

CHAPTER 1

GENERAL INTRODUCTION

Ever since the publication of Theo Colborn's book *Our Stolen Future* (Colborn et al., 1996), interest in the so-called endocrine disruptors has captured the imagination of scientists and lay persons worldwide. Colborn and numerous other researchers claim that there is accumulating evidence that humans and both domestic and wildlife species have suffered adverse health consequences as a result of exposure to environmental compounds that interact with the endocrine system.

While these problems have been identified primarily in animals exposed to relatively high levels of organochlorine compounds, including DDT and its metabolites, PCBs and dioxins, as well as to naturally occurring plant estrogens, concern has focused on whether similar effects are occurring in the general human population.

Suffice it to say, however, a number of *in vitro* and *in vivo* studies have established the potential of some of these compounds to bind with hormone receptors within cells and generate a pleiotropic response. The latter may be in the form of cellular proliferation or differentiation.

On the other hand, endocrine disruptors may also alter animal and/or cell physiology in other ways, such as by influencing hormone receptor levels, by affecting the synthesis of natural hormones, or by acting as competitive antagonists in receptor binding. They can also affect hormone production by altering cholesterol availability, steroidogenesis, or feedback loops in the hypothalamus – pituitary – gonad (HPG) axis. However, for most associations reported between endocrine disruptors and their effects, the specific mechanisms of action are poorly understood.

Among the more relevant findings gleaned from scientific literature are as follows:

- a. Human prenatal exposure to PCBs can cause lower birth weight and shorter gestation, and have been correlated with deficits in IQ and memory as well as delayed neuromuscular development (Yamashita and Hayashi, 1985; Hsu et al., 1985; Rogan et al., 1988; Guo et al., 1994; Guo et al., 1995; Jacobson et al., 1990; Jacobson et al., 1992; Jacobson and Jacobson 1996).
- b. Laboratory animal exposure to certain endocrine disruptors during development can produce structural and functional abnormalities of the reproductive tract as well as affect fetal development (vom Saal 1995; Cummings 1997; Hammond et al., 1979; Gray et al., 1994; Malby et al., 1992abc; Morrissey et al., 1987; Sharpe et al., 1995).
- c. Numerous wildlife studies show associations between reproductive and developmental anomalies and exposure to endocrine disruptors (Jobling et al., 1998; Schmidt 1997; Guillette et al., 1994, 1995, 1996; Guillette and Crain 1996; Colborn et al., 1993; Fox 1992; Facemire et al., 1995).
- d. Prenatal exposure of laboratory animals to endocrine disruptors can affect the developing nervous system (Lilienthal et al., 1990; Lilienthal and Winneke 1991; Daly 1991; Daly et al., 1989; Schantz et al., 1989; Seegal 1996).
- e. Wildlife exposed to organochlorines exhibit suppression of the immune system (Grasman et al., 1996; de Swart et al., 1994, 1996; De Guise 1994, 1995).

While much of the work so far has focused on *in vivo* experiments, this series of studies is aimed at determining the impact of synthetic and naturally-occurring

endocrine disruptors as well as food factors on animal cells *in vitro*. *In vitro* assays have an advantage over *in vivo* experiments because of their rapidity, relatively low cost and reproducibility. These assays also allow a larger number of samples or compounds to be screened simultaneously. *In vitro* assays are also excellent models for investigating the mechanism of action of endocrine disruptors and their interaction with endocrine-response pathways (Committee on Hormonally Active Agents in the Environment, 1999).

In this work, focus has centered primarily on alkylphenolic compounds, with special emphasis on the most commonly used alkylphenol, nonylphenol; the phytoestrogens genistein and daidzein, which are found in soy and soy products; and the edible fungus *Agaricus blazei* Murill, which is known for its anti-cancer properties.

The experimental techniques used in these studies are as follows:

- Lactate dehydrogenase (LDH) assay - A decrease in cell viability is often associated with a damaged cell membrane, leading to the release of enzymes, such as LDH, into the medium. This short-term cytotoxicity assay determines the amount lactic acid dehydrogenase that cells release following exposure to possible cytotoxic compounds.
- DNA fragmentation assay – Cells undergoing apoptosis often exhibit DNA fragmentation in a very specific pattern, producing fragments that are multiples of 180-200 base pairs. This appears several hours before cell viability begins to decrease. This assay determines apoptosis or programmed cell death in cells exposed to test compounds.

- Acetylcholinesterase (AChE) assay – Acetylcholinesterase is the enzyme that hydrolyzes the neurotransmitter acetylcholine in the neuromuscular junction. Certain compounds can suppress or enhance AChE production, and this has a corresponding impact in cholinergic synaptic transmission. This assay determines the amount of AChE that neural cells produce after exposure to test compounds.
- Cell proliferation assay – Mitogenic compounds can bind to certain receptors in cells and cause cell proliferation. To provide an estimate of the number of cells exposed to test compounds after a given period, the yellow water-soluble tetrazolium dye, MTT, was used for this assay. MTT is reduced only by live cells.
- Protein kinase C assay – Protein kinase C is an enzyme that plays a significant role in many signaling pathways within cells. The activity of this enzyme, which phosphorylates other proteins, can help explain some of the observed phenomena in *in vitro* studies, such as cell proliferation and/or differentiation.
- Western Blotting – This experimental procedure involves isolating total proteins from cells and separating them via sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After which the proteins are blotted onto a PVDF membrane and the protein of interest is then detected using a labeled probe. This assay determines the expression of proteins of interest.
- Northern Blotting – While western blotting can detect proteins, northern blotting, on the other hand, can detect RNA strands of

interest. The procedure involves careful isolation of total RNA, separating them using agarose gel electrophoresis, blotting them onto a PVDF membrane, and then probing them using a labeled DNA or RNA probe. This assay detects the expression of target mRNAs.

The first study (Chapter 2) focused on synthetic xenoestrogens. Alkylphenols are a group of compounds with established mitogenic effects on human breast cancer cells. They are likewise ubiquitous in the ambient environment, being components of detergents and plastics. In fact, humans are in contact with alkylphenols almost every day without their knowledge. Studies, however, on the impact of these compounds on neuronal cells is limited; hence, this research. Among others, results showed the inhibitory effect of alkylphenols on the AChE activity of neuronal cells.

The author then wanted to determine whether naturally-occurring endocrine disruptors also elicit the same response. Hence, attention was focused on the soy isoflavones genistein and daidzein (Chapter 3). Among others, results showed that both phytoestrogens were found to enhance the AChE activity of PC12 cells at very low concentrations by binding to the estrogen receptor (ER). In other words, the phytoestrogens elicited an opposite effect.

The preceding studies showed that while synthetic xenoestrogens such as alkylphenols can affect the function of neuronal cells by inhibiting AChE activity, natural xenoestrogens, such as genistein and daidzein, which are also food factors, can cancel this effect by enhancing AChE. Hence, it is possible that food factors can mitigate the harmful effects of endocrine disruptors as shown here. The author therefore wanted to relate this finding using a different model, this time using breast

cancer cells and food factors from an edible mushroom that is known for its anti-cancer and anti-tumor promoting activity (Chapter 4).

The initial hypothesis is that compounds from the edible mushroom *Agaricus blazei* Murill (ABM) will also mitigate the mitogenic effects of the known endocrine disruptor nonylphenol. The findings, however, were totally unexpected. Results showed a synergistic effect between *p*-nonylphenol and the aqueous extract (AE) from ABM in promoting MCF7 cell proliferation, and that the extract enhanced the expression of the c-Jun protein in MCF7 cells.