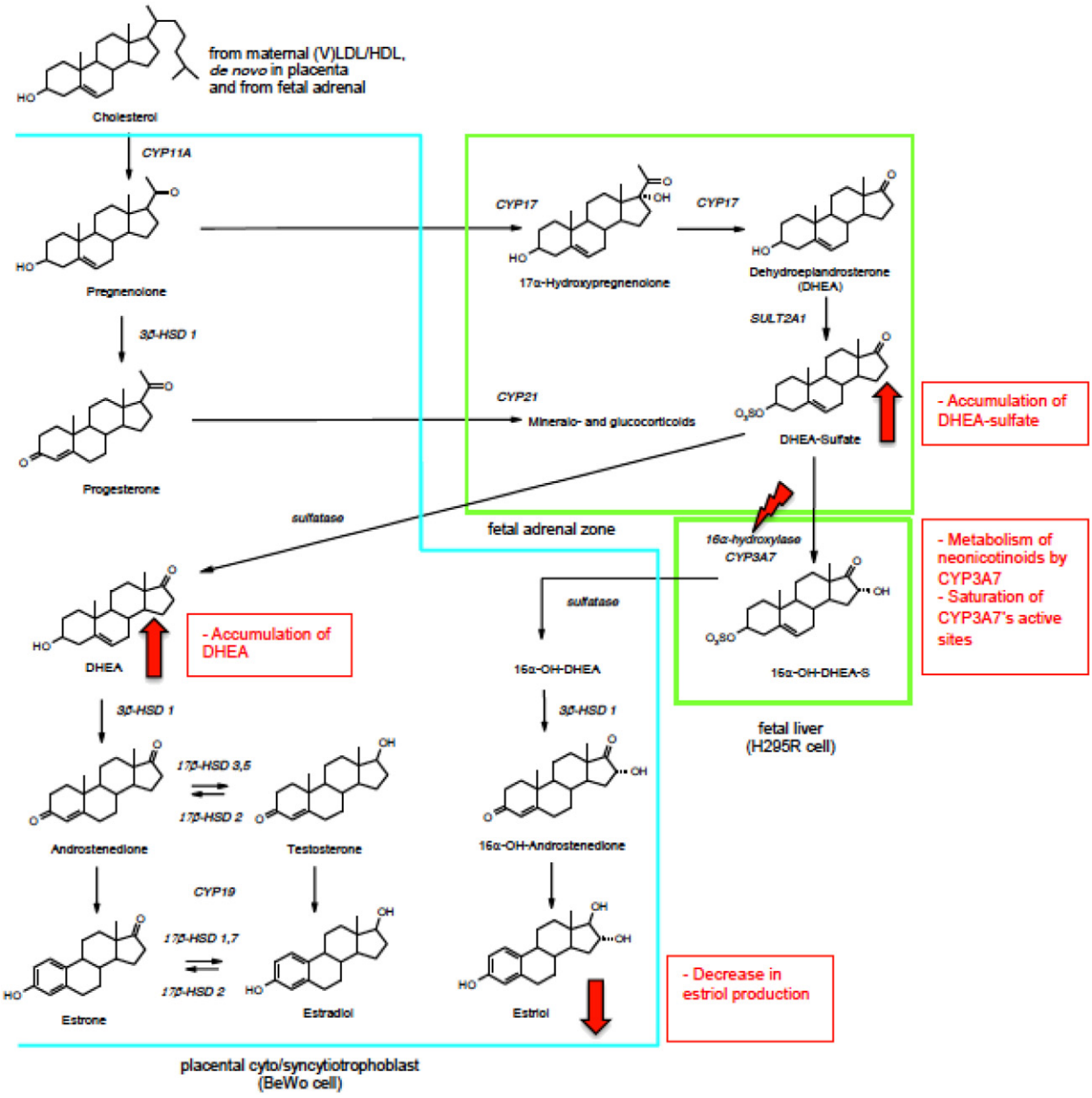

Fig. 7. Steroidogenic pathways in the co-culture model of fetoplacental steroidogenesis, and the proposed targets of neonicotinoid insecticides (in red) leading to decreased estriol production and increased levels of DHEA.

endocrine disrupting potential of neonicotinoid insecticides. Given the importance of estrogen synthesis during pregnancy and the association between environmental contaminants and birth outcomes, the use of physiologically relevant screening tools such as our co-culture model of fetoplacental steroidogenesis is paramount for a credible evaluation of the potential health risks posed by such chemicals.

Conflicts of interest

The authors declare to have no conflicts of interest.

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RESEARCH HIGHLIGHT

Effects of neonicotinoids on promoter-specific expression and activity of aromatase: Implications for the development of hormone-dependent breast cancer

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Aromatase (CYP19) is the key enzyme in the biosynthesis of estrogens. In humans, it is expressed in a tissue- and promoter-specific manner. In hormone-dependent breast cancer, CYP19 is overexpressed through the activation of several additional promoters (PII, I.3 and I.7) that are normally inactive in the healthy mammary gland. In the normal mammary gland, low basal CYP19 expression is regulated by the I.4 promoter, which is also active in adipose tissue. Here, we highlight our recent study of the effects of neonicotinoid pesticides on the promoter-specific expression of CYP19 in various human *in vitro* models. We also discuss the implications of endocrine disruption by environmental chemicals for the development of hormone-dependent diseases, such as breast cancer.

Keywords: Aromatase; neonicotinoids; promoter-specific expression; estrogen; H295R; breast cancer

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Introduction

In Canada, breast cancer represents 26% of all cancer diagnosis in females [1]. About 70% of breast cancers are estrogen-dependent, and aromatase (CYP19) is overexpressed in this type of cancer. Aromatase is the key enzyme in the final step of biosynthesis of estrogens. In hormone-dependent breast cancer, estrogens stimulate cancer cell proliferation [2] by activating estrogen receptor signalling pathways.

CYP19 is present in a variety of tissues and its expression is regulated in a promoter-specific manner (Fig 1). In pre-menopausal women, estradiol synthesis *de novo* occurs mainly in the ovaries, via the activation of the PII/I.3 promoters of CYP19. In post-menopausal women the ovaries are no longer functional and estradiol levels drop dramatically. However, low levels of circulating estrone are produced from adrenal androgen precursors by the adipose tissue, where CYP19 is mostly expressed by the I.4 promoter which has low basal activity [3]. In hormone-dependent breast cancers, estrogen biosynthesis is critical for an estrogen rich, tumor-promoting microenvironment. More precisely, fibroblast cells in the stroma that surround the epithelial tumor cells, known as cancer-associated fibroblasts (CAFs), are responsible for the majority of estrogen biosynthesis in

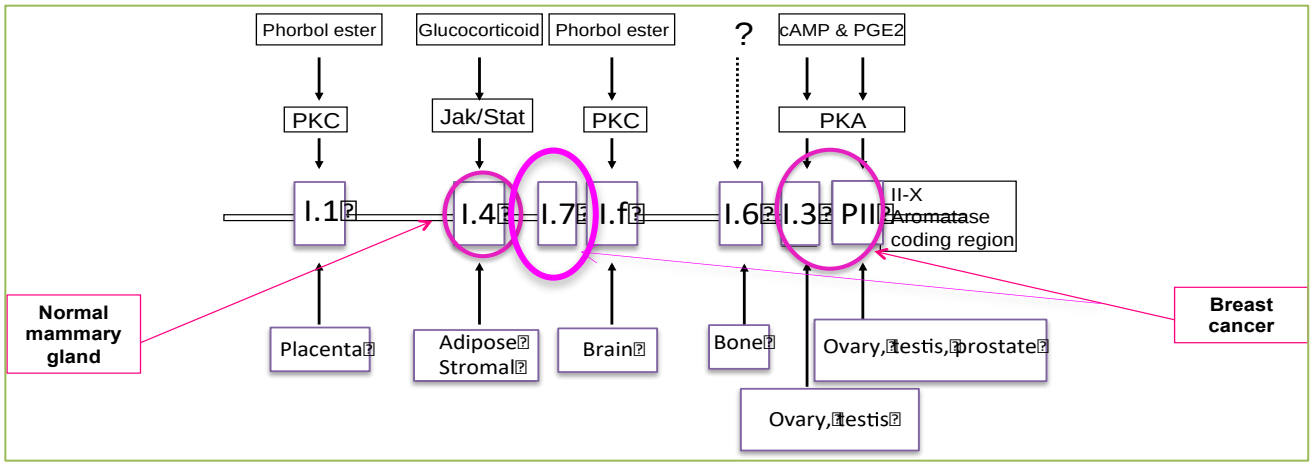

Figure 1. Tissue and promoter-specific organization of the CYP19 gene. Reprinted with permission [23].

close proximity to the tumor [4, 5]. Normal fibroblasts express CYP19 via promoter I.4 [4]. However, in CAFs, a promoter-switch occurs, where I.4 promoter activity is inhibited and promoters PII, I.3 and I.7 are activated [4, 6]. The mechanisms underlying this promoter-switch are still unknown, but it is thought to be partially due to increased prostaglandin E_2 (PGE_2) production by the epithelial tumor cells [4]. Moreover, the tumor cells also secrete cytokines, such as TNFα and IL-11 that promote a desmoplastic reaction, which involves the accumulation of CAFs and inhibition of the normal differentiation of CAFs into adipose stromal cells [7] (Fig 2).

Endocrine disruptors are chemicals that interfere with the synthesis, transport, metabolism or receptor activation of natural hormones. It is now well established that exposure to environmental contaminants may increase the risk of developing hormone-dependent breast cancer [8, 9] due to their estrogenic activity. However, studies investigating the proestrogenic mechanisms of endocrine disruptors mainly focus on estrogen receptor activation [10, 11]. Far less work has looked at the potential effects of environmental chemicals on key enzymes of steroidogenesis, such as aromatase. Almost nothing is known about the potential effects of endocrine disruptors on the tissue- and promoter-specific expression of CYP19, although such effects would have far reaching implications for human health, such as the development of breast cancer.

Exposure to atrazine, a widely used herbicide, induces CYP19 expression, aromatase activity and estrogen biosynthesis in human cell lines [12, 13, 14, 15, 16], but little is known about "emerging" contaminants such as neonicotinoid insecticides. Neonicotinoids are the most commonly used insecticides worldwide, and are applied as coatings to the seeds of corn, canola, soybeans and the majority of fruits and vegetables. Neonicotinoid pesticides exert their effect by binding to the nicotinic receptor of insects, where they act as agonist of the postsynaptic nicotinic acetylcholine receptor [17]. While effects of neonicotinoids on natural pollinators, such as honey bees, have been widely studied, little is known about their endocrine disrupting potential in humans. Nonetheless, a number of studies have demonstrated that the neonicotinoid imidacloprid induces fragmentation of seminal DNA and lowers sperm count [18] in male rats, whereas in female rats it decreases ovarian weight and alters luteinizing hormone and progesterone levels [19]. Moreover, half-lives of neonicotinoid pesticides in soil may exceed 1000 days [20]. A recent study conducted in Boston, MA, revealed that 100% of fruits and 72% of vegetables purchased from local grocery stores had detectable levels of one or more neonicotinoids [21]. Given the environmental persistence of neonicotinoids, their potential to bioaccumulate and presence in the human diet, chronic exposure to neonicotinoids and their potential health effects in humans is a real concern.

In our recent study, we investigated the effects of three widely used neonicotinoid pesticides (thiacloprid, thiamethoxam and imidacloprid) as well as the herbicide atrazine on the promoter-specific expression of CYP19 mRNA and aromatase catalytic activity in H295R human adrenocortical carcinoma cells. H295R cells are a well established *in vitro* model for the study of steroidogenesis [22, 23, 24, 25]. Indeed, H295R cells express aromatase regulated by two breast cancer-relevant CYP19 promoters: PII and I.3. In our study, we developed robust and sensitive real-time quantitative RT-PCR methods to measure the transcript derived from each specific CYP19 promoter. To do so, we paid particular attention to the validation of primer pairs using standard curves and our choice of reference genes. A series of reference genes were evaluated for each cell line and for each pesticide treatment using the Minimum Information for Publication of Quantitative Real-Time PCR Experiments or MIQE guidelines [26, 27]. At least two suitable

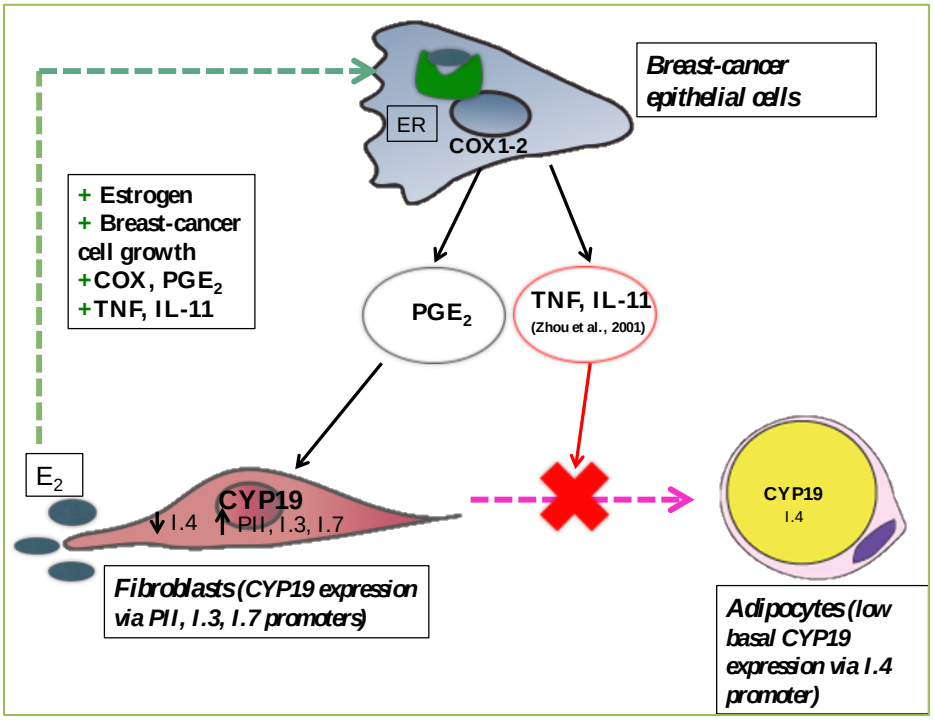

Figure 2. Cell-to-cell interactions in hormone-dependent breast cancer [34, 35]. Epithelial cancer cells produce PGE_2, which may induce a switch in *CYP19* promoter usage from I.4 to PII, I.3 and I.7 in fibroblasts, leading to increased local synthesis of estrogens. Epithelial cancer cells also synthesize cytokines (TNFα, IL-11) that contribute to the accumulation of undifferentiated fibroblasts in the tumor microenvironment (desmoplastic reaction).

reference genes were used to normalize levels of promoter-specific CYP19 mRNA expression. The choice of reference genes is of critical importance since there should be minimal variability in their expression among treatments. We validated previously published results showing that atrazine induces PII/I.3-mediated CYP19 expression and aromatase catalytic activity in a concentration-dependent manner in H295R cells, by activating the cAMP/protein kinase A signalling pathway [15, 16]. We also demonstrated that thiacloprid and thiamethoxam, at environmentally-relevant concentrations (0.1-10 μM) [20], induce PII/I.3-mediated CYP19 expression and aromatase catalytic activity, but unlike atrazine, the neonicotinoids produced biphasic or non-monotonic concentration-response curves. In H295R cells exposed to 0.1 and 0.3 μM thiamethoxam, PII/I.3-mediated CYP19 expression was strongly increased, up to 15-fold compared to control. In H295R cells exposed to 0.3 μM thiacloprid a strong increase in mRNA levels of the CYP19 coding region was also observed, whereas the effect on PII/I.3-derived transcript levels was weaker. This suggests the possible presence of other aromatase promoters in H295R cells. In our study, we also determined the effects of atrazine and neonicotinoid pesticides on aromatase catalytic activity, which as functional endpoint is more physiologically relevant than changes in mRNA levels. We found that the changes in mRNA expression corresponded with similar changes in enzyme activity in H295R cells exposed to atrazine, thiacloprid and thiamethoxam; imidacloprid had no effect on either endpoint. To our knowledge, we are the first to assess the endocrine disrupting effects of neonicotinoids related to the promoter-specific regulations of CYP19 expression and aromatase activity [12]. Since aromatase is overexpressed in hormone-dependent breast cancer by a unique CYP19 promoter usage which contributes greatly to the overproduction of estrogens in the tumor microenvironment, these results highlight the need to further investigate the endocrine-disrupting potential of neonicotinoids, to which we may be exposed chronically at relatively low concentrations.

The biphasic or non-monotonic responses that we observed with the neonicotinoids are not uncommon in toxicological studies. A good example of a biphasic concentration-response effect is typified by the action of bisphenol A, which binds to the estrogen receptor at lower concentrations, but will also bind to the androgen receptor at higher ones [28]. The mechanisms by which neonicotinoids selectively stimulate specific CYP19 promoters remain unknown and are currently under our investigation. Differential intracellular signalling factors that regulate CYP19 expression are likely targeted by the neonicotinoids. As example, increased intracellular levels of cAMP are

required to phosphorylate cAMP-response element-binding protein (CREB), which can then bind to cAMP-response elements (CREs) located in the regulatory regions of several genes involved in steroidogenesis, such as the mitochondrial steroidogenic acute regulatory protein (StAR) [29]. StAR is a transport protein that facilitates entry of cholesterol into the mitochondria, an essential first step in the initiation of all steroidogenesis [30]. The regulatory region of these CREB-responsive genes may also contain GATA-responsive elements, and phosphorylation of GATA factors such as GATA-4 may also be induced by intracellular cAMP levels, thus further enhancing the activation of factors that promote steroidogenesis [31].

In hormone-dependent breast cancer, the overproduction of estrogen is associated with an inhibition of normal I.4 promoter and an overexpression of PII, I.3 and I.7 CYP19 promoters in the stroma surrounding the epithelial tumor cells. We are currently working on a novel *in vitro* breast cancer model that allows us to determine this unique CYP19 promoter-switch. Our preliminary results in this cell-based model indicate that environmentally-relevant concentrations of imidacloprid and thiacloprid induce this CYP19 promoter-switch and result in elevated aromatase catalytic activity. We are also developing a co-culture model by placing this 'promoter-switch capable' cell system in close communication with estrogen-responsive breast cancer cells to reproduce the typical microenvironment of an estrogen-dependent breast tumor. In this co-culture model we will be able to assess the effects of neonicotinoid pesticides on estrogen biosynthesis and promoter-specific CYP19 expression as well as on other tumor promoting (growth and inflammatory) factors within a physiologically relevant tumor microenvironment. Similar co-cultures have been developed to mimic the tumor micro-environment and cellular interactions between fibroblasts and cancer epithelial cells [32, 33], although these models have as draw back that they require freshly isolated human fibroblast or use normal cell lines that propagate more slowly. It has also never been demonstrated whether these co-culture models are capable of undergoing a CYP19 promoter-switch in response to chemical exposures.

In conclusion, atrazine and certain neonicotinoid insecticides exert endocrine disrupting effects *in vitro* by altering the promoter/tissue-specific expression of CYP19 and its catalytic aromatase activity. Our novel *in vitro* screening tools will help in assessing the risk that certain chemicals may pose by causing tissue-specific disruption of estrogen biosynthesis, which is of particularly importance to women's health.

Conflicting interests

The authors have declared that no conflict of interests exist.

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Abbreviations

Camp: Cyclic adenosine monophosphate; CAF: cancer-associated fibroblast**;** CRE: cAMP-response elements; CREB: cAMP response element-binding protein; CYP19: Aromatase cytochrome P450 19**;** IL-11: Interleukin 11**;** MIQE: Minimum Information for Publication of Quantitative Real-Time PCR Experiments; PGE_2: Prostaglandin E_2**;** StAR: Steroidogenic acute regulatory protein**;** TNFα: Tumor necrosis factor alpha.

Author contributions

ECB helped with the design and coordination of the study. ECB carried out all the experiments (real-time qPCR, catalytic activity and cytotoxicity assays) and drafted the manuscript of the highlight study and the Research Highlight. JTS obtained the funding, provided the materials, designed the study and co-wrote and revised the highlighted manuscript; JTS revised the Research Highlight.

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