# Evaluating the Endocrine Disruptive Properties of Common Environmental Pollutants

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## Establishment and validation of a cellular model system to measure estrogenic effects

We have established and validated a tissue culture cell model system to measure estrogenic effects using different methods. Human breast cancer cells (MCF7, T47D, MDA-MB-231) are used to determine if pollutants can substitute for the natural hormone (estrogen) in promoting cell growth. A modified version of the T47D cell line (called T47D-Kluc) harbouring a stably integrated copy of a luciferase reporter gene under control of a promoter containing estrogen response elements, is used to see if any estrogenic effects observed act via the estrogen receptor.

Transcriptome (gene expression) profiling has been performed using the estrogen dependent MCF7 cells to test the involvement of other hormonal signaling pathways (including other non-classical estrogenic pathways) and confirm any endocrine disruptive effects.

We have established collaborations with the Integrated Systems Toxicology Division of the US Environmental Protection Agency, to detect the transcriptome (gene expression) profile signature of estrogen receptor activation in breast cancer cells. In addition, we have established a collaboration with the faculty of sciences of the Universidad Austral de Chile, in order to model the interaction of pollutants at the active site of estrogen receptor α and calculate their binding energy.

## Endocrine disruptive effects of Bisphenol A alternatives

Plasticizers with estrogenic activity, such as bisphenol A (BPA), have been reported to have potential adverse health effects in humans, especially in fetal and infant stages. BPA presence in breast tissue leads to poor clinical outcomes for breast cancer patients. Due to mounting evidence and public pressure BPA is being phased out by the plastics manufacturing industry and is being replaced by other bisphenol variants in “BPA-free” products.

We have tested 6 of these BPA alternatives (bisphenol S, BPS; bisphenol F, BPF; bisphenol AP, BPAP; bisphenol AF, BPAF; bisphenol Z, BPZ; bisphenol B, BPB). They were all able to substitute for the natural hormone in promoting cell growth through estrogen receptor ESR1. Surprisingly, we found that bisphenol AF, bisphenol B and bisphenol Z, contained in “BPA-free” plastics, were more estrogenic than BPA.

The use of selective inhibitors of oestrogen receptors (such as tamoxifen) was employed to determine their role in any toxic, endocrine disruptive chemical (EDC) effects observed, and the influence on chemotherapeutic agent efficiency. The addition of the potent estrogen receptor antagonist ICI 182,780 antagonized the activation of ER by estradiol and bisphenols confirming reporter gene expression via this receptor. However, estrogenic effects of some BP derivatives, such as BPB and BPZ, were not completely antagonized by ICI addition, suggesting estrogenic activation mechanisms independent of ER. This can have profound consequences on the efficiency of chemotherapeutic agents which could be inefficient in inhibiting tumor growth caused by this type of pollutants.

A transcriptome (gene expression) profiling using both microarray (gene chip) and RNA sequencing (RNA-seq) methods, has been performed using the estrogen dependent MCF7 cells to test the involvement of other hormonal signaling pathways (including other non-classical estrogenic pathways) and confirm any endocrine disruptive effects. The signature of estrogen receptor alpha activation was studied in collaboration with a research team of the United States Environmental Protection Agency. All transcriptome profile alterations resulting from exposure to BPA and all 6 BP alternatives achieved statistical significance and exhibited a pattern highly similar to that of the biomarker.

We reveal that BPA-free products are not necessarily safer. The clinical relevance in hormone-dependent breast cancer progression should be investigated. A global push to remove all bisphenols from consumer products would be necessary to protect the health of the population.

These results were published on the 7$^{th}$ of June, 2017, in the journal of the Society of Toxicology, Toxicological Sciences.

***Reference:*** *Robin Mesnage, Alexia Phedonos, Matthew Arno, Sucharitha Balu, J. Christopher Corton, and Michael N Antoniou.Transcriptome profiling reveals bisphenol A alternatives activate estrogen receptor alpha in human breast cancer cells. Toxicological Sciences. DOI: 10.1093/toxsci/kfx101*

**Endocrine disruptive effects of glyphosate/Roundup**

Three different glyphosate-based herbicide (Roundup) formulations, pure glyphosate, and one adjuvant mixture, have been tested in the cell culture systems described above.

Using an MTT (color change assay based on activity of the enzyme succinate dehydrogenase), subtle cell proliferative effects were detected with some of the Roundup formulations at non cytotoxic levels in MCF7 cells. In addition, we observed a cell proliferative effect with glyphosate alone albeit at a higher concentration to that of Roundup in both MCF7 and to a lesser degree in T47D cells. The investigation of direct estrogen receptor activation using the T47D-Kluc cells indicate that glyphosate alone but not Roundup is able to activate estrogen receptors and stimulate luciferase gene activation suggesting an endocrine disrupting capability for this compound. Activation of the estrogen receptor was confirmed by

adding the potent estrogen receptor antagonist (ICI) into the mixture, which effectively blocked the stimulating effects of glyphosate.

MCF-7 cells were treated with the test compounds for 48 hours. Transcriptome profiles were performed by microarray analysis or RNA-sequencing. Gene expression profiles of glyphosate-exposed MCF-7 cells were reflective of proliferative effects, but did not overlap with that of an ERα gene expression biomarker signature.

Furthermore, the chemical behaviour of glyphosate within the ligand binding domain of ERα was modelled using molecular dynamic simulation in order to evaluate the binding energy of complexes. The calculation predicts that glyphosate binding energy to ERα LBD is weak (−4.10 kcal mol-1) and unstable, compared to estradiol (-25.79 kcal mol-1).

Based on the results of the molecular modelling and ERα biomarker, we hypothesize that the increase in ERE-luciferase reporter gene expression caused by glyphosate, is due to a ligand-independent mechanism. A possible route for this ligand-independent activation is via cellular signalling pathways (such as the cAMP-dependent protein kinase (PKA) pathway) that modulate the balance between cell proliferation and apoptosis. We showed that T47D-Kluc cells treated with 500 µM of 3-isobutyl-1-methylxanthine (IBMX), an activator of the PKA pathway which raises cAMP levels, stimulate ERE-mediated transcription of the luciferase reporter gene suggesting this as a possible route of glyphosate activation. However, further studies would be needed to determine if glyphosate modulates the PKA pathway.

Our results demonstrate glyphosate is a weak activator of ERα in hormone-dependent human breast cancer cells. Our findings indicate that glyphosate is activating ERα through a ligand-independent mechanism. However, further studies would be needed to determine if glyphosate modulates the PKA pathway or another signalling pathway. The clinical relevance in hormone-independent breast cancer progression should be investigated.

These results are currently under review with the journal *Food and Chemical Toxicology*, a publication is expected by the end of the summer 2017.

***Reference:*** *Robin Mesnage, Alexia Phedonos, Martina Biserni, Matthew Arno, Sucharitha Balu, J. Christopher Corton, Ricardo Ugarte, and Michael N Antoniou. Evaluation of estrogen receptor alpha activation by glyphosate-based herbicide constituents. Submitted to Food and Chemical Toxicology.*

**Evaluating the Combined Effects of Endocrine Disruptive Pollutants**

A range of pesticides used in combination with glyphosate is being tested on human breast cancer cell lines. These include 2,4-D and its metabolite 2,4-DCP, dicamba, quizalofop-ethyl, quizalofop-p-ethyl,

glufosinate-ammonium, imazethapyr, imazamox, isoxaflutole, mesotrione, bromoxynil, chlorimuron ethyl, metolachlor, acetochlor, atrazine.

Preliminary results suggest that some of these pesticides activate estrogen receptor alpha. In particular, the metabolite of 2,4-D (2,4-DCP), which could be accumulated by the new generation of GM crops, has been found to be more toxic than its parent molecule and potentially endocrine disruptor.

In a second step, the no-effect dose of each compound, considered as safe if they are evaluated as single chemicals, will be combined in other to see if low concentrations of these pollutants can have combined effects on the activation of estrogen receptor alpha.

However, the completion of these experiments and generation of results have been delayed due to unforeseen technical problems. A high background hormone stimulating activity, possibly caused by the presence of a contaminants leaching from plasticware used in cell tissue culture, prevented the measurement of estrogenic effects. Several steps were undertaken in order to reduce this high background, including the replacement of the different plastics used in our lab. However, the assays are still not stable and an extensive troubleshooting investigation is currently ongoing.

In the meantime, we decided to generate our own reporter cell line by using technologies employed in gene therapy strategies in order to increase the expression and the stability of the luciferase gene reporter. Stable and high level transgene expression are essential for the efficient and rapid production of clonal cell lines. We used lentiviral vectors carrying the luciferase transgene under control of estrogen response elements and a ubiquitous chromatin opening element (UCOE) genetic regulatory element. Since their discovery in 1999, UCOEs and their sub-fragments have found applications in areas of biotechnology requiring stable, reproducible, and high levels of gene expression. MCF-7 cells are currently transduced with the lentiviral vector described above in order to create a new reporter cell line more sensitive than the currently used T47D-Kluc cells.

**Expected completion date:** End 2017.