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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

Élyse Caron-Beaudoin,[1,2] Rachel Viau,[1] and J. Thomas Sanderson[1]

[1]INRS - Institut Armand-Frappier, Université du Québec, Laval, Quebec, Canada
[2]Department of Occupational and Environmental Health, School of Public Health, Université de Montréal, Montreal, Quebec, Canada

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

Address correspondence to J.T. Sanderson, INRS-Institut Armand-Frappier, 531 boulevard des Prairies, Laval QC H7V 1B7, Canada. Telephone: (450) 687-5010. Email: thomas.sanderson@iaf.inrs.ca, or É. Caron-Beaudoin, Département de santé environnementale, et santé au travail, École de santé publique, Université de Montréal, C.P. 6128, succursale Centre-ville, Montréal H3C 3J7E, Canada. Telephone: 1-514-343-6111 #30445. Email: elyse.caron-beaudoin@umontreal.ca

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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).

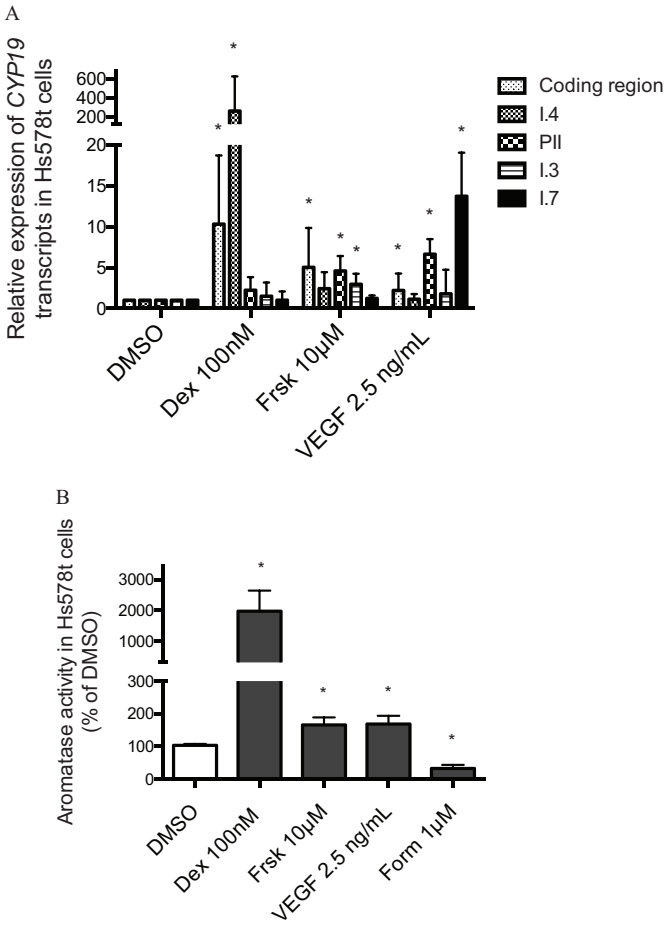

Figure 1. (*A*) Relative expression of *CYP19* coding region (nonpromoter-specific or total), and I.4, PII, I.3, and I.7 promoter-derived *CYP19* transcripts in Hs578t cells (fold DMSO control). (*B*) Aromatase catalytic activity in Hs578t cells exposed to dexamethasone (DEX) 100 nM, forskolin (Frsk) 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

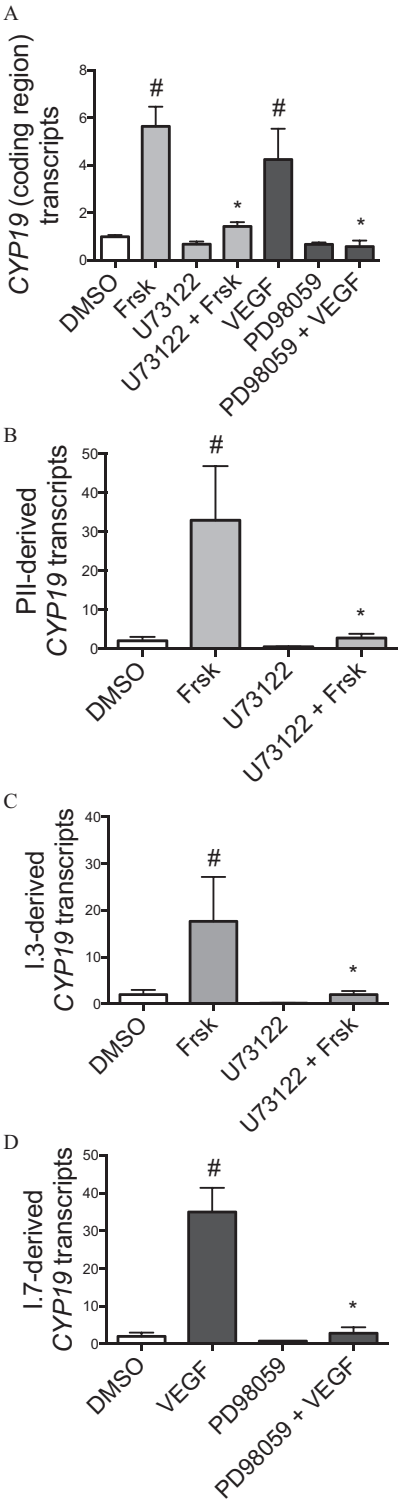

Figure 2. Relative expression of (*A*) *CYP19* coding region (nonpromoter-specific or total), and *CYP19* transcripts derived from promoters (*B*) PII, (*C*) I.3, and (*D*) I.7 in Hs578t cells (fold DMSO control). Cells were exposed for 24 h to 10 μM forskolin (Frsk) or 2.5 ng/mL VEGF, inducers of PII/I.3 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

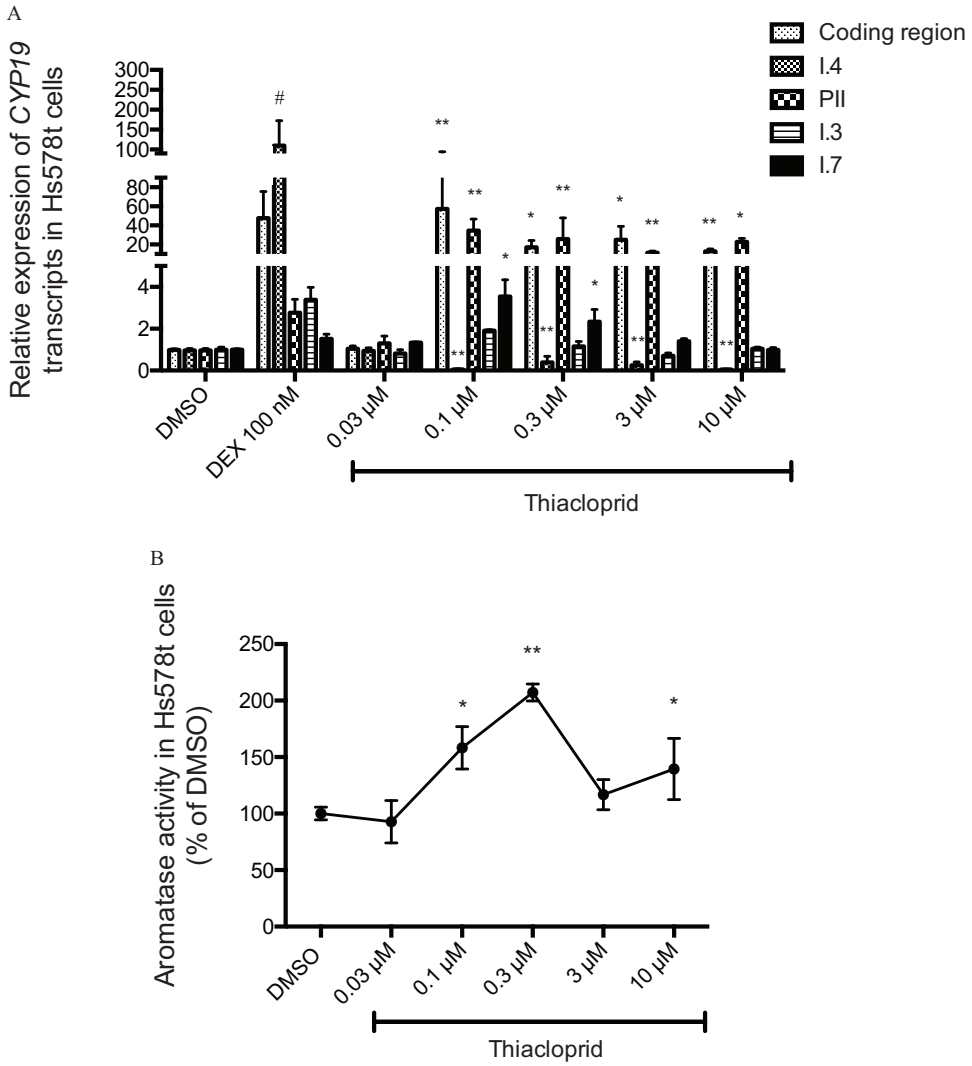

Figure 3. (*A*) Relative expression of *CYP19* coding region (nonpromoter-specific or total), and I.4, PII, I.3, and I.7 promoter-derived *CYP19* transcripts in Hs578t cells (fold DMSO control). (*B*) Aromatase catalytic activity in Hs578t cells exposed to thiacloprid (0.03, 0.1, 0.3, 3, and 10 µM). 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-

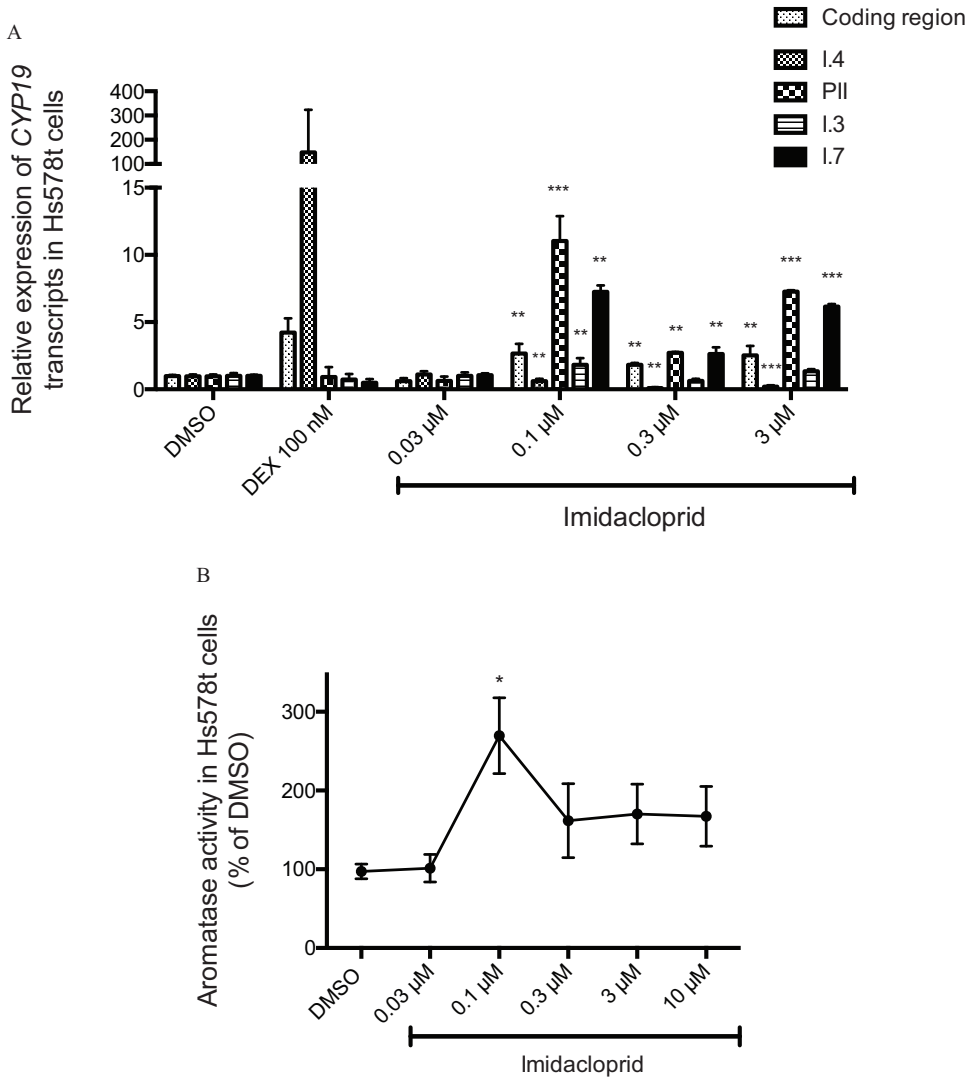

Figure 4. (*A*) Relative expression of *CYP19* coding region (nonpromoter-specific or total), and I.4, PII, I.3, and I.7 promoter-derived *CYP19* transcripts in Hs578t cells (fold DMSO control). (*B*) Aromatase catalytic activity in Hs578t cells exposed to imidacloprid (0.03, 0.1, 0.3, and 3 µM). DEX (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*

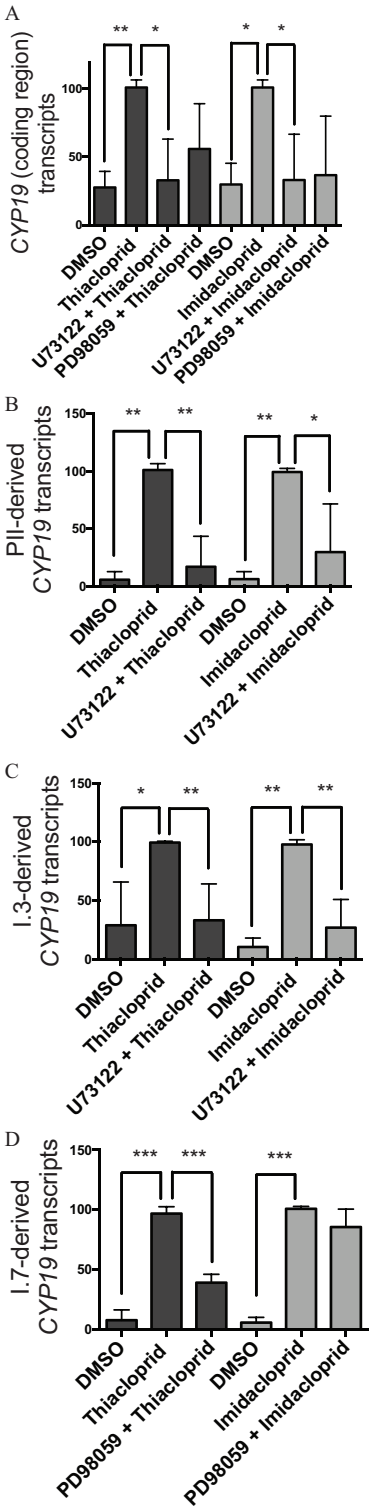

Figure 5. Relative expression of (*A*) *CYP19* coding region (nonpromoter-specific or total), and *CYP19* transcripts derived from promoters (*B*) PII, (*C*) I.3, and (*D*) I.7 in Hs578t cells exposed to thiacloprid (0.1 μM) or imidacloprid (0.1 μM) in the presence or absence of selective inhibitors 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

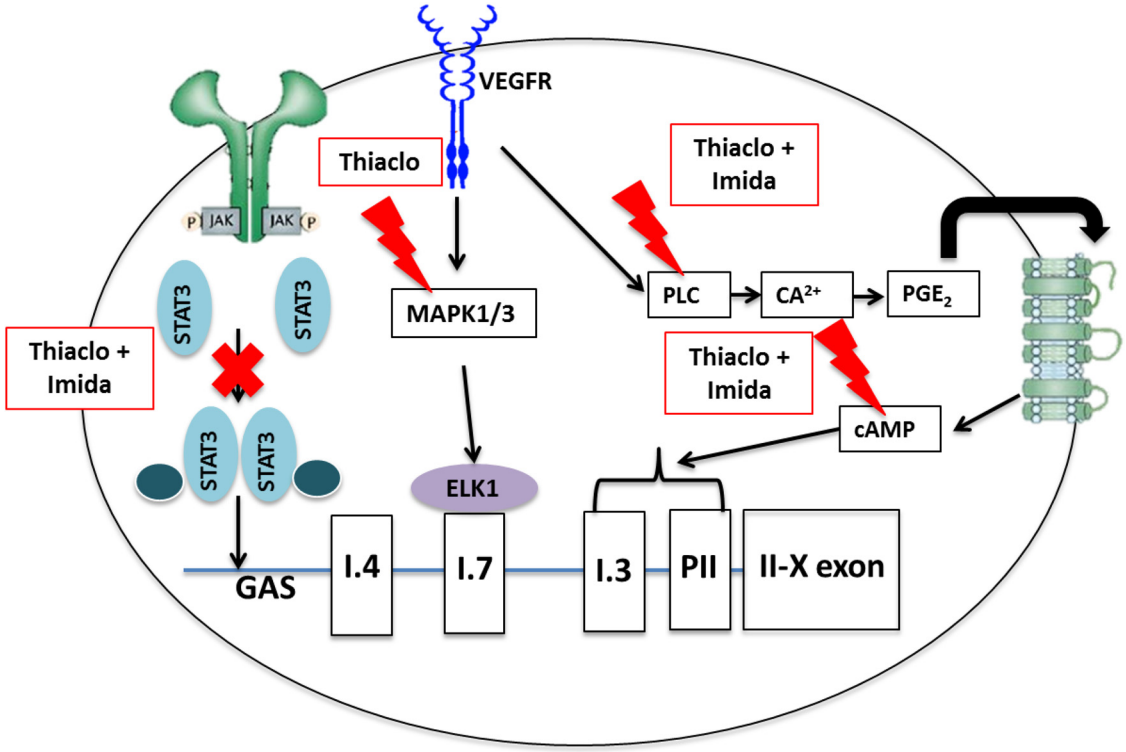

Figure 6. Proposed signaling pathways implicated in the promoter-specific expression of *CYP19* in Hs578t cells. It is proposed that thiacloprid and imidacloprid block the JAK/STAT3 pathway, which regulates I.4 promoter activity via a yet unknown mechanism. In addition, based on the effects of pathway 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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