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Where do we go from here: Challenges and the future of endocrine disrupting compound screening and testing

Running head: Future challenges for EDC screening and testing

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ABSTRACT

Worldwide concern about the impacts of endocrine disrupting compounds on both human and environmental health has led to implementation of multiple screening and testing programs. In most cases these programs have focused on impacts to the estrogen, androgen and thyroid hormone (EAT) signaling pathways. The goal of the presentations in session five of the Society of Environmental Toxicology and Chemistry (SETAC) North America Focused Topic Meeting: Endocrine Disruption (February 4 – 6, 2014) was to discuss moving beyond EAT pathways to address current challenges and identify future approaches for the expansion of screening and testing programs. The session was chaired by Drs. Gerald A. LeBlanc and Vickie S. Wilson and included five presentations. Dr. Gerald A. LeBlanc provided insight on non-EAT endocrine targets that are known to be susceptible to endocrine disrupting compounds. Dr. Seth Kullman gave an overview of emerging technologies that hold promise for the screening of chemicals for interaction with EAT and other endocrine pathways. These were followed by two presentations on the current status and future promise of computational (Dr. Kevin Crofton) and *in silico* (Dr. Patricia Schmieder) approaches for screening and ranking chemicals for endocrine activity. Dr. Miriam Jacobs culminated the session with an overview of the current understanding of the role of epigenetics in endocrine regulation and approaches for evaluating chemicals for their ability to disrupt the epigenetic regulation of endocrine processes.

Key words: Endocrine disruption, endocrine targets, EDC screening, in silico, epigenetics

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INTRODUCTION

The ability of some exogenous chemicals to disrupt normal endocrine function has been well demonstrated. In response, significant efforts are underway to develop screening and testing strategies aimed at detecting and characterizing hazard resulting from exposure to endocrine disrupting chemicals (EDCs). This effort is not trivial. The endocrine system consists of an extensive network of signaling pathways with hundreds of potential targets of disruption. Individual pathways within the network have evolved to respond to different chemical regulators (hormones) and, accordingly, have diverse susceptibilities to exogenous chemicals. Species differences in hormones and their receptors render interspecies extrapolations of chemical susceptibilities challenging. Nonetheless, the endocrine system regulates diverse processes that are critical to an individual's health and population sustainability (*e.g.*, development, growth, metabolism, reproduction). Approaches and assays are urgently needed to screen and test chemicals for such activities.

Efforts, to date, have largely focused upon three pathways within the endocrine network: estrogen, androgen, and thyroid hormone (EAT) signaling with the utilization of a relatively limited repertoire of assays and endpoints for each pathway. The goal of session five of the Society of Environmental Toxicology and Chemistry (SETAC) North America Focused Topic Meeting: Endocrine Disruption (February 4 – 6, 2014) was to chart a course for the sagacious expansion of screening and testing efforts. The session was chaired by Drs. Gerald A. LeBlanc and Vickie S. Wilson. Novel endocrine pathways, with known susceptibility of disruption by exogenous chemicals were introduced by Dr. Gerald A. LeBlanc. Dr. Seth Kullman complemented this presentation with a discussion of emerging technologies that hold promise for the screening of chemicals for endocrine disrupting activity. Drs. Kevin Crofton and Patricia

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Schmieder provided updates on advances in the use of computational and *in silico* approaches for the screening and ranking of chemicals for endocrine disrupting activity. Lastly, Dr. Miriam Jacobs provided an overview of epigenetic regulation of gene expression, the involvement of epigenetics in endocrine regulation, the potential susceptibility of epigenetic mechanisms to chemical interference, and assay approaches that could be applied to the screening and testing of chemicals for epigenetic interactions.

This session, ideally, will stimulate discussion for a more inclusive EDC screening and testing paradigm, utilizing technologies for the rapid and effective screening of chemicals, that will prove more efficacious in identifying chemicals that can disrupt endocrine function at relevant exposure levels and preventing such exposures from occurring.

SESSION PRESENTATION SUMMARIES

Emerging Targets of Endocrine Disruption: The Xenocrine System, by: Gerald A. LeBlanc.

The 2012 Organization for Economic Co-Operation and Development (OECD) monograph entitled *State of the Science on Novel In Vitro and In Vivo Screening and Testing Methods and Endpoints for Evaluating Endocrine Disruptors* (OECD 2012) describes results of the formative task of identifying endocrine signaling processes that have known susceptibility to disruption and assays for the evaluation of such disruption. Among the pathways recommended for future priority consideration were the peroxisome proliferator activated receptor (PPAR), vitamin D receptor (VDR), and the retinoic acid receptor (RAR) signaling pathways. Noteworthy, is that none of these pathways utilize a classical hormone. Rather, activating ligands tend to be of exogenous origin related to diet (i.e., PPAR – dietary fatty acids, VDR–

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dietary vitamin D, RAR - dietary vitamin A). Furthermore, the three signaling pathways utilize a common receptor, the retinoid X receptor (RXR), to partner with the primary receptor to form an active dimeric transcription factor. RXR also can serve as an independent, ligand-activated transcription factor.

The existence of a common ligand-binding component (RXR) to the three pathways provides a scenario for possible inter-pathway interactions (Fig. 1). For example, a ligand to RXR may simultaneously activate one signaling pathway, suppress another, and have no effect on the third. Little is currently known of the toxicological consequences of such ligand-mediated inter-pathway interactions. However, it is highly likely that RXR-binding xenobiotics would elicit multiple effects by impacting the signaling of multiple RXR-dependent pathways. For example, tributyltin is a potent RXR agonist and is known to elicit diverse effects spanning reproductive and developmental abnormalities, immunosuppression, and adipogenesis.

The provision of two distinct ligand binding sites, one on each receptor protein subunit, within the receptor complex provides the opportunity for intra-pathway interactions between ligand to the primary receptor and ligand to the RXR (Fig. 1). For example, the PPAR ligand clofibrate can independently activate the PPAR:RXR receptor complex, as can the RXR ligand 9-*cis* retinoic acid. In combination, these ligands can synergistically activate the receptor complex. Such intra-pathway interactions provide a mechanism by which exposure to chemical combinations can result in greater cellular responses than would be predicted by exposure to the individual chemicals.

This signaling network, the "xenocrine system", regulates various aspects of metabolism and development. Disruptions in the network can have implications on several endemic disease conditions in human populations (*e.g.*, obesity, diabetes, metabolic syndrome) and energy-

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dependent processes in wildlife populations (*e.g.*, growth, development, and reproduction). Potential disruptors of this network include chemicals that function as agonists/antagonists of the primary receptors, agonists/antagonists of the RXR, modulators of multi-functional co-activators that contribute to receptor activity, and modulators of intracellular receptor levels.

Emerging Technologies to Assess Endocrine Disruption: Overview of New Assays and Approaches That Show Promise in Evaluating the Endocrine Activity of Chemicals, by: Seth Kullman

This presentation examined novel technologies and approaches to assess endocrine active chemicals. Specifically, this presentation related new advances in EDC function beyond standard EAT pathways that are well represented within the EPA/OECD endocrine screening programs. The foundation of the discussion was based upon a recent OECD detailed review paper (DRP) *State of the Science on Novel In Vitro and In Vivo Screening and Testing Methods and Endpoints for Evaluating Endocrine Disruptors* (OECD 2012). A short synopsis of the detailed review document was provided highlighting DRP content and focus: 1) categorizing the DRP as a literature-based document designed to identify assays/methods to assess for EDC activity and function with an emphasis on non-EAT pathways, 2) demonstrating both *in vitro* and *in vivo* assay that are sufficiently developed for standardization (not validated), 3) illustrating the importance of neuro-endocrine axis and the key role of nuclear receptor binding and transactivation as primary molecular initiating events and 4) emphasizing the value of presenting pathways in the context of adverse outcome pathways that link molecular initiating events with apical outcomes.

The thyroid hormone (TH) signaling pathway was presented as an example to illustrate

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hormone axis complexity and putative sites of action for TH disruptors. Specific mechanisms of chemical action were discussed including disruption in thyrotropin-releasing hormone and thyroid-stimulating hormone synthesis and signaling, inhibition of iodine uptake into the thyrocyte, synthesis of T4/T3 by thyroperoxidases, modification in hormone transport-blood binding proteins, hepatic metabolism of T4/T3, disruption of deiodinase, alteration in cellular uptake/excretion of thyroid hormones, and direct interaction of compounds with the TH and RXR receptors as direct agonists or antagonists. Specific examples detailed novel assays to assess chemical-target interactions and activities. Further attention was given to notable assays developed to assess molecular initiating events associated with thyroid receptor (TR) transactivation/repression including TR expression, protein-protein interactions between TR and the nuclear receptor co-regulators (co-repressors, co-activators), TR-DNA binding, TR agonists and antagonists, and heterodimerization of TR with RXR. To further detail specific mechanistic inferences, TR-DNA binding interaction studies were presented as an example highlighting novel assays to assess chemicals that may disrupt fundamental nuclear receptor (NR) function. Results from two publications were discussed (Ibhazehiebo et al., 2011, Iwasaki et al., 2008) demonstrating the utility of a NR:HRE association/dissociation assay to inform NR-DNA interactions. Results of assay performance were described in context of specific chemical agents that disrupt TR signaling through this mechanism.

Select assays from the literature were collated to provide a larger context and to illustrate their utility in establishing an adverse outcome pathway (AOP) for thyroid disrupting compounds and how linking exposure impacts and molecular initiating events to biological outcomes at higher levels of biological organization could inform disruption of the thyroid axis. Several assays were discussed in context of their potential to inform macro-molecular interactions,

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cellular responses, tissue/organ level responses or organism level responses. Discussion emphasized the ability of AOP's to effectively translate and link mechanistic information at the molecular level (*i.e*. chemical target) to meaningful apical endpoints at the organismic level such as neurobehavioral function.

Application of high throughput screening (HTS) technologies was additionally discussed in context of how current HTS programs including ToxCast and Tox21 could inform endocrine disruption. Multiple *in vitro* cell-based assays were highlighted with a focus on nuclear receptor transactivation including the *Attagene* Factorial Trans reporter assay, *NCGC* β-lactamase reporter gene assay and competitive receptor binding assays. Attention was also drawn to the need to incorporate *in vivo* translational models for validation of cell-based HTS assays. Several novel assays exploiting the use of endocrine sensitive transgenic reporter lines in zebrafish and medaka were demonstrated. These systems emphasized the utility of incorporating comparative *in vivo* approaches that leverage the advantages of alternative models in combination with emerging high-throughput and computational technologies that may facilitate extrapolation of chemical action to human toxicity studies.

The conclusion reiterated the importance of establishing and validating assays that inform EDC function and activity beyond EAT. Emphasis was placed on the ability to incorporate assays into defined adverse outcome pathways with the ability to identify novel molecular initiating events and link these activities to apical endpoints. The importance of integrating high throughput assays informative of EDC function was also discussed in context of developing a global assessment of endocrine active compounds. And finally, the necessity to utilize translational *in vivo* models was also highlighted to better establish means to extrapolate cell-based data to human toxicity.

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Using High-Throughput Methods to Conduct Risk-based Prioritization of Chemicals for the EDSP, by: Kevin Crofton

A major challenge for regulatory authorities is assessing risk for the large universe of untested chemicals for which there is human or ecological exposure. This chemical universe has been estimated to be 30K-50K unique substances. In particular, the U. S. EPA Endocrine Disruption Screening Program (EDSP) is required to test 5K-10K chemicals due to pesticidal use or their potential to contaminate drinking water. The U.S. EPA Computational Toxicology program is developing methods and models to prioritize this large chemical universe for further testing based on estimation of risk. The prioritization framework includes consideration of hazard, exposure and dosimetry. Hazard estimation combines *in vitro* high-throughput screening (HTS) assays plus QSAR and docking models. The U. S. EPA's ToxCast program, together with the U.S. Interagency Tox21 program, has generated data for over 8,500 chemicals using a variety of HTS assays for endocrine activity (*e.g.* ER, AR, TR). Additionally, a large-scale multinational effort is developing and evaluating QSAR and docking models to use and extend the HTS data, initially for the estrogen receptor. Results of this modeling effort will, in turn, drive further *in vitro* testing. U. S. EPA's ExpoCast program is developing quantitative exposure prediction models based on chemical properties and use patterns. These models allow rapid estimates of exposure potential for thousands of chemicals. Finally, for dosimetry, we are using a combined *in vitro* and modeling approach (called Reverse Toxicokinetics (RTK)) to provide quantitative estimates of concentration-to-dose scaling. By combining quantitative *in vitro* potency estimates from ToxCast, concentration-to-dose scaling from RTK, quantitative exposure values from ExpoCast, and estimates of uncertainty, we can provide quantitative risk metrics at

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the pathway level (estrogen, androgen, thyroid) for hundreds to thousands of chemicals. The first use of these estimates will be in prioritizing chemicals for inclusion in the EDSP Tier 1 assay battery.

Developing In Silico Approaches to Chemical Prioritization for ED Testing Within an AOP Context, by: Patricia Schmieder

There are several key things the U.S. EPA must consider in implementing the Endocrine Disruptor Screening Program (EDSP), particularly with regard to the use of predictive tools to wisely screen chemicals and identify which are most likely to initiate toxicity due to interference with endocrine pathways. The presentation, summarized herein, described important aspects of building a predictive tool specifically for prioritization of the EPA's EDSP chemical universe of ~10,000 chemicals (US EPA 2012*).* Key considerations in tool development are clarity of the programmatic application of the tool; (*i.e*., to determine the order in which EDSP universe chemicals are to be screened) and defining the domain of application where the regulatory decisions are needed so that the tool is developed to cover those types of chemicals. The OECD principles for QSAR validation serve as a guide to ensuring scientific soundness, transparency and maximum utility of the tool (US EPA 2012, OECD 2007). An estrogen receptor expert system (ERES) developed for ED chemical prioritization following these guidelines was presented to an EPA Scientific Advisory Panel (SAP) in 2009 (US EPA, 2009*)* and again in 2013 (US EPA 2013a) as it was expanded to cover the EDSP chemical universe. The ERES was developed in the context of an ER-mediated adverse outcome pathway (AOP) (Hornung et al 2014, Schmieder et. al 2004, Schmieder et. al 2014, US EPA 2009, and US EPA 2013a) using *in vitro* assays at the molecular and tissue levels of biological organization (see Figure 1 in

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Schmieder et. al 2004). The *in vitro* assays used to develop the ERES were specifically optimized to identify the types of low affinity binding that are characteristic of EDSP chemicals, whereas off-the-shelf assays developed for different types of chemicals and a different purpose were found to be limited (Schmeider 2004, US EPA 2013a, US EPA 2013b).

An extensive database was developed using the *in vitro* rainbow trout ER binding and liver slice gene activation assays (Hornung et al 2014, Schmeider 2004) which are considered the gold standard in detecting ER activation for this AOP (US EPA 2013b). Once the chemical universe for tool application was defined, a systematic process was used to develop the database by testing the boundaries of where chemical-ER binding occurs and where there is no binding. By striving for a mechanistic understanding of ER binding grounded in theory and confirmed in practice, it was possible to define seven major 'effects-based chemical categories' (Figure 2; I to VII) where the effect in question is binding to the ER and potentially initiating an ER-mediated adverse outcome pathway (Hornung et al 2014, Schmieder et. al 2004, Schmieder et. al 2014, US EPA 2009, US EPA 2013a). The coding of logic rules in a decision tree was automated into an ERES that not only provides a prediction of ER binding potential but also allows the user to examine the empirical evidence within an effects-based chemical category to which an untested chemical is assigned based upon its similar chemical features and properties pertinent to ER binding that the untested chemical shares with the training set chemicals (US EPA 2013a, US EPA 2013b). Applying the ERES to the initial chemical inventories it was built to prioritize resulted in only ~5% of the chemicals being flagged as potential ER binders (US EPA 2009). The expansion of the ERES to cover the discrete chemicals in the more recently defined EDSP universe (OECD 2007) was demonstrated for the 2013 SAP review (US EPA 2013a) where again only 5-10% of the universe is prioritized for further examination based upon potential to

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initiate an ER-mediated AOP. The approach, found to be transparent, scientifically sound, and relevant to EPA needs by SAP panels (US EPA 2009, US EPA 2013a) and an OECD expert consultation (OECD, 2009) is equally applicable to building prioritization tools for additional ED pathways (US EPA 2013b).

Endocrine Disruptors and the Epigenome: Potential Regulatory Applications for Chemical Safety Testing, by: Miriam N. Jacobs

Epigenetic modulations underlie critical developmental processes and contribute to determining adult phenotype. Alterations to the phenotype, due to exposure to environmental insults during sensitive periods of development, are mediated through alterations in epigenetic programming in affected tissues. This presentation evaluated the potential role of chemical-induced epigenetic modifications to endocrine signaling pathways during sensitive windows of exposure as a mechanism of endocrine disruption, and examined potential methods for assessing such disruption, building upon the Annex to OECD 2012 and Greally and Jacob (2013) including table references therein. Potential targets of disruption along putative AOPs associated with the signaling pathways were identified as were assays that showed promise in evaluating the target in a screening and testing program. *In vitro* methods are used where possible, and animal experiments are used only when *in vitro* methods were inadequate. Monitoring such epigenetic changes in response to toxicant exposure may provide a valuable tool for predicting adverse later life outcomes. Indeed, preliminary work in which embryonic stem cells (ESCs) have been differentiated into primordial germ cells (PGCs) in the presence of 17-beta estradiol and dibutyl phthalate suggests that altered microRNA expression centered around PPAR alpha and the reproductive system may be involved in such chemically-induced epigenetic toxicity. Unlimited

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stocks of clonal PGCs can be differentiated from ESCs in the presence of environmental chemicals. These clones can be used to investigate mechanisms of chemically-induced epigenetic changes that may lead to altered phenotypes in unexposed future generations. MicroRNAs are important epigenetic regulators that might mediate the adverse effects of environmental chemicals across multiple generations and the in vitro PGC system is a valuable tool for investigating such mechanisms of multigenerational epigenetic inheritance.

A more robust basis for Test Guideline (TG) recommendations, however, is still needed. Although there is evidence to suggest that epigenomic dysregulation might mediate effects of exposures to endocrine disruptors, it is uncertain as to whether these changes are truly predictive of adverse outcome(s). Adverse effects observed in the OECD transgenerational assays could be used to inform future tests specifically designed to investigate the epigenetic mechanism of action. Collection and identification of RNA/DNA for genomic and epigenetic analysis from the higher level tests would assist in the mechanistic understanding of the epigenetic contributions to an adverse outcome pathway as well as making better use of the animal tissues. Follow-up studies should include both an epigenetic as well as a genomic component to differentiate between the contributions of potentially compensatory mechanisms.

Other particularly pertinent test method models to develop include the zebrafish. The use of zebrafish for studies of reproductive and developmental screening of endocrine active substances is now well-established. Zebrafish appear to represent a suitable model organism available for studies replicating the effects of endocrine active substances and endocrine disruptors on vertebrates and aquatic wildlife. This model also has great potential for rapid, reliable, and less expensive exploration of the role of epigenetics, aging, senescence, and cancer outcomes in relation to endocrine endpoints. This area is increasingly important to address in

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293 regulatory toxicology, but the current higher level (level 5) *in vivo* TGs are unable to address it
294 for reasons of high cost, extended time, and the humane concerns related to the extension of such
295 tests.

296 There are clearly well supported reasons for further development of the zebrafish model
297 which has already been successfully applied by many laboratories. It could be further developed
298 to specifically address epigenetic endpoints in relation to endocrine activity and phenotypic
299 consequences in the model, to assess the quantitative and predictive capabilities for later adverse
300 outcomes. It might also be a useful model to assist in the discussion on the treatment of
301 functional genomics in TGs. A better understanding of the mechanisms and consequences of
302 epi-mutations is vital for assessing the risk of environmental human exposures.

303 **CONCLUSION**

304 In summary, the topics presented during session five of the SETAC Focus Topic
305 Meeting: Endocrine Disruption highlighted important factors to consider as we move forward
306 with the screening and testing of endocrine disrupting compounds. While the traditional focus of
307 these screens has been on classic EAT pathways, it is clear that other endocrine signaling
308 processes are susceptible to disruption. Utilization of assays that address such disruption may be
309 relevant in understanding xenobiotic contributions to adverse conditions in human and wildlife
310 populations. Furthermore, the inclusion of novel assays that provide additional information on
311 the EAT pathways should be considered, such as those that disrupt nuclear receptor function.
312 Other assays that can evaluate the effects of chemicals on the epigenome, particularly those
313 involving zebrafish, should also be considered as the role of epigenetic modulations becomes an
314 area of increasing importance. In addition, modifications to existing assays to include genomic-

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and epigenetic-relevant endpoints can further expand the information that can be obtained from animal tissues used in higher level tests.

As the number of suggested screening assays increases to address these new potential targets, the need to devise rapid screening methods becomes increasingly important. Thoughtful and strategic utilization of high throughput screening assays in conjunction with alternative models and computational technologies could add significant value to the overall effectiveness of this approach and provide a method for rapidly screening hundreds to thousands of chemicals; and framing these data within the context of adverse outcome pathways will provide the most effective approach in the prioritization of chemicals. Therefore, the utilization of transparent, scientifically sound, and relevant tools to assess these pathways will be key as we move forward in this effort.

ACKNOWLEDGMENTS

The authors would like to thank the following collaborators: John M Greally from Albert Einstein College of Medicine and Emma L Marczylo from Public Health England. The authors would also like to thank Drs Justin Conley and Mary Ann Ottinger for providing technical reviews of this manuscript

Disclaimer: This manuscript has been reviewed in accordance with the policy of the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views or policy of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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Figure 1. Pleotropic consequences of RXR ligand binding. Xenobiotic ligand binding (triangle) to the RXR subunit may stimulate differential dimerization with partner receptor subunits (**A)** resulting in the differential activation of signaling pathways. In addition, combinations of RXR ligands with partner receptor subunit ligands (circles) can result in multiple intra-pathway regulatory activities (**B**).

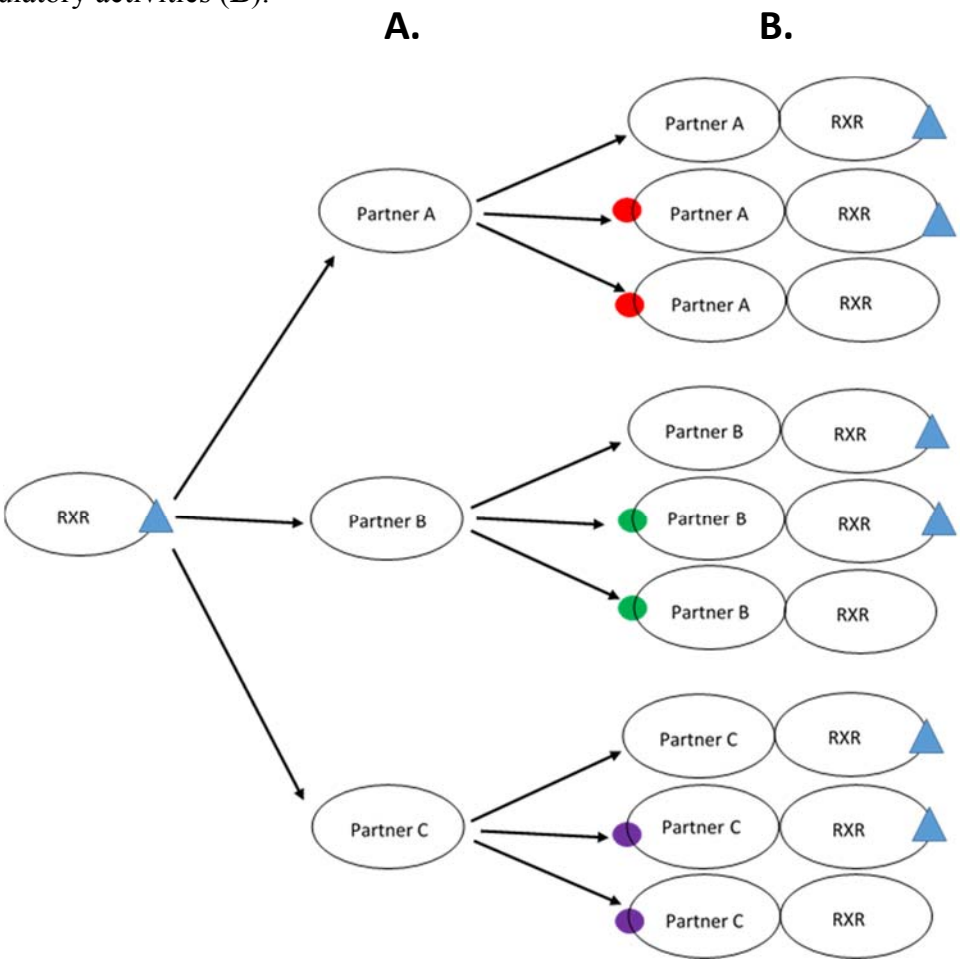

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Figure 2. The rule-based ERESv1 for predicting binding potential for low affinity chemicals to the rtER. The decision tree contains seven major nodes and multiple effects-based chemical categories within the nodes. The ERES is built on rainbow trout ER binding data using the cyto rtERαß in combination with rainbow trout tissue slice gene activation data.

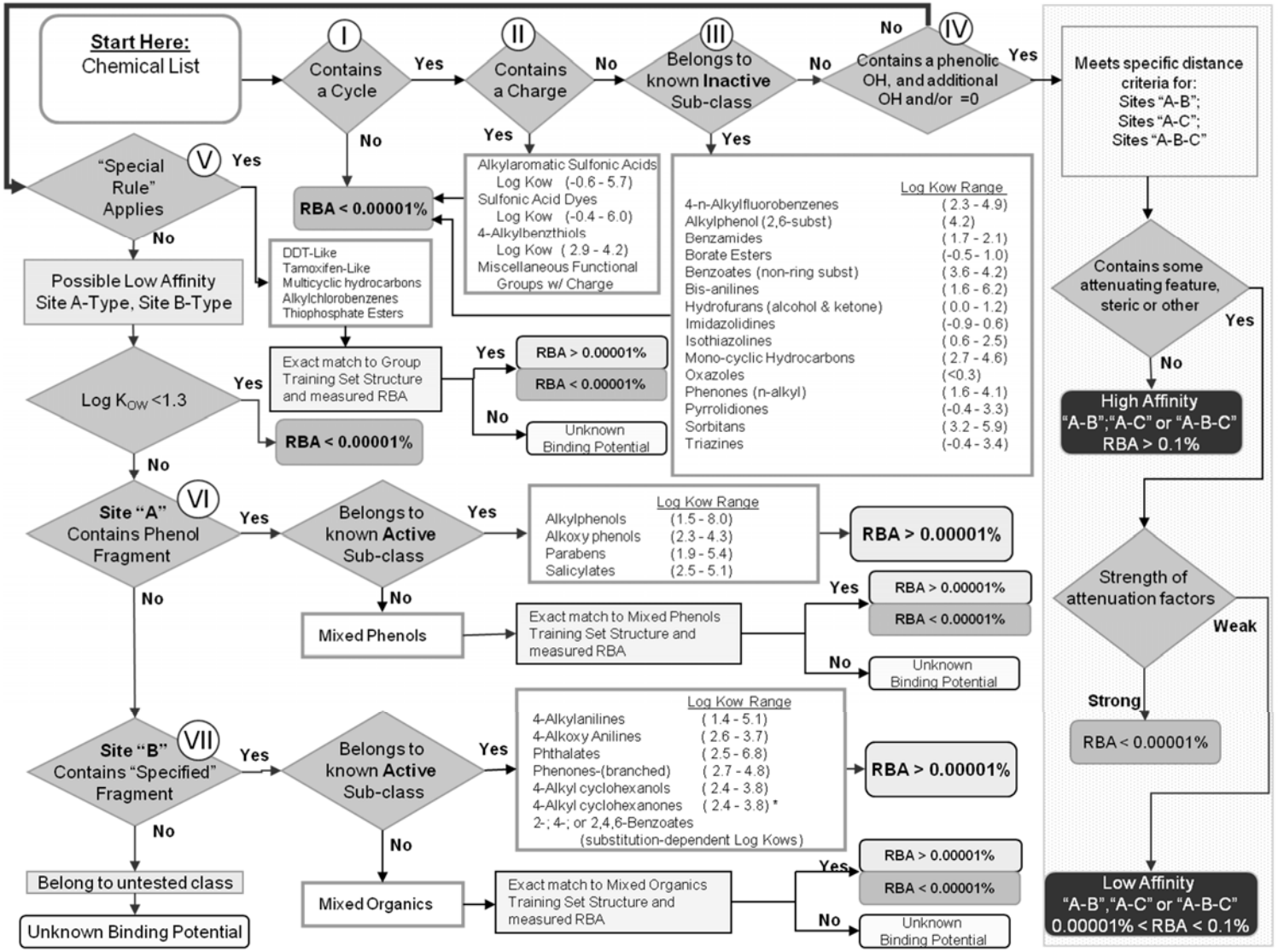

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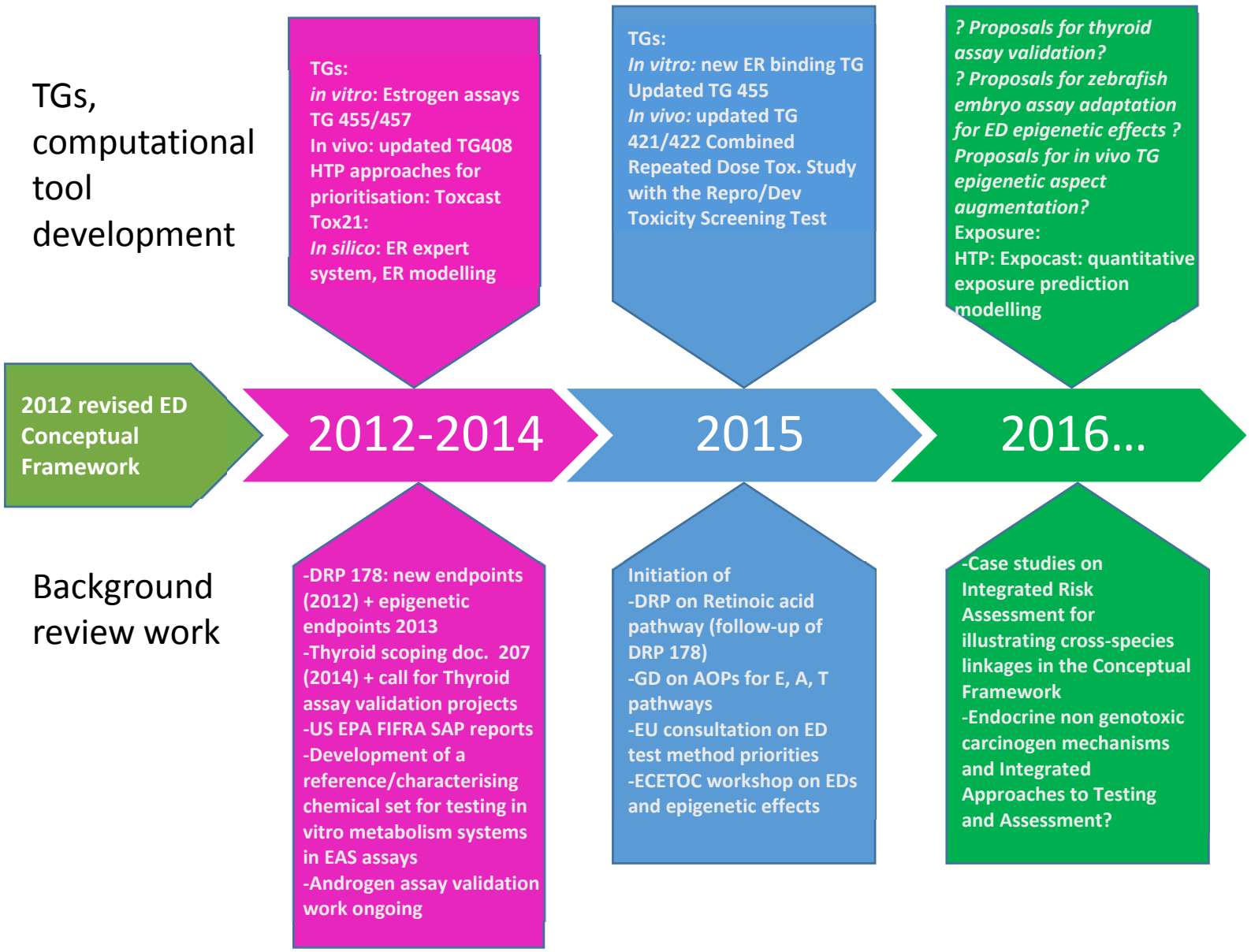
Figure 3. Current OECD (and related stakeholder activities) Workplan Timeline for Future of Endocrine Disruptor Screening and Testing



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