International Research Journal of Natural and Applied Sciences
ISSN: (2349-4077)
Impact Factor 5.46 Volume 8, Issue 2, February 2021
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Green Chemistry Different Approach &Fundamental Goals

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Introduction :

As noted by Anastas and Warner, most efforts at roducing risk so human health tom chemicals have focused on reducing the probability and magnitude of expoures. That approach works, until it fails. Failure, unfortunately, is virtually inevitable, because of accidents and practices not part of the intended use of a product. There are a multitude of examples of unintended exposures including socidents like the accidental release of methyl isocyanate gas at Bhopal, BP's Deepwater Horizon oil spill, the recycling of electronic waste by children in China and India, and household dust in Califomis containing flame retardance.

Green chemistry takes a different approach. One of its funda mental goals is to synthesize chemicals that are not hazardous for human health and the environment. To achieve this goal efficiently, chemists must be able to assess potential hazards of the chemicals that they develop.

We use the word "hazard' deliberately "hazand is embedded in groen chemistry as one of the two determining elements of risk. It is commonly accepted that risk is a function of inherent. hazard and exposure. Green chemistry deals with risk by seeking to eliminate inherent hazard rather than by controlling exposure. Ideally this assessment would take place as carly in the design process as feasible so that decisions can be made whether to pursue further development. If a hazard is identified, the chemist can opt either to cease development of that chemical or to manipulate the molecular structure to design against harand

In an ideal world, it would be possible to predict with confi- dence the potential toxicity of new molecules based on their aructure and physical characteristics. Well-known weaknesses in these approaches, however (note for example the Structure Activity Relationship Panados' discussed below), render this approach not just inadequate, but potentially misleading. In this endeavor, such potential for false positives and false negatives is unacceptable. Actual biological experiments are therefore necessary

Because chemists typically are not trained in toxicology or other relevant fields, developing the means to achieve this goal requires collaboration between environmental health scientists and green chemists. This collaboration, systematically applied and constantly adjusted to reflect new scientific discoveries, would help lead to a new generation of inherently safer chemicals.

In this paper we explore how chemists can apply principles and tests from the environmental health sciences to identity potential endocrine disruptors. Specifically, we propose a five- tiered testing protocol, TiPED We begin with computational approaches as the fastest and the least expensive asays. Sub- sequent tiers involve increasingly specialized

tests to determine the potential for endocrine disrupting characteristics of a chemi- cal under development. Some of the assays are based on known mechanisms of action; some are designed to catch disruptions for which the mechanisms or receptors are as yet unknown. We present the overall structure of the protocol with assay examples that could be used in each tier.

We noted above that actual biological experiments are necessary for predicting toxicity. This is especially the case with endocrine disruption because of the complex signaling eventsGreen Chemthat control endocrine activity within and between cells, tiss and organs. We discuss this issae in greater detail as we discuss the strengths and weaknesses of our different tiers.

We present the tiers in a logical sequence for a chemist design ing a new chemical from the simplest approach (and least expensive through the more complex (and often more expen sive). We recognize, however, that different users will have different needs. A user can start anywhere in the system, not necessarily with Tier 1. An academic research chemist drawing a molecule de nove will have different issues and questions than an industrial chemist with a molecule already in hand; the former would be more likely go through the protocol in a linear progression. The latter might use assays in a later tier to get a quick read on the likelihood of potential problems. Some users may want a straight "harm no likely harm" answer, abandoning failed molecules rather than developing them into products Others, after getting a "harm" realt, might pursue a series of increasingly specific assays to identity mechanisms of biological action so that they might redesign the product To iterate, though presented here in a linear fashion for the sake of new chemical design, other users can enter the system where it best meets their needs.

To support the tiered assay system, we also identify a suite of principles that should be used to guide implementation (dis cussed after summary of TIPED). These principles focus both on general concems about toxicity testing as well as unique charac teristics of endocrine disrupting compounds (EDC) that makes their detection particularly challenging

At this stage. TiPED is a scientific framework in progress This paper presents the overall strategy, its scientific rationale and the principles that govem its design and implementation The formal protocol itself will be presented on the Til'ED website (www.TIPEDinfo.com). The website will undergo formal peer review and invite constant input from EDC specialists and chemists who use it.

Scientific understanding of endocrine disruption is advancing rapidly New mechanisms of endocrine disruption, new targets for EDC action and new ways to measure the effects of EDCs are being reported regularly. Any effective testing protocol must evolve as new scientific discoveries are reported. The guiding principles behind this testing protocol, however, remain constant.

We choose to focus on endocrine disruption for three season First, the body of evidence that has emerged from the past 20years of research on this class of mechanisms has grown, indicat ing it is a serious public health issue. Second, it is clear that the current pondigm focused on exposure, instead of hazard, has failed to protect public health tim endocrine disruption Measurements by the US. Centers for Disease Control and Pre vention document widespread exposure to multiple EDCs at levels that current scientific research suggests may not be safe Third, despite a 1996 Congressional mandate to develop toxicity assays for EDCs, the United States Environmental Protection Agency (US. EPA.) has made little progress in implementing the use of EDC assays in the regulatory process. With this focus in mind, we invited leading experts in endocrine disruption science to collaborate with leading green chemists to develop a testing protocol that could be used by chemists as a voluntary not

regulatory design tool (Table $11). This allowed us to focus on scientific issues, rather than regulatory debates.

1. What is endocrine disruption ?

The endocrine system uses chemical signals bonnes-to direct development and reproduction, regulate body function and metabolam, and influence behavior and immunity In its binad est sense, endocrine disruption takes place when an agent alters hormone signaling or the response to hormone signaling, and in se doing alters some aspect of the organisen under hormonal conmol. According to the Endocrine Society, the world's authori tative scientific association of clinical and nach endocrinolo gists, an endocrine-disrupting chemical (EDC) is an exogenous chemical, or mixture of chemicals, that can interfere with any aspect of homoor action.

Endocrine distaption can be caused by diverse mechanisms Homones work by hinding with protein receptors in the cell membne, the cytoplasm or the muclous. Binding initiates gene activity or physiological processes (depending upon the receptor, in location, hormone concentration, and the developmental state of the cell tissue/organisms) that are part of and sential to normal organismal function EDCs work by interfering with that sipating process. They are not necessarily stractually similar to hormones; many, but not all, are lipophilic

Mechanisms of action include the EDC binds to the receptor and adds to the normal signal; the EDC binds to the receptor and blocks the normal signal, the EDC affects hormone synthesi (increasing or decreasing the amusent of natural hormone that is available for signaling). the EDC alters hormone metabolism or hormone munsport and storage within bodily time again, increasing or decreasing hormone amount and/or the EDC affects the levels of mature hormone receptor via disruption or modulation of gene expression, folding, or transport.

A central part of the phenomenon of endocrine disruption is receptor hinding, which depends upon the molecular confor mation of the hormone and its recepturs, Molecular structure is a good, but imperfect predictor of whether hinding will occur chemists can use information about structure both to predict potential hand described below) as well as to guide manipu lation of a chemical's structure to avond harand

A crucial aspect of hormone action is that it takes place a extremely low concentrations For an estreges, for example, typical physical levels of the biologically-active form of an estrigen are extremely low, in the range of 10-900 pg ml (high parts per quadrillion to low parts per trillions. This is possi ble became of the specificity of hormone binding to its receptar, and is biologically necessary because of the large number of sig naling molecules present at any one time. Specificity and exte sitivity make it possible for an enormous number of signaling molecules to co-exist in circulation without disrupt ing each ther's signaling. The specificity also evolved, presun ably, to reduce ur avoid disruption by exogenous compounds with which orgiiems have had evolutionary experience.

Within the past century, over 80 000 new chemicals have bees synthesized and used in ways that have rosulted in widespread human expoures A sahset of these chemicals are tosia of these toxic chemicals are toxic due to endocrine diingin. A small number of these chemicals have been created explicity to alter homene signaling, eg, the estrogenic drug diethylstilbes trol and many pesticides (se target species). Other chemicals have molecular structures that unintentionally bear sufficient resemblance to hormones such that they are capable of hinding. with varying degrees of affinity to hormone receptues, or of mer acting at the molecular level with other molecules involved in hormonal activity, Ofen EDCs are much less potent than the ondigenous hermones in binding with receptors. An increasing number of examples appearing in the pect-viewed literature. however, show that in some signaling

pathways exogenous hormune-mimics can be equipotent and capable of provoking biological responses at psicomoiar (pM) levele or lower.

Most early research on EDCs focused on the effects of disrup tion of sexual reproduction interactions with the estrogen and androgen nuclear noeptors. Evidence gathered over the past decade now shows that the mechanisms and endpoints vulner ahle se endocrine disruption ate muts header than originally understood. Indend, EDCs are now known to affect metabolism. diabetes, obesity, liver function, bone function, immune fine ton, learning and behavioe via a pinuply of receptor systems and signaling pathways. In addition, the actions of EDCs on reproduction are now known to go far beyond maclear sex steroid hormone receptors. In principal, there is virtually no endocrine signaling vyse or hormone pathway immune to disruption (Fig. 1)

Fig. I The dicrine system is comprised of the hypotalamus, pui sary, adrenal gland, pantyl pal gland, thyroid, paess, and podactics glands Our is and guns sach as the liver, heart, and adipoutisse have sondary endocrine fonctions and may also be targel by EDC Endocrine glands accrutea homme, which is cal throughout the body sia the blood, and muy bond to its specite upor intar ons For instance, omogen a related by the n binds estrogen roeprs (Ro and E dinibuted throughout the body and brain.

The majority of research on EDCs has examined the come quences of their interactions with sucher hormone receptors (NRs), especially estrogen receptors alpha and beta (ERA, ER the androgen receptor (AR), among others. NRs are a supertam ily of transcription factors, proteins that can bind to DNA and influence the expression of neathy gones NRs play central coles in development, physiology and disease. In humans, there are somme 4 dentified Nits Many others remain orphans meaning that their endogenous ligands have not yet been identfied. When activated, NRs undergo conformational changes that allow recruitment of coregulatory molecules and the chromatin modifying machinery of the cell. The ultimate action of NRs is to influence the transcriptional machinery of target penes. NR also interact with other intracellular signaling pathways. Examin- ing how chemicals hind to these receptors can provide important infirmation concoming their endocrine disrupting potential. There are in vitro assays, some of which can be performed as part of high throughput, screening systems that can confirm chemical binding to the majority of NRs. The strengths and weaknesses of in vire tools in predicting hazard will be dis cussed helow in the section on Tier 2

Endocrine disruption also takes place outside the cell nucleus Many natural steroid hormones hind to cell membrane-bound receptors, which in turn partner with a variety of well-known sig- naling cascade proteins. Recent evidence demonstrate that EDC may exert hormonal effects in these non-nuclear hormone receptors as well. Rather than acting as transcription factors, membrane harmone receptors act in intracelhilar signaling molecules to affect phosphorylation and calcium thus within a cell. Disruption of this pathway is another way by which EDCx may alter endogenous homone actions.

Thus, EDCs can act sie multiple pathways and receptor-based mechanisms (Fig. 2). At higher doses they may also exent recep for independent actions via more traditional mechanisms of tesi-organisms city. Their effects are species, time and cell-specific, and are influenced by metabolism.

2. Testing for endocrine disruption

The complex biology of endocrine disruption means that u single areas or single approach can be used to identify chemi- cals with EDC characteristics Instead, a combination af approaches is necessary, including computational methods as well as both in vitro and in vivo testing. Compared to current practice, a carefully composed battery of assays can

dramatically reduce the likelihood that a newly developed chemical will later be found to be an EDC

In vivo methods can text for many types of EDC activity. Actual endocrine disruption, however, involves perturbing the action of one or more hormones within a whole organism Today's in vitro and computer models de not incorporate the complexity that this involves. For this reason, in vive assays will also be nocessary.

Two additional chancteristics of the endocrine system must inform a strategy to detect potential EDCs. First, like undogen aus hormones, EDCs may display non-monotonic dese-response curves. This means that effects observed at low dose levels may be completely unpredictable, and indeed the opposite, of effects observed at high levels. Multiple mechanisms undertie the non-monotonicity of endocrine systems. Thus it is critical to assess chemicals over a wide concentration mange in vits and wide dose ringe in viso to determine whether they have EDC characteristics.

Second, the effects of an exposure to EDC vary with the life stage in which it is experienced (Fig, 3) Thus, the consequences of exposure during periods of development (fital, childhood and adolescence, including puberty) can vary among periods and may also yield very different effects compared to expansin adulthood. While adult exposure to EDCs can certainly be an important factor in adverse health outcomes, lay times in devel opment are likely to be more sensitive to endocrine disruption.

Adverse effects during periods of developmental transition are likely to occur at concentrations of the chemical that are far boliw levels that would be considered huruful in the adul These vulnerable life stages, including fetal, childhood, and puls ertal development, are of particular concem because it is during these stages that the individual is changing physiologically and morphologically. These periods of transition are marked by massive changes in the endocrine envinnment as the new phenotype (or body plan) is being developed.

This heightened sensitivity during developmental transitions results from multiple factors. Most important, the organizational activities of hormones (formation of organs, hmin organization, a) are not reversible, whereas the activational activities (re lation of suproduction, imune systems modulation, etc.) the prevail in adulthood and reversible. Second, the protective moch animms available to the adult such as DNA repair mechanisms, a competent immune system, detoxifying enzymes, liver metab olum excretion, and the blood/brain barrier are not fully func tional in the fetus or newbom. Third, the developing organism has an increased metabolic rate as compared to an adult or aged and this, in some cases, may result in increased or reduced toxicity.

Lastly, any strategy designed to test for EDC activity must cumine organists during different developmental stages because the suite of endogenous hormonos present during develop iment vary from one stage to another. A developing organism may he at a stage when it would not normally be exposed to a certain honnone and thus, exogenous exposure to an EDC that acts upon that hormone's recepter or signaling system will acti vate a pathway that should not be active at that life stage. There fure, prenatal exposure to environmental factors can modify normal cellular and tissue development and function through developmental programming, such that the individual may have a higher risk of reproductive pathologies and metabolic and hormonal disorden later in life.

Principles guiding protocol development and use

Several principles have framed the development of TIPED and will continue to guide its development in the future. Table 1 summarizes the overarching principles guiding our design of the EDC testing protocol. We briefly elaborate on each of the prin ciples below.

Overarching principles :

The first principal comes from green chemistry. Green chemists design against hazard.57 The earlier in the design process that hazard can be discovered, the more likely it is that downstream problems will be minimized, if not avoided entirely. This can have material benefits for the chemist and his/her company.

The second principle, on current scientific understanding, con trasts our protocol with standardized approaches used in regulat ory toxicology. As noted above, the standardized assays upon which traditional toxicological approaches are based are often decades old. They rarely reflect the quality and modernity of assay tools used in scientific research funded by the National Institutes of Health, including the National Institute of Environ mental Health Sciences. The old approaches are insensitive and largely incapable of dealing with EDCs. Ignoring current science would result in chemists producing yet another generation of hazardous chemicals.

That said, the assays we recommend have been chosen because multiple laboratories have successfully used them. They can require specialized knowledge and skills, but are not so

Table 1 Overarching principles guiding design of TiPED

- Chemical hazard must be considered at all stages of molecular design and synthesis.
- Assays used should reflect current scientific understanding, and the protocol should be reviewed regularly to incorporate new scientific discoveries and tools.
- The assays within each tier should span a comprehensive range of EDC mechanisms of action.
- While in silico and in vitro assays offer less costly starting points, in vivo assays are necessary to conclude that a chemical is unlikely to have EDC activity.

arcane that only a single, or small number of, laboratories, would be capable of implementing them. The second half of the prin ciple acknowledges the fast pace of scientific discoveries in the field of endocrine disruption. New modes of action requiring new assays will certainly be discovered. Incorporating this evol ving knowledge into the protocol is essential.

The third principle, a comprehensive range of EDC mechan isms, reflects the need to look for more than one or two EDC modes of action. This is because single chemicals can act through multiple mechanisms. The absence of action through one mechanism cannot be taken as evidence of no action through another mechanism. A case in point is BPA. It is an estrogen via both genomic and non-genomic pathways, an anti androgen, a thyroid hormone antagonist, and a peroxisome pro liferater-activated receptor (PPAR) agonist.

The fourth principle acknowledges that the current state of in silico and in vitro assays do not sufficiently incorporate the complexity of an endocrine system functioning in a living organ ism, and especially that of a developing organism..

Evaluating EDC assays :

In all likelihood, the chemist him/herself will not be performing the assays, but instead will be working in partnership with environmental health scientists or with a contract laboratory. Because this is not the chemist's field, yet their research is dependent upon the test findings, it is impor tant for the chemist to have some ability to gauge the quality and reliability of the work being done. This is especially the case for EDCs because of the complexity of the science.

With this in mind, we offer a set of principles that chemists can use to select and evaluate EDC assays (Table 2). Each principle is briefly elaborated upon below.

The first principle is designed to select assays that have proven reliable among different laboratories, to have well defined per formance standards and to avoid assays that test for poorly defined endpoints and hence are open to arbitrary and variable interpretation.

The second principle should guide experimental design. Nega tive controls are essential to establish an effect. Positive controls are needed to demonstrate that the experimental system is appro priately sensitive and free of contamination or other confounding variables. The positive control must be used at an appropriate concentration or dose to demonstrate the sensitivity of the assay in terms of being capable to detect effects of low doses of EDCs. Prior use of insensitive strains of rodents in EDC tests without positive controls has led to significant confusion in the

Table 2 General principles for selection and evaluation of EDC assays for Green Chemists

- Each assay should be reliable, relevant, meet performance standards and use well-defined endpoints.
- Experimental design should employ concurrent negative and positive controls and blanks to confirm that the experimental system is free from contamination and that it is appropriately sensitive.
- A dynamic testing range should be established, and testing should be carried out over the full range, including high and low doses.
- Some in vivo tests should be structured to reveal the consequences of developmental exposures on health and function later in life, through all life stages.
- Some in vivo tests should not assume knowledge of the mechanism/ pathway of action.

  peer-reviewed literature. Controls must be run concurrently because of the potential for temporal variation in unintended contamination (e.g., changes in composition of rodent chow from batch to batch or inadvertent contamination of lab).

The third principle acknowledges a fundamental feature of endocrine disruption, that high dose effects do not necessarily predict low dose effects, i.e., non-monotonicity in the dose response curve.

The fourth principle addresses another key feature of endo crine disruption, that developmental exposures can lead to effects that are initially subtle, for example changes in epigenetic programming, but ultimately highly adverse, e.g., cancer in adulthood.

The fifth principle is designed to widen the reach of the assays beyond currently known mechanisms of endocrine disruption. We have identified several in vivo versions of high-throughput screening that do not assume the mechanism of EDC action but instead look broadly at developmental disorders following early life exposure in fish and amphibians.

Evaluating laboratories :

As with the choice and evaluation of specific assays, assessment of laboratory practice per formance and capabilities in experimental environmental health science is outside the expertise of most chemists. In Table 3 we list six important criteria that should be addressed explicitly in the choice of collaborators/contract laboratories.

The first criterion specifies that the laboratory must demon strate it can replicate the appropriate performance of the assay(s) as carried out by other laboratories and that it is capable of repeatedly performing the assay successfully.

The second criterion specifies that the laboratory must be willing to share all relevant information about the laboratory and its methods and practices, as well as all relevant data on assay performance.

The third criterion focuses on animal husbandry practices by the laboratory. Poor animal husbandry is not only unethical, it introduces additional variability in the experiments that can mask effects, making it more difficult to confirm or reject EDC activity. The laboratory should share information about its hus bandry practices and benchmark those against industry standards.

The fourth criterion stipulates that power analyses should be performed in preparation for the full assay. Power analysis is a statistical tool that provides guidance, based on preliminary data, on the sample size necessary to find a statistically significant result given the magnitude of the effect and the variance inherent in the data. Use of power analysis is especially important in

Table 3 Criteria to guide evaluation of laboratories

A lab must:

- Demonstrate intra- and inter-laboratory repeatability.
- Demonstrate transparency in reporting.
- Utilize effective, safe husbandry practices; high mortality/morbidity rates in controls are unacceptable.
- Employ power analysis of preliminary results to design methods.
- Utilize standard protocols and solutions/reagents/cultures/etc., where they are available.
- Undergo external review and audit on regular basis comparable to NSF/NIH external reviews.

in so studies to ensure the sample size is large enough to detect an effect but not so large that an excessive number of animals are used.

The fifth criterion addresses replicability and reliability Standard protocols, well established in the pect-reviewed liters time, should be followed in carrying out the assays and variations in assay performance must be avoided. Use of standard solu tions reagents/cultures/etc., will help avoid inadvertent contami nation and unexpected biological variability

The sixth criterion external review and add will provide the chemist overall assumance of the labonnery's quality.

Using TiPED with known EDCs verification of methodology :

When we first started the process of developing this tiered approach to screening new chemicals, we identified several known EDCs (chemicals or classes of chemicals) that work through different mechanisms and are known to have widely different effects on exposed cells, animals or humans. Using these examples, we identified a repertoire of assays that we expected would be sufficient to detect known endocrine disrupt ing activities. To continue this thought-exercise, we then ident ified published studies that determined whether the

TIPED assays described above (or similar ones) have been used success fully with these six known EDC's (Table 891)

Clearly, some of these EDCs would be identified by several computational assays in Tier 1. BPA and phthalates, for example, have been tested with both QSAR and molecular docking assays, and both of these methods indicate that these chemicals bind to nuclear hormone receptors. Other EDC's, such as per chlorate and strazine, would likely "pass" the first tier. Testing BPA farther with TIPED, it would also be identified as an EDC in Tiers 2, 3, 4 and 5. Thus, a chemical like BPA, with mechan isms that span several NRs, would be easily identified by this tiered screening protocol In contrast, perchlorate and atrazine might make it to Tiers 3 or 4 before they are identified as EDCs Yet the proposed assays are clearly robust enough that these chemicals would not make it to market, providing supportive evidence that the TiPED screens will be sufficient to identify putative EDC,

Currently we intend to place EDC test protocol in the public domain. The institutional home for it is still to be determined, but it will likely be either an academic or government institution. Whenever it is located the "home" for TIPED will be a place where detailed protocols for assays will be found along with lists of available online databases and tools. In addition there will be trained personnel to answer questions and provide general gui- dance and referral to labs that can contract to do specific assays The design and creation of the protocol has been overseen by a Scientific Advisory Committee comprised of experts from both chemistry and biology (Table SIT). Future management of the protocol will also require oversight and regular reexamination of the assays in light of scientific advancement.

The latter point is critically important in order to avoid sub mitting future chemical innovation to insensitive salety tests (ot worse, giving approval to chemicals that future scientists learn to be EDCs), the assays and tiers of the protocol must be reviewed and updated on a regular basis. The continuing role of the Scie tific Advisory Committee will be essential to this process and will keep the protocol on the leading edge of EDC science.

Summary and conclusions :

TIPED provides tools that can guide the development of inher ently safer materials by avoiding chemicals likely to disrupt the endocrine system. Using the assays in the protocol carly in the design process to detect potential EDCs, chemists can choose not to purvue development of a candidate chemical that has EDC characteristics Alternatively, they can use mechanistic data from the assays to guide redesign of the chemical, with the goal of retaining desired material characteristics but avoiding action through identified EDC mechanisms

In an effort to prevent novel EDCs from being produced in appreciable quantities, we focused solely on scientific issues to provide chemists with a set of guiding principles and tools that will enable them to stem production of chemicals with EDC potential. The goal of this ground-up approach, termed TIPED, is to identify hazand early in the design process using a systema tic series of assays that build upon one another. We wish to emphasize that this tiered protocol was not designed as a one size-fits-all tool Depending upon their unique situation, a chemist may have good reason to start at any point within the protocol, not necessarily with Tier 1.

A positive test at any step in the process is an indication of potential endocrine disruptor activity and thus provides the chemist an opportunity to modify the chemical under develop ment. The endocrine disruption screening assays comprised in each tier are based on the best and most up-to-date science collectively TIPED is designed to cover all known aspects of endocrine disnaption.

Beyond serving as a tool for chemists, this paper highlights the need for a transformation in the field of toxicology, advan eing this science from an exclusively reactive, analytical one

to include a significantly preemptive arm. For new chemicals, and perhaps old ones as well, toxicology, at least as it is associated with commercial chemicals, has to become much more a colla borative undertaking between the chemist and scientists who can evaluate toxicity in real time both theoretically and experimen tally. It is our hope that collaborative efforts such as these, which lie at the interface of endocrine disruption and green chemistry, will help lead to a new generation of inherently safer chemicals

Acknowledgements :

We are grateful to the following scientists who participated in a two day retreat at Cavallo Point, California to help us develop und refine our thinking on this investigation: Paul Anastas, Evan Beach, Amy Cannon, Chad Ellis, Julie Haack, Andreas Kortenkamp, CJ. Li, Nicolas Oles, Sarah Vogel, Adelina Voutchkova and Wim Thielemans. The work was supported by grants from NIEHS (grant no. IRI3ES016981-01), the Cedar Tree Foundation, the Johnson Family Foundation, the Kendeda Fund, the Marisla Foundation, the John Merck Fund, and the Passport Foundation. We thank Julie Jones for outstanding logistical and organizing efforts without which this collaboration

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