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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

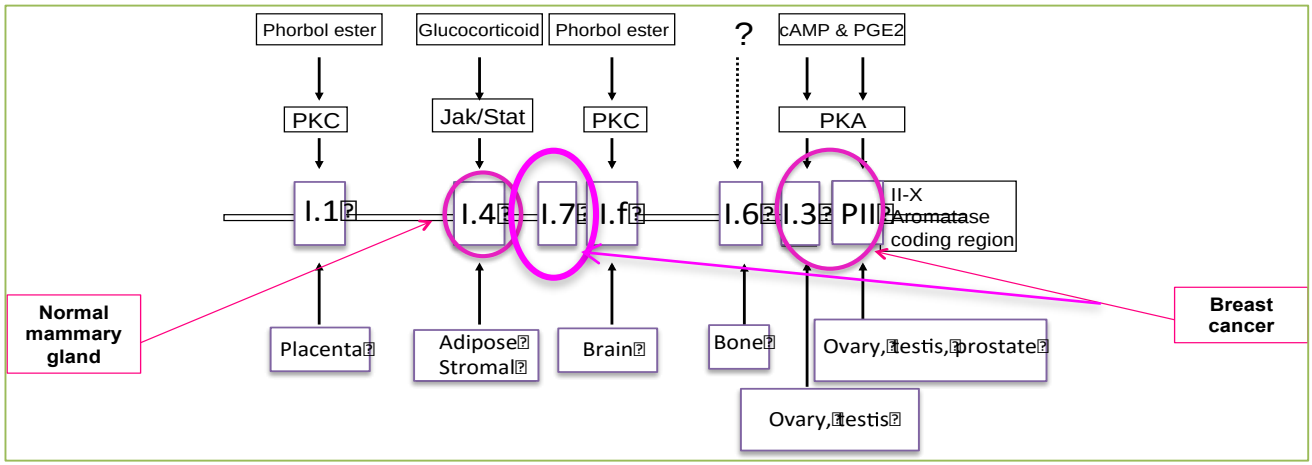

Figure 1. Tissue and promoter-specific organization of the CYP19 gene. Reprinted with permission [23].

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

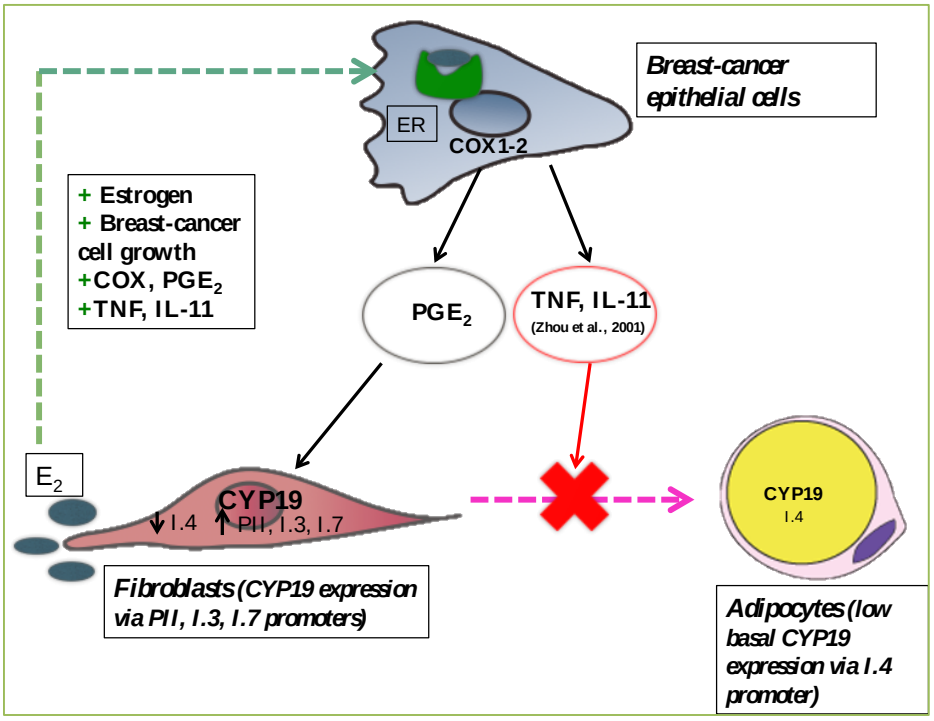

Figure 2. Cell-to-cell interactions in hormone-dependent breast cancer [34, 35]. Epithelial cancer cells produce PGE_2, which may induce a switch in *CYP19* promoter usage from I.4 to PII, I.3 and I.7 in fibroblasts, leading to increased local synthesis of estrogens. Epithelial cancer cells also synthesize 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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