

Chapter 4

Ligand-Mediated Toxicology: Characterization and Translational Prospects

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Abstract In biochemistry a ligand is a molecule that binds to a receptor or other biomolecule that forms a complex and produces a biological effect. We live in a world of exposure to exogenous ligands and there are three long-established strands of biological research that have investigated the actions of biologically active dietary or environmental compounds on animals, including humans.

First, in agricultural and animal husbandry research, there is extensive documentation of the many thousands of phytochemicals that mediate plant-animal interactions many of which produce toxic effects at certain levels of exposure in terms of dose, duration and window of sensitivity. In a broader biological context, many of these relationships have been formalized by concepts of co-evolution of plants and animals.

Second, there is a long history of toxicology research showing a wide range of adverse effects that often result from exposures to hormones and other modulators of endocrine function, especially during developmentally sensitive intervals. In recent decades much of this research has been encapsulated by a single term of “endocrine disruptors” even though many ligand-mediated actions are not endocrine *per se* or definitively adverse.

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Third, nutritional and functional foods research have demonstrated a wide range of health benefits produced by consumption of many phytochemical ligands through mechanisms that are not truly distinct from those that seem to mediate the adverse effects noted in either the animal husbandry or developmental toxicology research arenas.

We are confident that environmental and public health can be enhanced by defining how such exposures to exogenous signaling molecules (ligands) pose both risks and benefits. Therefore, it is time to modify the scientific perspective and nomenclature to encompass these several streams of investigation such that both risks and benefits of exposure to dietary and environmental ligands may be characterized and translated into sound strategies to address environmental and public health concerns. Fetal alcohol spectrum disorder (FASD) is an illustrative case for translational toxicology-informed interventions where there is a viable prospect for a multi-component nutritional health benefit to reduce occurrence or severity of FASD. Health promoting interventional options must include reduction(s) in exposures to ligands that incur health risks but when exposures cannot be adequately reduced or preempted, we should also define and employ active mechanistically-based mitigative interventions.

Keywords Translational toxicology • Endocrine disruption • Epigenetics • Ligand • Receptor mediated toxicity • Xenosensors

4.1 Living in a World of Ligands: Translating Exposures to Exogenous Signaling Molecules into Human Health Benefits and Risks

In biochemistry a ligand is generally defined as a molecule (usually small), that binds to a receptor or other biomolecule that forms a complex and produces a biological effect. Our complex environment is laced with ligands to which animals, including humans, are continuously and episodically exposed. Endogenous ligands are “part and parcel” of our *milieu interieur* while naturally-occurring and man-made exogenous ligands are major elements of our *milieu exterieur*. At all times across the lifespan, growth impacts and functional/homeostatic effects of exposure to such exogenous ligands may occur. Effects of such exposures on differentiation events might also occur at any time in an animal’s life, but such organizational impacts are more likely to be robust during developmentally-sensitive intervals (“developmental windows”). Our challenge for human health is not merely to document the occurrence of such effects but to also to characterize the risk – non-effect – benefit profile of such exposures and further to ascertain how prevention or intervention may be used to enhance environmental and public health while reducing exposures to ligands that incur health risks. We will consider how current

toxicologic approaches might be reoriented into a more translational scientific endeavor by reflecting on the broad range of exposures from a perspective that we will call ligand-mediated toxicology.

4.2 Traditional Toxicology

Toxicology studies the potential adverse effects of exposures, especially xenobiotics, on various forms of life including human beings. Modern toxicology goes beyond documenting adverse effects to the study of molecular toxicology, performance of safety evaluations and risk assessments as ways to translate basic toxicology into public health or public policy interventions. Two main principles underlie all descriptive toxicity testing: (1) the adverse effects produced by an agent in laboratory animals are applicable to humans and (2) high dose exposures of a toxicant to animals may be required in order to discover possible hazards in humans. The first toxicity test performed on a new chemical is acute toxicity which gives a quantitative estimate of lethality (lethal dose or lethal concentration that kills 50 % of exposed animals, LD₅₀ or LC₅₀). Acute studies are used to compare the toxicity of agents, to identify target organs and other clinical manifestations of acute toxicity, to establish the reversibility of the toxic response and to provide dose-ranging guidance for other studies. Subacute toxicity testing is performed to obtain toxicity data for a chemical after repeated exposure as well as to establish doses for subchronic toxicity. Subchronic exposure can be of different durations but 90 days is the most common duration. Long term or chronic toxicity studies are performed similarly to subchronic toxicity studies except that the duration of exposure is greater than 90 days and up to the lifetime of the animal. Chronic toxicity studies are performed to assess the cumulative toxicity of chemicals (Eaton and Gilbert 2010).

4.3 Mechanisms of Toxicity

The quantitative characterization of how toxic effects occur is important for an evaluation of the potential hazard posed by a chemical. It is valuable to understand the mechanisms responsible for the manifestation of toxicity. The understanding of mechanism of toxicity is of both practical and theoretical importance. Selective or altered toxicity may be due to different or altered exposure and delivery, target molecules, biochemical alterations, cellular and molecular repair and the mechanism of circulatory and thermoregulatory reflex in the adaptation of the toxic events. The significance of the chemistry of a toxicant for its delivery and reaction with the target molecule as well as the biochemistry, cell and molecular biology, immunology and physiology of the exposed organism in its response to the action of the toxicant are equally important. An organism has specific mechanisms to counter the delivery of

the toxicant, to detoxify the toxicant, to reverse the toxic injury by repair mechanisms and to develop an adaptive response. There are several fundamental steps in any mechanism of toxicity such as delivery from the site of exposure to the target involving absorption (versus presystemic elimination), distribution to and away from the target, excretion versus reabsorption and activation as a part of toxication versus detoxication and, ultimately, reaction of the active toxicant(s) with the target molecule(s). Various types of reactions may occur including effects of toxicants on target molecules and toxicity not initiated by reaction with target molecules; cellular dysfunction and resultant toxicities involving toxicant-induced cellular dysregulation and toxic alteration of cellular maintenance including repair or dysrepair involving molecular repair, cellular repair, tissue repair, repair failure and toxicity resulting from dysrepair (Gregus 2010).

4.4 Translational Toxicology

The nature of toxicology has always been driven by a translational sense of purpose with respect to human health. The earliest toxicological studies carried out were to assess the effects of plant and animal toxins on human subjects (Gallo 2008). However there are still gaps in the transformation of human toxicology into a translational discipline in the contemporary biomedical sense of the term. There is a gap between laboratory toxicology research and testing for cellular, molecular and pathological outcomes in cell systems and animal models and epidemiological and ecosystem studies, which examine exposed populations for disease. The current approach may or may not correlate the exposure to a toxicant with a clinical outcome, but this almost never leads to identification of a specific protective, corrective or compensatory therapy that may be used in currently intoxicated individuals or those who may be exposed in the future. The claims of association of cause-and-effect are often no better than a tentative association of exposure and effect. If there is inadequate evidence to show either adverse effects due to exposure to a potential toxicant or that there is no plausible risk due to exposure, then there is little or no prospect for intervention of any sort beyond application of the “precautionary principle.” Therefore in strategic terms, the main objectives of translational toxicology are to advance environmental health sciences in order to: (1) transform research and development programs to better link exposures to disease occurrence or outcomes; (2) link target tissue/organ/system outcomes in animal models to animal biomarkers to human biomarkers to human target organ outcomes; and (3) enhance predictive use of animal and human biomarker data by development of computational models (Hale et al. 2009). Therefore we will reconsider how current knowledge from toxicological research and epidemiological studies can be seen as a contemporary translational biomedical sub-discipline of ligand-mediated toxicology in which developmental exposures may impact health unfavorably or favorably across the human lifespan.

4.5 Translating Biomarkers

In clinical toxicology potential biomarkers that may have utility in translational toxicology research include chemical and molecular markers such as clinical chemistry and hematology profiles, and hormone levels (Hale et al. 2009) as well as imaging techniques such as radiology, ultrasound, computerized tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET). In preclinical toxicology, histopathology remains a key component in assessing target tissue responses to exposures while new genomic, transcriptomic, proteomic and metabolomics technologies as well as next generation sequencing methods generate data sets that can serve as biomarkers in toxicological investigations across the lifespan (West et al. 2010). Global metabolite profiling allows the measurement of numerous chemicals in minimal amounts of biological material (Patti et al. 2012) and is applicable to translational toxicity research. Within genomics and RNA signaling, micro RNAs that are known to be key regulators of metabolism are available in accessible human blood to quantify as either signaling molecules or disease biomarkers (Rottiers and Naar 2012). Additionally, other molecular and cellular biomarkers such as the levels of leukocyte mitochondrial DNA copy number can also be used in translational studies (Kim et al. 2012).

For biomarkers to be useful in predicting and monitoring drug safety in a clinical population, temporal response must be compared clinically and preclinically. Thorough preclinical qualification ideally includes studies of acute and chronic dosing, a range of toxicants with varying mechanisms of injury in the target organ, negative control compounds, and several toxicants known to affect other organs to assess the specificity of the biomarker. Limitations exist to how we can study a biomarker's behavior in a clinical population. Histological examination of tissue is not possible in most circumstances; scheduled sampling is more complex in an outpatient population; and ethical issues prevent the deliberate administration of toxicants to humans. However, strategies for qualifying biomarkers in humans can take advantage of marketed therapeutics with known toxicities. For example, a clinical study for qualifying nephrotoxicity biomarkers considered several clinical populations with known kidney injury before deciding to monitor cardiac catheterized patients with an unfortunately high event rate of contrast dye-induced nephropathy (Fisch 2012). Considered were patients with shock trauma, aminoglycoside-treated cystic fibrosis, and cisplatin-chemotherapy. The goal of toxicology testing in pharmaceutical development is to identify and predict safety issues to inform the risk-benefit decision about using a drug for a particular indication. The safety assessment is not always fully predictive, and limitations exist in extrapolating findings from animal studies to clinical studies. Therapeutic dosing prior to clinical trials is the accepted paradigm of safety testing. This depends on the ability to extrapolate to humans from the drug's activity and dose-response in the animal model.

Although, specific pharmacokinetic and pharmacodynamic parameters and a standard battery of toxicological end points are routinely measured, many factors that affect the comparative disposition of the drug in preclinical model versus

human are rarely fully elucidated. Post-sacrifice microscopic histopathology plays a central role in identifying drug-induced lesions in diverse tissues in animal studies; extrapolating these findings to clinical trials requires a noninvasive, monitorable end point (Euling et al. 2008). However, studies in healthy, genetically homogeneous animals can hardly be expected to predict low-incidence toxicities that arise clinically as a result of individual human differences in response due to genetic variations in metabolism or drug–target pathways or to coexisting conditions and subsets of disease phenotypes. Extrapolation from preclinical to clinical following the working principles of translational toxicology research is required.

It has also been proposed that peroxisome proliferation could be used as a biomarker of exposure to a variety of pollutants in environmental pollution assessment such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), chlorinated herbicides, chlorinated solvents, perfluorinated acids, phthalate ester plasticizers, phenoxyacetic acids, alkyl carboxyl acids and tetrazoles (Woods et al. 2007). The increase in the volume and number of peroxisomes is usually accompanied by induction of specific peroxisomal enzymes including all three enzymes of the peroxisomal β -oxidation pathway, namely, acyl-CoA oxidase (ACO), peroxisomal hydratase-dehydrogenase-isomerase or multifunctional enzyme, and thiolase. Enzyme induction occurs through transcriptional activation of the corresponding genes by peroxisomal proliferator activator receptors (PPARs). The mechanism by which humans and nonhuman primates are resistant to peroxisome proliferation is not known. Humans possess a functional PPAR α , indicating that the lack of receptor is not responsible for the lack of peroxisome proliferation upon treatment with fibrate drugs.

4.6 Receptor Mediated Toxicity: Xenosensors, Nuclear Hormone Receptor Activation, Actions of Hormonal Ligands

Living organisms including humans are exposed to thousands of exogenous chemicals, both man-made (synthetic, xenobiotic) and of natural origin (prominently phytochemicals). For survival, organisms have developed systems for handling these potentially harmful exogenous substances. When a subject (human, wildlife, farm animal, etc.) is exposed to a chemical, these processes typically either eliminate and/or transform the chemical within the exposed subject. If a chemical is modified by the exposed subject, it interacts with sensors called xenosensors which prepares the subject for the elimination of the chemical from the system. The exposure to chemicals leads to activation of enzymes referred to collectively as xenobiotic metabolism (monooxygenases, conjugation enzyme and transporters) which creates increased centers of hydrophilicity, followed by subsequent conjugation with either glucuronic acid or sulfate to prepare the drug or chemical for its excretion from cell

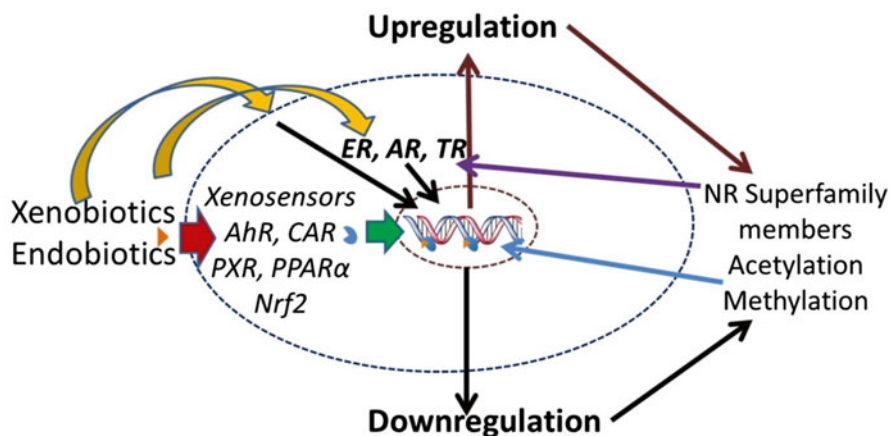


Fig. 4.1 Xenosensing and xenobiotics mediated possible effects via gene regulation. *AhR* Aryl hydrocarbon receptor, *CAR* Constitutive androstane receptor, *PXR* Pregnane X receptor, *PPARα* Peroxisomal proliferator activated receptor- α , *Nrf2* Nuclear factor (erythroid-derived-2) like 2 (NFE2L2)

using multi-drug resistant transport processes. During the process of metabolism, the chemicals either become less toxic or may acquire characteristics which allow further interaction with cellular constituents. Compounds like PAH and halogenated hydrocarbons (HHC) bind to aryl hydrocarbon receptor (AhR) which not only serves as a xenosensor but also acts as receptor and activates the monooxygenase system for creating hydrophilic centers and conjugation with hydrophilic compounds for elimination. One mode of action of endocrine disruption is the ability of some chemicals to either directly or indirectly disrupt such nuclear hormone receptor signaling. In addition to man-made chemicals, nature also produces a number of compounds which possess the ability to bind and either disrupt or activate nuclear receptor superfamily members. Figure 4.1 depicts this general type of mechanism of receptor mediated toxicity.

A number of receptors sense the presence of xenobiotics in the cell and induce a cascade of events leading to neutralization and excretion. However, in many cases the metabolism of xenobiotics can give rise to toxic metabolites or free radicals/reactive oxygen species (ROS) that can cause further oxidative injury to the cells. The metabolism of xenobiotics can also perturb production and metabolism of certain hormones in the body. Therefore in some instances receptors that induce the elimination of toxicants may themselves mediate some of their toxic effects.

Certain environmental toxicants can also bind to receptors other than the xenosensors (Ruegg et al. 2009). In particular the receptors for the female sex steroids (estrogen receptors, ERs) have been identified as targets for many synthetic and natural compounds. Interactions of exogenous substances with hormone receptors influence a number of other steps in physiological signaling which have been termed

“endocrine disruption.” One common and widely-used definition was formulated as follows:

An environmental endocrine disruptor was broadly defined as an exogenous agent that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes. (Kavlock et al. 1996)

Note that this definition did not require consideration of whether any effects that may result from a compound capable of this mode of action be assessed as adverse or even potentially beneficial. Additionally over time and with ongoing usage, the designation of “endocrine disruptor” has broadened to such an extent as to often not relate to endocrine (hormonal) actions at all, and we accept that this is the established usage of the term. However, given the abiding need to consider both risks and/or benefits of exposures to dietary and environmental chemicals and the utilitarian value of including a broad spectrum of highly specific but non-hormonal modes of action, we will use “ligand-mediation” as the broader core concept.

Endocrine disruption has been associated with major health threats in the western world, such as development of diabetes and obesity, decreased fertility, and with the occurrence of hormone-associated cancers. Several of these chemicals of concern are persistent organic pollutants (POPs) because they are resistant to environmental degradation. They are thus persistent over a long time in the environment, can contaminate drinking water and food, and can be biomagnified via the food chain. Organochlorine pesticides, like DDT (dichloro diphenyl trichloroethane), and gamma hexane are quite persistent. A number of the man-made environmental chemicals identified as ER disruptors were pesticides. Symptoms in humans working with the manufacture of DDT led to the identification of DDT as an ER agonist. Other pesticides that activate ER are the DDT metabolites DDD (dichlorodiphenyl-dichloroethane) and DDE (dichlorodiphenyldichloroethylene), methoxychlor, and dieldrin (Lemarie et al. 2006). In addition, DDE and the fungicide vinclozolin can also affect the function of other hormone receptors by acting as androgen receptor (AR) antagonists (Kavlock and Cummings 2005), while vinclozolin can further antagonize the activity of progesterone receptor (PR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR) *in vitro* (Molina-Molina et al. 2006). Chemicals intentionally produced besides pesticides include biocides, plasticizers, and food additives and cosmetics. Many of these substances are structurally related to steroid hormones and may thus act on the respective hormone receptor(s). Non-persistent compounds such as phthalates and bisphenol A are additives used in the plastics industry. These compounds leach from plastic containers to water and liquids. These chemicals may also interact with nuclear receptors ER and AR while bisphenol A can also bind to thyroxine receptor (TR).

Endocrine-disrupting chemicals (EDCs) can have effects at low doses that are not predicted by effects at higher doses (Vandenberg et al. 2012). Low-dose effects are those that occur within the range of human exposures or those observed at doses below the range used in traditional toxicological studies. These effects may depend upon non-monotonic dose–response relationships. A nonlinear relationship may be

seen between dose and effect where the slope of the curve changes sign somewhere within the range of doses examined. It is noteworthy that non-monotonic responses and low-dose effects are remarkably common in studies of natural hormones and EDCs. In most instances where there is a scientifically valid health concern, the developmental toxicology concept of endocrine disruption must still be “put to the test” and advanced to an active translational (protective, mitigative or compensatory) step in which: (a) human exposure(s) occur, (b) there is a plausible mode of action such that the exposure might elicit developmental effects, (c) human health outcomes are assessed and (d) both passive [reduce exposures] and active [nutritional, sociobehavioral or pharmaceutical interventions] health risk intervention strategies can be developed for exposed individuals.

4.7 Endocrine Disruption and Epigenetic Changes After Exposure to Chemicals

The range of mechanisms of endocrine disruption is not highly constrained. Studies with EDCs like vinclozolin, bisphenol A and polychlorinated biphenyls demonstrate disruption of the hypothalamic-pituitary axis (Vandenberg et al. 2012; Bergman et al. 2012; Zoeller et al. 2012), to disruption of nuclear hormone receptor signaling (Bergman et al. 2012), to epigenetic changes transmitted from exposed individuals to their fetuses and even to subsequent generations. This latter mechanism of endocrine disruption, epigenetic changes, (Bollati and Baccarelli 2010) is gaining prominence as an important mode of action in developmental toxicology. Epigenetics is the study of heritable changes in gene expression without changes in the DNA sequence of the genome. Modification of DNA by methyltransferases leads to decreased charge on DNA causing compaction of chromatin which is not suitable for active transcription. It is known that methylation of CpG islands which are present in promoters of genes results in gene silencing. In addition chromatin associated proteins, i.e., histones, can be modified by acetylation, methylation, phosphorylation, sumoylation etc. Acetylation of histones is known to relax the chromatin and activate transcription. *In utero* exposures to bisphenol A, a constituent of polycarbonate plastics, has been implicated in breast and prostate cancer (Bollati and Baccarelli 2010). Experimentally, it has been demonstrated that bisphenol A exposure causes hypomethylation. Exposure of bisphenol A to pregnant mice results into altered methylation in CpG islands of the forebrain. In addition, it leads to shifting of agouti coat color as a result of decreased DNA methylation (Walker and Gore 2011). In rats, bisphenol A exposure increases methylation of several prostate specific genes while decreasing the methylation of phosphodiesterase 4 variant 3 (PDE4D3). PCBs have also been demonstrated to decrease DNA methylation enzyme activities (Dnmt1 in hypothalamus and liver and Dnmt1 and methylation of 16 genes in liver) in postnatal day 21 in offspring (Walker and Gore 2011).

Transfer of epigenetic changes has been demonstrated by animal experimentation. It has been demonstrated that exposure to EDCs *in utero* leads to epigenetic changes in fetuses which leads to altered gene expression and these epigenetic changes may be passed on to the next generation (Walker and Gore 2011). Thus exposure of fetuses in pregnant female rats to vinclozolin during the sex determination phase results in transgenerational spermatogenic defects and prostate enlargement (Anway et al. 2005, 2006a, b; Anway and Skinner 2006; Chang et al. 2006). Other environmental exposures may activate endogenous toxicologic mechanisms that could lead to epigenetic changes. Studies have demonstrated that workers who are exposed to particulate matter exhibit reduced CpG methylation of inducible nitric oxide synthase. Many chemicals when detoxified produce oxidative stress which in turn may cause DNA damage and this can in turn hinder methyltransferase and binding of methyl groups to DNA. Under mild oxidative stress DNA methylation machinery malfunctions, leading to hypomethylation of genes (Collotta et al. 2013) and this can lead to altered methylation of cytosine residues in DNA. Hypothetically, DNA methylation and chromatin modification can change the activity of vital genes. Correcting methylation via chemicals may prove a good prospect to counteract environmental chemical-induced epigenetic effects. In the case of hypomethylation, methyl donors like folic acid can be used to block epigenetic changes. Genistein, a phytohormone which blocks BPA-induced hypomethylation might also be employed. These are only preliminary findings but preclinical and clinical trials can be envisioned. We need to determine if some nutritional or other generally-regarded-as-safe (GRAS) agents could potentially limit environmental chemical-induced epigenetic changes and whether such effects could have a salutary impact on human health.

Changes in acetylation of histones which can open chromatin and increase the transcriptional activity of genes have also been observed with EDCs including persistent organic pollutants, arsenic metal and pesticides (herbicides, insecticides and fungicides). Increased acetylation of histone and neurodegeneration was observed with dieldrin in N27 cells (Song et al. 2010). Another herbicide, paraquat was also observed to increase acetylation of histone *in vitro* using N27 cells (Song et al. 2011). Histone modification in brain is linked to sexual differentiation of brain. In males, the bed nucleus of the stria terminalis in the hypothalamus is larger compared to females. Increasing acetylation by inhibiting the deacetylase activity with valproic acid in androgenized female results in femanization of the bed nucleus of stria terminalis (Walker and Gore 2011). A number of agents which alter the activity of bromodomain proteins (which sense epigenetic changes) are being developed and may be able to reverse and rectify epigenetic changes due to various chemical exposures including EDCs.

In addition to methylation and acetylation as epigenetic changes, gene regulation at the transcriptional level is also affected by micro-RNAs (miRNA) (Yang and Zhou 2013). miRNAs are short 22–24 nucleotide long having sequence complementarity to the 3′-untranslated sequences of mRNA. miRNAs binds to 3′-untranslated sequences as well as other regions of mRNA to regulate mRNA level and thus controlling the gene expression. miRNA is an evolving field and it is believed that

one miRNA can regulate several mRNAs and several miRNA can regulate one gene. A good amount of data documents changes in miRNAs expression patterns in diseases and after exposure to chemicals (Berezikov 2011; Wang et al. 2009). It is proposed that miRNAs can serve as signalling molecules besides their role as transcriptional regulators (Russo et al. 2014). If changes in the expression pattern of miRNAs are transferred from mother to fetus due to exposure to environmental chemicals, the pattern of key gene expression may affect the development of the offspring. By performing miRNA arrays after exposure of porcine kidney epithelial cells with the organophosphate pesticide, dichlorvos, alterations in miRNA expression were observed (Li et al. 2011). Changes in miRNA expression profiles were observed in zebrafish after exposures to several pesticides (Wang et al. 2010). Changes in miRNA profiles have been linked to development of cancer. Cancer causing chemicals including the fungicides triadimefon and propiconazole, and arsenic have been found to alter miRNA expression profiles in mouse liver and in human lymphoblastoid cells. To our knowledge, studies on transgenerational changes in miRNA due to exposure with chemicals including ECDs have not been performed. Thus it is imperative to document the miRNA expression patterns (over 1000 miRNAs in humans are known) in unexposed subjects for comparative purposes. Then the possibility of effects of altered miRNA mediated toxicity can be visualized and a translational approach can be imagined.

4.8 Broadening the Translational Perspective About Exogenous Ligands (Exogenous Signaling Molecules): Risks, Benefits, Neither or Both?

While there are numerous well-documented hormone receptors, the range of potential biomolecules with which ligands may interact in highly specific ways is far greater, exceeding two thousand different intracellular molecular species. The full range of potential modes of action is captured in the “Guide to Receptors and Channels (GRAC)” (Alexander et al. 2011; Pawson et al. 2014). This compilation of the major pharmacological targets is divided into seven sections: G protein-coupled receptors, ligand-gated ion channels, ion channels, catalytic receptors, nuclear receptors, transporters and enzymes. The most straightforward access to this resource is the website, <http://www.guidetopharmacology.org/> which was originally created as a collaboration between The British Pharmacological Society (BPS) and the International Union of Basic and Clinical Pharmacology (IUPHAR), and is now supported with funding from the Wellcome Trust. Within this searchable database, in addition to listing of thousands of known ligands, thousands of biomolecules that are known to interact with small molecular ligands are listed. The categories of biomolecules include the following:

- G protein-coupled receptors
- Ion channels
- Nuclear hormone receptors

- Kinases
- Catalytic receptors
- Transporters
- Enzymes
- Other protein targets

All of these biomolecular targets present potential mechanisms by which ligand-mediated toxicity could occur. While some targets such as enzymes might seem less likely to robustly affect developmental events, numerous other targets in the several categories could plausibly impact development and functional status over the lifespan in either adverse or beneficial ways.

The current list of ligands (Pawson et al. 2014) within the website includes multiple categories of chemicals as follows:

- Approved [drugs] (current or previously approved for clinical use in humans)
- Synthetic organic chemicals
- Metabolites
- Natural products
- Endogenous peptides
- Other peptides
- Antibodies
- Labeled (for research applications)

Especially when we add that many compounds are known to have pleiotropic actions, the potential number of ligand-biomolecule signaling effects are astronomical, and we must accept that we must link exposures of interest to attendant modes of action and with resultant “phenotypes” that are a definable functional or structural outcome plausibly implying adverse or beneficial health consequences.

4.9 Dietary and Environmental Ligands: Simple Insights from Animal Husbandry and Co-evolution of Plants and Animals

When we consider potential effects of exogenous molecules that act as ligands on human development, it makes biological and pharmacological sense to consider that some such effects may be either adverse (Hughes and Tansey 1998) or beneficial (Adlercreutz 1998) as is well-documented for livestock (Cheeke 1989) and wild animals (Belovsky and Schmitz 1991). A paradigm for considering the human situation regarding such a Janus-like consequence of developmental exposures to exogenous ligands may derive from consideration of co-evolution of plants and animals (Harborne 1982; Thompson 1994). The chemical warfare contest between plants and their animal predators captures the two-faced nature of animal exposures to plant derived ligands. On one hand, massive amounts of scientific data demonstrate that thousands of phytochemicals produced by plant secondary metabolism limit or

deter herbivores via specific and general mechanisms of toxicity (Cheeke 1989; Belovsky and Schmitz 1991). On the other hand, many of the same classes of phytochemicals have been shown to provide health promoting benefits when present at modest levels in the diet of animals and humans (Adlercreutz 1998; Hughes and Tansey 1998; Polya 2003). Particularly for omnivores like humans (Hughes and Dhiman 2002), dietary diversity has been convincingly shown to be health-promoting especially regarding diverse intake of colorful and flavorful plant-based foods which are rich in biologically active phytochemicals. Therefore, when we consider the actions of xenobiotic exogenous ligands and their potential effects on developmental events in humans, we must assess their modes of action as well as manifestations of persistent changes in biological function that can be judged as adverse, beneficial or nil (inconsequential). In this context we include as xenobiotic exogenous ligands all man-made compounds with specificity of actions on mammalian signaling pathways. Thus it is essential that all exposures including environmental contaminants, pharmaceuticals, over-the-counter medications, herbal and supplement products, lifestyle drugs of use or abuse, household products, etc. merit thorough assessment. Furthermore, mode of action alone cannot be sufficient to determine presence or absence or degree of risk to human development. Understanding what exposures (in terms of dose, duration, window of exposure etc.) incur human developmental risk is the critical basis upon which alternative protective strategies must be based. For adverse exposures that can be obviated, exposure should be reduced or eliminated. For the many exposures which are unforeseen or unavoidable, thorough understanding of the effects of the antecedent ligand exposure and the induced persistent functional change(s) will be essential for the translational steps of proposing, assessing and implementing mitigative or restorative therapeutic treatments.

Early in life humans are exposed to numerous environmental factors that may influence developmental processes and subsequently health across the entire ensuing lifespan. During developmentally sensitive windows such as *in utero*, neonatal and peripubertal periods, the young human may be and sometimes is inevitably exposed to complex exogenous factors such as psychosocial stressors, maternal metabolic *in utero* environment, hypertensive disorders of pregnancy which may subject the fetus to placental insufficiency, inadequate or inappropriate nutrition, and other mixtures for which the toxicopathologic pathways are poorly understood. The translational prospects for developing mitigative or restorative interventions for adverse effects that may have been induced by those categories of exposures is certainly daunting because the specific molecular, cellular and organizational perturbations are not well characterized. In contrast to the staggering complexities posed by several of these developmental exposures, other exposures to compounds that act as ligands in interactions with biomolecules offer some prospects for more specific countervailing interventions. In brief, if exposure to an exogenous ligand acts through a particular molecular signaling pathway then the prospect for identifying a therapy that may antagonize or circumvent the induced signal should be more straightforward. If the signaling perturbation during a developmentally sensitive window is known with a high degree of precision and certainty, then therapeutic

mitigation of that signal during that developmental window can be specifically assessed and possibly implemented. Additionally if ligand exposure during a sensitive developmental window is shown to have occurred, understanding of the affected signaling pathway may also allow more precise compensatory therapy at some later stage in life.

As mentioned earlier, one area of developmental toxicology that has been extraordinarily controversial over the last two decades is the field defined as “endocrine disruptors” (Kavlock et al. 1996; Olsson et al. 1998). There are core elements to the controversy that relate to the prospects of adverse developmental effects of variable levels of exposure to hormone like compounds via interactions with highly specific hormone receptors and non-monotonic dose response curves.

On one hand, there seems to often be limited recall regarding decades of historical studies that investigated potential toxicities attributed to hormonal exposures which built the foundation for our more contemporary research regarding hormone mediated developmental effects (McLachlan 1980, 1985; Kincl 1990) and show the extent to which adverse effects can be manifested. On the other hand, the designation of “endocrine” has often been generalized to such an extent that any apparent mode of action, even those that may be totally unrelated to the long-established “action at a distance” notion that underpins endocrinology as a field of research and medicine has been included as endocrine disruption rather than simply and more generally, developmental toxicology. Additionally, some use of nomenclature in this field suggests that any compound that affects any signaling biomolecule is “endocrine” and *de facto* is hazardous. It seems likely that some transient perturbations in signaling pathways may be inconsequential depending upon whether they occur and persist in a critical developmental window and whether or not there is an adverse impact on functional status especially later in life. It is our viewpoint that the field is now confounded by the way in which current nomenclature is used by both those who appear to hold entrenched positions in this controversy as well as the many objective open-minded scientists and physicians who continue basic, epidemiologic and clinical research. Accordingly, we propose that the study of developmental effects of hormone and hormone-like compounds should be seen as a subtopic encompassed within the broader field of ligand mediated pharmacology which we call “ligand-mediated developmental toxicology.”

4.10 Nutrition and Functional Foods: Intentional Exposures to Dietary Ligands

In our present effort to consider both the health risks and benefits of exposures to exogenous ligands, the dynamic field of functional foods research appears to be carrying nutritional research into the realm of therapeutics by assessing intentional exposures to enhanced levels of dietary ligands with the anticipation of health benefits (Martirosyan and Liu 2014; Martirosyan 2014). A number of definitions of

“functional food” have been advanced and debated by investigators, institutions and regulatory bodies in several countries. Functional food development is driven by efforts to prevent, mitigate or treat various diseases and is based on anticipated actions of bioactive compounds (predominantly ligands). Widely-recognized examples of health benefits of bioactive compounds include the following:

- Lutein and zeaxanthin for cataract prevention and for macular degeneration
- Beta-carotene and lycopene for skin protection from ultraviolet radiation injury
- Lutein and lycopene for cardiovascular risk reduction
- Lycopene for prostate cancer risk reduction

Many more therapeutic uses are currently under active research and development (Martirosyan and Liu 2014).

Some modes of action of bioactive compounds in functional foods include activation of antioxidant defenses, modulation of signal transduction pathways, changes in cell survival-associated gene expression, changes in cellular proliferation and differentiation and enhancement of mitochondrial integrity and function (Martirosyan 2014).

Given the breadth and depth of knowledge that is being developed in the field of functional foods, we think that there is great potential for defining generally-regarded-as-safe (GRAS) interventions for mitigation of adverse developmental effects that would be attributable to ligand-mediated toxicant actions.

4.11 Illustrative Therapeutic Opportunities in Ligand-Mediated Toxicology

“Fetal therapy” is a treatment administered to the fetus or via the mother with a primary indication to improve perinatal or long-term outcomes for the fetus or newborn (Paulis et al. 2013). Due to concern about unknown consequences of fetal exposures, it is often presumed that no pharmaceutical interventions should be given to pregnant women for the purpose of enhancing lifelong well-being of the mother’s offspring. Besides immunizations, a few obstetrical therapies are clearly “fetal therapy” including care of maternal acute and chronic diseases as well as specific obstetrical conditions such as pre-eclampsia and gestational diabetes, and this includes administration of antenatal corticosteroids to enhance fetal lung maturity when premature delivery is anticipated. There are a few additional treatments that are often provided to pregnant women where a primary aim is to effect fetal benefit that would obviously or likely manifest later in the lifespan (Hughes et al. 2013a, b). What are the prospects for developing others to address other clinically important disorders that result from adverse effects of developmental exposures? We will consider two general cases where ligand mediated signaling mechanisms may present opportunities to impact developmentally-driven metabolic disorders in offspring, and one specific exposure as a “strawman” entity that may illustrate the prospect for pairing of exposure reduction with protective/mitigative intervention.

4.12 Childhood Effects of Maternal Metabolic Milieu on the Fetus

It is now well demonstrated that the maternal metabolic milieu in which the fetus develops has a profound effect on the subsequent risks of several childhood diseases (Hughes et al. 2013a) including obesity, hypertension, early onset of type II diabetes mellitus, cardiovascular disease and immune-mediated disorders such as asthma. A common theme and potential organizing principle is that activated inflammatory pathways during fetal life seem central in mediating many of these adverse effects. Accordingly, signaling pathways that may modify inflammation are worthy candidates for investigation as potentially protective interventions. One such prospect is liver X Receptor α , a nuclear transcription factor that regulates lipid metabolism. Recently, it has been shown that activation of LXR α with synthetic ligands has anti-inflammatory effects in atherosclerosis and chemical-induced dermatitis.

Obesity is a worldwide problem and its prevalence is increasing rapidly (Danaei et al. 2011). It is caused by the storage of excess calories as triglycerides in adipose tissue (Popovich et al. 1997), which is associated with insulin resistance; type 2 diabetes, hypertension, hyperlipidemia, cardiovascular disease, stroke and non-alcoholic steatohepatitis (Saltiel et al. 2001; Visscher and Seidell 2001). LXRs are potential drug targets for obesity, dyslipidemia and atherosclerosis. Earlier studies have shown that the synthetic LXR agonist GW3965 lowers cholesterol levels in both serum and the liver, inhibits the development of atherosclerosis in mouse models (Joseph et al. 2002; Kratzer et al. 2009), and improves glucose tolerance in diet-induced obesity and insulin resistant mice by regulating genes involved in glucose metabolism in the liver and the adipose tissue (Lafitte et al. 2003). On the other hand, LXR antagonists, such as 5 α , 6 α -epoxycholesterol-3-sulfate, block the formation of plaques of atherosclerosis by inhibiting LXR function (Song et al. 2001).

Remarkably the liver-X-receptors have shown anti-inflammatory ability in several animal models of respiratory disease. The effect of LXR agonist in allergen-induced airway remodeling in mice was studied by Shi et al. 2014. Ovalbumin-sensitized mice were chronically challenged with aerosolized ovalbumin for 8 weeks. The LXR agonist failed to attenuate the inflammatory cells and Th2 cytokines in bronchoalveolar lavage fluid. But the application of LXR agonist reduced the thickness of airway smooth muscle and the collagen deposition. It is concluded that LXRs may attenuate the progressing of airway remodeling, providing a potential treatment of asthma (Shi et al. 2014). The effect of the LXR α agonist T0901317 on lung inflammation in a rodent model of hemorrhagic shock shows that it increased nuclear LXR α expression and DNA binding while also inhibiting activation of NF- κ B, a pro-inflammatory transcription factor, in the lung. A study suggests that LXR α is an important modulator of the inflammatory response and lung injury after severe hemorrhagic shock, likely through the inhibition of the NF- κ B pathway (Solan et al. 2011).

In summary, LXR-mediated signaling pathways illustrate the potential for identifying and developing therapies that could mitigate both metabolic and immunologic diseases of childhood.

4.13 Prospective Interventions for Free Radical-Mediated Pathways

Various antioxidants have been shown or presumed to have a wide range of health benefits but broad public health recommendations have often been confounded by contradictory results or unexpected outcomes in studies. Nonetheless we think that the biology of free radicals as mediators of injury remains critically important to understand and for development of protective interventions. We wish to illustrate such prospects by reference to current research with one natural product (kuding tea made from the leaf of Broadleaf Holly which belongs to the *Folium Llicis Latifoliae* family) and an established pharmaceutical drug (N-acetylcysteine).

Recently, several clinical studies have focused on kuding tea effects on lipid lowering, body weight reduction and blood glucose lowering in patients with metabolic syndrome. Animal studies have shown that the phenolic constituents and phenyl ethanoid glycosides of kuding tea exhibit significant antioxidant activities *in vitro* (Zhu et al. 2009). The extracts of kuding tea prevent the development of obesity, hyperlipidemia and glucose tolerance in high-fat diet-fed C57BL/6 mice, and inhibit the transactivities of LXR β (Fan et al. 2012). Kuding tea has both preventive and therapeutic roles in metabolic disorders in mice induced with high-fat diets (Fan et al. 2012). The effects appear to be mediated through the antagonism of LXR β transactivity. Kuding tea may be a useful dietary therapy and a potential source for the development of novel anti-obesity and lipid lowering drugs in humans.

N-acetylcysteine (NAC) is a low molecular weight compound administered to neutralize the deleterious effects of free radicals. NAC raises the intracellular concentration of cysteine and hence of reduced glutathione (GSH), which acts as an important endogenous antioxidant. Moreover, NAC has direct scavenging properties *in vitro* against hydroxyl radicals and hypochlorous acid (Brunet et al. 1995). NAC is rapidly absorbed following oral dosing and reaches its peak concentrations in the blood within 1 h. After passing through the intestine and liver, NAC is transformed into a variety of metabolites. Consequently, NAC has been shown to have multiple therapeutic benefits (Marthler and Keresztes 2004). NAC has been used in clinical practice since the 1960s when it was introduced as a mucolytic agent for the treatment of respiratory diseases, such as chronic bronchitis and cystic fibrosis. Since the late 1970s, NAC has been administered as an antidote for the therapy of acute acetaminophen intoxication (Van Shooten et al. 2002). During the last decade, numerous *in vitro* and *in vivo* studies have suggested that NAC has beneficial medicinal

properties including inhibition of carcinogenesis, tumorigenesis, and mutagenesis, as well as the inhibition of tumor growth and metastasis (Kumamoto et al. 2001). Currently, NAC is being studied for use in many disorders, such as chronic obstructive pulmonary disease (COPD), contrast-induced nephropathy, influenza, idiopathic pulmonary fibrosis and polycystic ovary syndrome (Millea 2009). Recent study showed that treatment with NAC may contribute to the restoration of non-enzymatic antioxidant reserves when administered to lead-exposed workers (Kasperczyk et al. 2014). When administered to workers chronically exposed to lead, NAC reduced lipid peroxidation in a dose-dependent manner. The simultaneously elevated concentrations of alpha-tocopherol may enhance the beneficial effects of NAC. However, the influence of NAC on the levels of uric acid, bilirubin, albumin and ferric reducing ability of plasma (FRAP) seems to be limited.

4.14 Fetal Alcohol Syndrome (FAS) and Fetal Alcohol Spectrum Disorder (FASD) as a Prototype “Endocrine Disruptor” Syndrome: Reduce Exposure and Mitigative Intervention

Alcohol (ethanol) is a well-known developmental toxicant that exerts its effects via mechanisms that fall within the definition of “endocrine disruptor.” The primary public health intervention to prevent FAS/FASD is and must be to avoid fetal exposure. Nevertheless, presently in the industrialized world, even in the socioeconomic “middle class,” 2.4–4.8 % of children are victims of FAS/FASD (May et al. 2014). This reality compels us to ask if there are mitigative therapies that could be given in pregnancy or after birth to minimize the adverse developmental effects or that could enhance or accelerate post-natal compensatory processes.

4.15 Antioxidant Therapy for Reduction of Oxidative Stress and Application of Modulators of Bromodomain Proteins to Alter Transcription Pattern for Reversing FAS/FASD

It has long been known that children exposed to alcohol *in utero* exhibit a range of abnormalities. The abnormalities include mental, cranofacial and immunological disorders. The severity of the symptoms relates to the amount, duration and timing (in fetal life) of alcohol consumption by the pregnant mother. A milder and lower level of exposure can lead to hyperactivity, anxiety and depression which will affect overall quality of future life. With severe exposures, deformities, mental retardation and impaired metabolic and immune function may be obvious (Momino et al. 2012).

Studies in animals also have established that fetal exposure with ethanol leads to fetal alcohol spectrum disorder (FASD). A number of mechanisms have been proposed to pinpoint how FASD occurs. Two proposed mechanisms are dysregulation of hypothalamic-pituitary-adrenal (HPA) axis function (Mead and Sarkar 2014) and epigenetic changes (Kleiber et al. 2014). Endocrine dysfunction relates to how HPA regulation is altered by alcohol by a loop mechanism (Mead and Sarkar 2014). Experimental studies with rats demonstrate that proopiomelanocortin (POMC) producing neurons which serves as precursor of a number of biologically active peptide were found to be affected (Boyadjieva et al. 2009; Hellemans et al. 2008; Sarkar et al. 2007). In rats, replacing β -endorphin-POMC producing cells has been shown to ameliorate alcohol-mediated effects. This observation suggests the need to measure the circulating levels of POMC in unexposed mothers who bear normal children. Consideration could then be given to whether rectification of possible FASD could be attained by use of recombinant POMC in exposed pregnant mothers to protect the fetus. In the present era of biotechnology, production of POMC for partial treatment and amelioration of FAS/FASD should face no major hurdle at a time when an ever expanding spectrum of biomolecules are being produced as therapeutic drugs (Smith et al. 2009). Finally, in any program to develop a fetal protective therapy, maternal genetic heterogeneity will likely be an important consideration since the number of pregnant mothers drinking alcohol is quite high compared to number of children who are diagnosed with FASD. Alcohol metabolism is linked to single nucleotide polymorphism of alcohol dehydrogenase (ADH) and that impacts alcohol metabolism.

Another mechanism for induction of FASD which is gaining support is the inheritable epigenetic changes to fetuses due to alcohol exposure through mothers. A number of processes involving DNA modification including methylation and acetylation as well as synthesis and secretion of micro RNAs (miRNA) have been shown to be affected by ethanol exposure. The genome is protected from changes in nucleotide sequence (mutations) by DNA repair enzymes to maintain integrity of the genome. Similarly, the epigenome is protected by bromodomain proteins (as mentioned earlier) which sense the changes in methylation and acetylation pattern (Sanchez et al. 2014). Methylation of CpG and CpG islands in promoters and histone acetylation regulate gene expression. Increased methylation of POMC promoter in fetal pups in rats and decreased acetylation have been observed with the promoter of POMC leading to decreased circulating levels (Govorko et al. 2012). This leads to a feedback effect on the hypothalamus-pituitary axis. An increase in POMC by feedback increases the POMC derived peptides, like endorphins and adrenocortical releasing hormone (ACTH) leading to increased cortisol, an indicator of stress. Experimentally, it has been demonstrated that effects of alcohol exposure to pregnant mothers is transmitted to the third generation. Modifying the activity of bromodomain proteins may be able to reverse alcohol-mediated epigenetic changes. In order to reverse alcohol-induced FASD, either the physiological level of POMC could be maintained by administration *per se* or the activity of bromodomain proteins could be modified to upregulate the synthesis and secretion of PMOC or a combination of both could be attempted. Currently, a number of

prospective agents which can modify the activity of bromodomain proteins are being developed and could be considered for use in clinical trials for mitigating occurrence of FASD (Sanchez et al. 2014).

Alcohol metabolism by Cytochrome P450 2E1 is induced in chronic alcoholics and produces superoxides and increases oxidative stress. In addition, alcohol metabolism by alcohol dehydrogenase (ADH) is decreased by fasting and malnutrition due to degradation of ADH and metabolism by CYP2E1, thereby increasing the oxidative stress in alcoholics and possibly among pregnant mothers who consume alcohol. As regards fetal effects, antioxidants are a good prospect for inhibiting some alcohol toxicity but it is unsettled as to which antioxidants may be most effective for this therapeutic application. Given the plausibility of benefit with likely safety, further research into use of antioxidants for FAS/FASD should be pursued in animal models and hopefully could advance into clinical trials. This general point-of-view has recently been robustly encouraged by a recent comprehensive and insightful review (Young et al. 2014). Young et al. detailed the range of metabolic and nutritional perturbations that appear to be involved in the occurrence of FASD. These investigators considered studies in animal models as well as human FASD-related research and propose that a modest set of nutritional supplementations including vitamin A, docosahexaenoic acid, folic acid, zinc, choline, vitamin E, and selenium could plausibly prevent or alleviate the development of FASD. These investigators (Young et al. 2014) emphasized that avoidance of exposure is the primary protective intervention; however, they also argued that further research should be conducted to assess the potential for reducing the occurrence or severity of FASD by determining optimized amounts of these nutrients to reduce specific detrimental outcomes of FASD and to define the most effective timing of such supplementation, e.g., first, second, third or all trimesters and/or into the early postnatal and childhood intervals.

Another class of biomolecules receiving great attention relative to FASD is micro RNAs (miRNA) as mentioned earlier. Changes in miRNA with FASD as related to neuronal development have been observed in rodents (Wang et al. 2009; Miranda 2012). It is possible that expression of miRNA could be altered via epigenetic genetic changes due to exposure to ethanol (Miranda 2012). Alteration in miRNA expression could significantly contribute towards FASD among children of women who abuse alcohol during pregnancy. Experimentally, alteration in miRNAs of miR-29c and miR-204 has been demonstrated in adult rat frontal cortex due to ethanol while changes in miR-29c and miR-30c were observed in fetal brains exposed through maternal ethanol. It is anticipated that these miRNAs can affect the HPA axis, thereby producing FASD. As aforementioned, miRNA based gene regulation is a recently discovered area, only a few agents have been developed for targeted therapy for controlling the activity of miRNA. Antagomirs are the chemicals which target miRNA and modify its activity. Only a few antagomirs have been developed and their safety for use in humans remains to be determined. There is a timely need for the development of agents which can modulate miRNAs involved in FAS/FASD.

4.16 Summary

If we set aside the design and testing of pharmaceutical compounds as exogenous chemical agents to intentionally influence mammalian physiology, there are three other long-established strands of biological research that have investigated the actions of biologically active dietary or environmental compounds on animals including humans.

First, in agricultural and animal husbandry research there is extensive documentation of the many thousands of phytochemicals that mediate plant animal interactions, many of which produce toxic effects at certain levels of exposure in terms of dose, duration and window of sensitivity. In a broader biological context, many of these relationships have been formalized by concepts of co-evolution of plants and animals.

Second, there is a long history of developmental toxicology research showing a wide range of adverse effects that often result from exposures to hormones and other modulators of endocrine function during developmentally sensitive intervals. In recent decades much of this research has been encapsulated by the single term of endocrine disruptors.

Third, nutritional and functional foods research have demonstrated a wide range of health benefits produced by consumption of many phytochemical ligands through mechanisms that are truly not distinct from those that seem to mediate the adverse effects noted in the animal husbandry and developmental toxicology research arenas.

Humans (and animals) live in a world of exposure to ligands. We are confident that environmental and public health can be enhanced by defining how such exposures to exogenous signaling molecules (ligands) pose both risks and benefits. It is timely to modify scientific perspectives and nomenclature to encompass these several streams of investigation such that risks and benefits of exposure to dietary and environmental ligands may be translated into validated strategies to improve environmental and public health. Health promoting interventional options must include exposure reduction(s) to ligands that incur health risks but we should also define and employ active mechanistically-based mitigative interventions when exposures cannot be adequately reduced or preempted.

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