Combined Exposures and Mixtures Program

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Problem Statement

Humans are continuously exposed to mixtures of chemicals. Challenges persist in characterizing exposure to mixtures, evaluating their toxicity and hazard, and assessing associated risk. Limitations in our understanding have led to inconsistent use of available mixture methods and significant uncertainties in their application. The lack of harmonized terminology and methods comparisons complicate the synthesis of information across disciplines and impede the use of mixtures data in decision-making.

Objectives

The Combined Exposures and Mixtures (CEM) Program is structured around the following three objectives:

1. Develop and apply a disease-centered systems biology approach for prioritizing mixtures for toxicological and hazard characterization to inform cumulative risk evaluation. This approach starts with the disease-of-interest and aims to identify and evaluate the joint effects of factors that contribute to disease development; it contrasts with component-based mixtures approaches that focus exclusively on structurally or mechanistically similar chemicals.
2. Develop and apply methods for complex mixture[1] testing and data interpretation to inform risk assessment of whole mixtures.[2] Methods include targeted and nontargeted chemical analyses, complex mixture read-across (i.e., determining sufficient similarity), polypharmacokinetics (i.e., pharmacokinetics of multicomponent exposures), and bioassay-guided fractionation to identify toxic constituent(s).
3. Apply component-based approaches by experimentally evaluating defined mixtures[3] and using predictive modeling approaches (e.g., dose addition, response addition) and compare the results with alternative whole-mixture evaluation.

[1] Complex mixture is broadly defined here as a mixture of many constituents with some unidentified fraction (e.g., effluent sample, diesel exhaust). The term complex mixture is used synonymously with *substances of unknown or variable composition, complex reaction products or of biological materials* (UVCB substances).

[2] Whole mixture is defined here as a complete mixture. A whole mixture can be simple (containing few constituents) or complex (see footnote 1). Whole-mixture approaches involve toxicity and risk evaluation of the complete mixture (i.e., the mixture of interest) and can be contrasted with component-based approaches that use toxicological data from some or all mixture constituents to estimate risk of the mixture.

[3] Defined mixture is defined here as a mixture in which all components are identified and quantified.

Rationale

Public Health Context

People are continuously exposed to mixtures of chemical and nonchemical stressors (e.g., psychosocial stress) throughout their lifetimes and there is clear evidence that combined exposures can have cumulative effects (e.g., asbestos and tobacco smoke on lung cancer). Because most toxicity studies and risk assessments address single chemical exposures, the scientific community may be underestimating the effects of exposure to multiple substances on human health and disease progression.

Alignment with Mission, Goals, Strategic Pipeline

The CEM Program is aimed at using a systems biology approach to predict which chemicals might act additively when present in mixtures, developing and refining whole-mixture evaluation and complex mixture read-across approaches, and strengthening predictive models of mixture toxicity by decreasing uncertainty in the application of component-based models. The Division of the National Toxicology Program (DNTP) mixtures portfolio offers a unique opportunity to inform literature-based evaluations in addressing human health effects from exposure to mixtures and is deliberately geared toward informing decision-making related to component-based and whole-mixture risk assessment. Assessing mixtures requires collaborative engagement across multiple scientific disciplines and multidisciplinary team science is a core value at DNTP. Approaches for complex mixture read-across applied in the CEM Program represent leading-edge science. Broad public health implications involved in mixtures research ensure that DNTP trainees involved in mixtures projects gain experience in translational research. Thus, the CEM Program is well aligned with the mission and goals of DNTP to incorporate the use of predictive models and emphasize the translational nature of our research.

Due to the diversity of projects, all aspects of the DNTP pipeline are actively engaged in the CEM Program. Examples of the utilization of early pipeline resources include in silico evaluation of individual chemicals in the Polycyclic Aromatic Compounds Mixtures Assessment Program (PAC-MAP), high-throughput screening to evaluate mixtures of endocrine active compounds, and the use of in vitro hepatocyte assays to evaluate multiple botanicals in the sufficient similarity assessment projects.

Stakeholder Interest and Engagement

The DNTP's strategic approach to mixtures research has been developed over many years in close coordination with the National Institute of Environmental Health Sciences (NIEHS) mixtures strategy. In brief, NIEHS embarked on a period of reflection on the state-of-the-science of mixtures research and engagement with experts beginning in 2010. The cross-divisional NIEHS Combined Exposure and Mixtures Working Group was formed in 2010 to gather scientists from DNTP, Division of Intramural Research (DIR), and Division of Extramural Research and Training (DERT) with an interest in mixtures research. The Working Group continues to meet regularly and serves as a forum for discussing and fostering collaboration on mixtures projects across NIEHS. Efforts to build an NIEHS mixtures research strategy culminated in a workshop entitled "Advancing Research on Mixtures: New Perspectives and Approaches for Predicting Adverse Human Health Effects" held in 2011 and in the incorporation of CEM

into the NIEHS Strategic Plan.[4,5] The 2011 workshop gathered mixtures experts from multiple disciplines (toxicology, epidemiology, statistics, exposure science, risk analysis) to discuss challenges in mixtures science and to prioritize research goals. Following this foundational effort, CEM research was included as a strategic goal in the 2012–2017 NIEHS Strategic Plan and in the current NIEHS Strategic Plan (2018–2023).

DNTP has also been actively engaged with stakeholders in efforts to bring clarity to mixtures terminology. Co-chairs of the NIEHS Combined Exposure and Mixtures Working Group (Cynthia Rider and Danielle Carlin) served as reviewers of the Agency for Toxic Substances and Disease Registry's Framework for Assessing Health Impacts of Multiple Chemicals and Other Stressors[6] and provided input on the definitions of mixtures-related terms. Additional efforts to clarify and harmonize terminology are evident in the 2018 book "Chemical Mixtures and Combined Chemical and Nonchemical Stressors Exposure, Toxicity, Analysis, and Risk," co-edited by Cynthia Rider and Jane Ellen Simmons (U.S. Environmental Protection Agency (EPA)).[7] The introduction contains definitions and clarifying examples, and a chapter by NIEHS PRIME grantee, Thomas Webster (Boston University), explores the contrasting views of mixture terms and approaches in epidemiology and toxicology.

Steps Taken to Engage Stakeholders

Stakeholders for the DNTP mixtures portfolio have been engaged on a project-by-project basis as well as on the program level. Examples of both the project-specific and program-level engagement are provided in the table below. DNTP has also engaged academic, industry, and government stakeholders through workshops on mixtures:

- 2011 Advancing Research on Mixtures: New Perspectives and Approaches for Predicting Adverse Human Health Effects[4,5]
- 2015 Statistical Approaches for Assessing Health Effects of Environmental Chemical Mixtures in Epidemiology Studies;[8] followed by the DERT's Powering Research Through Innovative Methods for Mixtures in Epidemiology (PRIME) Program (RFA-ES-17-001[9])
- 2016 Addressing Challenges in the Assessment of Botanical Dietary Supplement Safety[10,11,12,13]

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[4] Carlin *et al*. (2013) Unraveling the health effects of environmental mixtures: an NIEHS priority. Environ Health Perspect. 121(1):A6-8. https://doi.org/10.1289/ehp.1206182

[5] Rider *et al*. (2013) Mixtures research at NIEHS: an evolving program. Toxicology. 313(2-3):94-102. https://doi.org/10.1016/j.tox.2012.10.017

[6] ATSDR. (2018) Framework for Assessing Health Impacts of Multiple Chemicals and Other Stressors. Atlanta, GA: Agency for Toxic Substances and Disease Registry, U.S. Department of Health and Human Services, Public Health Service.

[7] Rider CV, Simmons JE. (2018) Chemical Mixtures and Combined Chemical and Nonchemical Stressors: Exposure, Toxicity, Analysis and Risk. Cham, Switzerland: Springer International Publishing. p. 556.

[8] Taylor *et al*. (2016) Statistical Approaches for Assessing Health Effects of Environmental Chemical Mixtures in Epidemiology: Lessons from an Innovative Workshop. Environ Health Perspect. 124(12):A227-A229. https://doi.org/10.1289/EHP547

[9] https://grants.nih.gov/grants/guide/rfa-files/RFA-ES-17-001.html

[10] Catlin *et al*. (2018) How similar is similar enough? A sufficient similarity case study with Ginkgo biloba extract. Food Chem Toxicol. 118:328-339. https://doi.org/10.1016/j.fct.2018.05.013

[11] Roberts *et al*. (2019) Finding the bad actor: Challenges in identifying toxic constituents in botanical dietary supplements. Food Chem Toxicol. 124:431-438. https://doi.org/10.1016/j.fct.2018.12.026

[12] Shipkowski *et al*. (2018) Naturally complex: Perspectives and challenges associated with Botanical Dietary Supplement Safety assessment. Food Chem Toxicol. 118:963-971. https://doi.org/10.1016/j.fct.2018.04.007

[13] Waidyanatha *et al*. (2018) Follow that botanical: Challenges and recommendations for assessing absorption, distribution, metabolism and excretion of botanical dietary supplements. Food Chem Toxicol. 121:194-202. https://doi.org/10.1016/j.fct.2018.08.062

- 2018 Understanding the Combined Effects of Environmental Chemical and Non-Chemical Stressors: Atherosclerosis as a Model (eFactor story[14])
- 2019 Converging on Cancer Workshop; convened to understand the joint action of multiple chemicals that interact via different molecular pathways leading to cancer (eFactor story[15])

Furthermore, the DNTP mixtures research strategy was presented to NTP and NIEHS advisory groups, international partners, and academic institutions:

- 2017 NTP Board of Scientific Counselors Meeting, "Strategies for Studying Combined Exposures and Mixtures" (presentation,[16] minutes[17])
- 2017 Texas A&M University, "Deconvoluting a Complex Problem: Mixtures Research at the National Toxicology Program"
- 2018 European Union (EU) Joint Research Centre, "Mixtures Research at the National Toxicology Program: Generating Data to Inform Regulatory Decisions"[18]
- 2018 National Advisory Environmental Health Sciences (NAEHS) Council Meeting, "Tackling Complex Problems: Combined Exposures and Mixtures Research at the National Toxicology Program"
- 2019 North Carolina State University, "Deconvoluting a Complex Problem: Mixtures Research at the National Toxicology Program"
- 2019 U.S. Food and Drug Administration (FDA) National Center for Toxicological Research (NCTR), "A Strategy for the Botanical Dietary Supplements Research Portfolio at NTP"

Ongoing and Continuing Interactions

Stakeholder	Issue	Role of Stakeholder
Environmental Working Group	Carcinogenic mixtures	User
University of California–Berkeley	Carcinogenic mixtures	Collaborator
California Environmental Protection Agency	Carcinogenic mixtures	User
Getting to Know Cancer	Carcinogenic mixtures	Collaborator
FDA National Center for Toxicological Research	Testing botanicals for safety	Partner
National Institute of Health (NIH), Office of Dietary Supplements	Testing botanicals for safety	Partner
NIH, National Center for Complementary and Integrative Health	Testing botanicals for safety	Partner
NIEHS Division of Intramural Research Investigators	Sufficient similarity of botanicals; statistical tools for mixtures data analysis	Collaborator

[14] https://factor.niehs.nih.gov/2018/5/science-highlights/atherosclerosis/index.htm
[15] https://factor.niehs.nih.gov/2019/6/science-highlights/cancer/index.htm
[16] https://ntp.niehs.nih.gov/ntp/about_ntp/bsc/2017/june/presentations/02rider_508.pdf
[17] https://ntp.niehs.nih.gov/ntp/about_ntp/bsc/2017/june/minutes20170629_508.pdf
[18] Drakvik *et al.* (2020) Statement on advancing the assessment of chemical mixtures and their risks for human health and the environment. Environ Int. 134:105267. https://doi.org/10.1016/j.envint.2019.105267

Stakeholder	Issue	Role of Stakeholder
Botanical Safety Consortium: FDA, Office of Dietary Supplement Programs; Health and Environmental Sciences Institute; Council on Responsible Nutrition; The Procter & Gamble Company; Amway	Testing botanicals for safety	Partner
Triangle Research Initiative on Household Energy Transitions	Health effects of cookstove emissions	Technical advisor, partner
EPA, Office of Research and Development	Cookstove emissions	Collaborator
EPA, National Center for Environmental Assessment	Testing request for complex mixtures	User
Health Canada	PAC-MAP	Collaborator
Texas A&M University	PAC-MAP	Collaborator
Oregon State University	PAC-MAP	Collaborator
RTI International	Cookstove emissions	Collaborator
Drexel University	Chemical and nonchemical stressors	Collaborator
European Union Joint Research Centre	DNTP mixtures strategy	Partner
Agency for Toxic Substances and Disease Registry	Mixtures terminology	Partner

Input Received

Support for the DNTP mixtures research strategy has been strong from institutional partners and within NIEHS. In particular, there has been a consistent message from stakeholders that DNTP is uniquely positioned to contribute to our understanding of the safety and hazards associated with botanical dietary supplement use. Input from stakeholders has been instrumental in the development of the research programs in the CEM portfolio. For example, input and advice from collaborators at Health Canada (e.g., Paul White and Alexandra Long) and EPA (e.g., Glenn Rice and Margaret Pratt) helped shape the PAC-MAP in vivo studies to avoid redundancy and be complementary with existing research efforts on this well-studied class of compounds with high public health importance. Participation in meetings at UC Berkeley with collaborators Martyn Smith (University of California–Berkeley), Lauren Zeise (California Environmental Protection Agency), and Leroy Lowe (Getting to Know Cancer) were critical in developing the 2019 Converging on Cancer Workshop and continue to inform progress on a research program to address the joint effects of chemicals on cancer.

Milestones and Metrics

Measures of progress toward achieving the program objectives are outlined below. For each objective, individual aligned projects are listed along with relevant milestones. Milestones are grouped by expected timeframe as short-term (1 year), medium-term (2–3 years), and long-term (4–5 years). All research objectives are expected to result in publicly available data housed in the Chemical Effects in

Biological Systems (CEBS) database and in publications of key findings in the form of NTP reports and/or peer-reviewed manuscripts.

Objective 1: Develop and apply a disease-centered systems biology approach for prioritizing mixtures for toxicological and hazard characterization to inform cumulative risk evaluation. This approach starts with the disease-of-interest and aims to identify and evaluate the joint effects of factors that contribute to disease development; it contrasts with component-based mixtures approaches that focus exclusively on structurally or mechanistically similar chemicals.

Related projects: Converging on Cancer and cardiovascular disease mixtures.

The Converging on Cancer project explores the hypothesis that environmental chemicals that target different cancer-related pathways contribute cumulatively to the development of cancer in a manner not accounted for by individual chemical evaluation. Development is ongoing of the Converging on Cancer project in collaboration with the Carcinogenesis Health Effects Innovation (Carci HEI) PMT. Similarly, the cardiovascular project aims to replicate the Converging on Cancer approach with a focus on cardiovascular disease.

- Short-term:
  - Identify team and begin scoping for disease-centered systems biology project development.
  - Build an adverse outcome pathway (AOP) network depicting hypothesis of how mixture components are expected to interact.
  - Develop project plan and complete internal review steps.
- Medium-term:
  - Evaluate the specific mixture hypothesis identified during AOP development (see short-term milestones, above) by testing individual chemicals and defined mixtures in an appropriate model and comparing results to those predicted on the AOP-derived assumptions.
- Long-term:
  - Actively engage and communicate with mixtures research practitioners and risk assessors (e.g., workshop, working group) to compare DNTP findings to those from parallel efforts (e.g., EuroMix, EPA), and to evaluate the viability of application of the disease-centered approach in risk assessment contexts.
  - With input from stakeholders, determine whether there is a sufficient scientific basis to support application of disease-centered systems biology approaches to risk assessment or more work is needed to fill knowledge gaps.

Objective 2: Develop and apply methods for complex mixture testing and data interpretation to inform risk assessment of whole mixtures. Methods include targeted and nontargeted chemical analyses, complex mixture read-across (i.e., determining sufficient similarity), polypharmacokinetics (i.e., pharmacokinetics of multicomponent exposures), and bioassay-guided fractionation to identify toxic constituents.

Objective 2.1: Apply targeted[19] and nontargeted[20] chemical analyses, in vivo bioassays, and literature review methods for complex mixture testing and data interpretation to inform risk assessment.

Related projects: Botanical testing program (e.g., *Garcinia cambogia*, black cohosh extract, *Echinacea purpurea*), Office of the Report on Carcinogens (ORoC) woodsmoke evaluation, Office of Health Assessment and Translation (OHAT) personal care products evaluations.

These projects involve adaptation of traditional testing and evaluation methods to whole mixtures. For example, evaluation of *Garcinia cambogia* in a 90-day rodent study is similar to that of a single chemical, but additional chemical analysis is required to extrapolate findings to different *Garcinia*-containing products. Similarly, the ORoC woodsmoke project will progress as a traditional systematic review for a single chemical but will require additional consideration of exposure variables for comparison across the literature (e.g., use of different methods of measuring the woodsmoke exposure as a whole mixture, quantification of different individual constituents of woodsmoke from study to study).

- Short-term:
  - Conduct data analysis and reporting for *Garcinia cambogia, Echinacea purpurea,* and black cohosh.
- Medium-term:
  - Finish the existing toxicity testing program of current botanical ingredient projects (e.g., completing in-life portion of studies) and report out results.
- Long-term:
  - Evaluate the body of DNTP research on botanical mixtures to determine its effect and utility in hazard characterization (e.g., state-of-the-science paper).

Objective 2.2: Develop methods for complex mixture evaluation including sufficient similarity,[21] polypharmacokinetics,[22] and bioassay-guided fractionation[23] to identify toxic constituents.

Related projects: Sufficient similarity methods development studies with botanicals, polypharmacokinetics methods development with *Ginkgo biloba*, toxic constituent identification using bioassay-guided fractionation of black cohosh.

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[19] Targeted chemical analysis approaches examine known chemicals using optimized analytical methods to quantify all, or a subset of, constituents of a complex mixture. Measured constituent(s) can be unique marker chemical(s) that are used to characterize a complex mixture (e.g., a subset of ginkgolides is used as a marker constituent to characterize Ginkgo biloba extract) or constituents with established toxicological activity (e.g., benzo(a)pyrene in cookstove emissions).

[20] Nontargeted chemical analysis methods can be used to 1) provide a qualitative chemical profile of a given mixture for comparison purposes, or 2) identify previously unknown compounds that may be associated with the complex mixture using existing chemical libraries and software applications.

[21] Sufficient similarity refers to a determination that two mixtures (e.g., two products containing a common botanical ingredient but produced using different manufacturing processes) are similar enough in chemical composition that toxicity data from one can be used to estimate the risk associated with the other, i.e., a read-across approach for complex mixtures.

[22] Polypharmacokinetics is an approach to simultaneously characterize the concentration-time profiles of constituents in complex mixtures, their metabolites, and changes to the endogenous metabolome following exposure using mass spectrometry-based techniques coupled with multivariate statistics.*

[23] Bioassay-guided fractionation first separates the complex mixture into fractions based onaccording to physicochemical properties (e.g., extracted using multiple diverse solvents). Next, each of the fractions is evaluated in a carefully selected and fit-for-purpose bioassay to identify the fraction(s) with biological activity. This process can be repeated until the fraction is simple enough to allow for isolation and identification of the active constituent(s).

* Xie G, Wang S, Zhang H, Zhao A, Liu J, Ma Y, Lan K, Ni Y, Liu C, Liu P et al. 2018. Poly-pharmacokinetic Study of a Multicomponent Herbal Medicine in Healthy Chinese Volunteers. Clin Pharmacol Ther. 103(4):692-702. 10.1002/cpt.784

- Short-term:
  - Generate and analyze data for black cohosh project.
  - Prepare manuscript for polypharmacokinetics project.
- Medium-term:
  - Complete methods development work on a targeted sufficient similarity project to evaluate market variability of a popular botanical ingredient that is not within the dietary supplement legislative framework (e.g., cannabidiol, kratom).
  - Develop a framework for making decisions when complex mixtures tools should be used.
  - Determine whether additional complex mixtures methods development is required.

Objective 2.3: Provide NTP research support for the Botanical Safety Consortium[24] – a public-private partnership aimed at developing a toolbox of in vitro assays for identifying hazards associated with botanical ingredients.

- Short-term:
  - Develop Botanical Safety Consortium program plan.
  - Identify the botanical library (i.e., botanical ingredients with known toxicity profiles) to be evaluated and recommend in silico and in vitro assays in target areas (hepatotoxicity, genotoxicity, reproductive and developmental toxicity).
- Medium-term:
  - Complete screening of botanical library in suite of in vitro assays.
  - Identify areas wherein assay coverage is inadequate for botanical safety evaluation.
- Long-term:
  - Develop recommendations for using chemical analysis and in vitro/in silico approaches for evaluating botanical ingredients.

Objective 3: Apply component-based approaches by experimentally evaluating defined mixtures and using predictive modeling approaches (e.g., dose addition, response addition) and compare the results with alternative whole-mixture evaluation.

Related projects: PAC-MAP evaluation of the assumptions of a component-based approach using PACs as an example by testing rodent in vivo and human in vitro assays.

- Short-term:
  - Complete and publish PAC-MAP in vivo work.
  - Initiate human in vitro immune assay in PAC-MAP.
  - Develop project plan building on the studies with individual per- and polyfluoralkyl substances (PFAS) to evaluate defined PFAS mixtures.

[24] https://botanicalsafetyconsortium.org/

- Medium-term:
  - Report findings on human in vitro immune assay in PAC-MAP.
  - Compare defined and whole-mixture approaches.
  - Scope and prioritize a project on high-impact, combined chemical and nonchemical stressors requesting ideas both to internal and external partners.
- Long-term:
  - Synthesize case studies on scientific basis for component-based approaches (i.e., state-of-the-science papers) and engage with stakeholders to evaluate continued reliance on this approach as the default in cumulative risk assessment.
  - Provide statistical tools for use in predicting mixture toxicity based on dose-response data from individual chemicals resulting from the PAC-MAP project to the scientific community.

Value Proposition and Summary

Our work on defined mixtures strengthens our ability to predict the effects of known environmental mixtures on the basis of their individual constituents, which will be useful for risk assessors applying these types of toxicological studies to human biomonitoring-based risk assessment. It will also contribute to decreasing the uncertainty involved in component-based risk assessment by providing hypothesis-driven research on the current assumptions (e.g., dose addition for estimating cumulative effects of like-acting chemicals, lack of interactions among chemicals within a class). Our work on defined mixtures approaches leverages the toxicological data that already exist for single chemicals to estimate effects of mixtures. It is anticipated that the lessons learned, and statistical tools developed during these projects, will be useful in research programs under other strategic focus areas that involve environmental mixtures (e.g., per- and polyfluoroalkyl substances, phthalates, and flame retardants) as well as combination pharmaceuticals (e.g., AIDS therapeutics). Future work will further build on this foundation by addressing combinations of chemical and nonchemical stressors.

DNTP is currently applying the concept of sufficient similarity of complex mixtures to its test article selection process for botanicals; further development of methods to evaluate sufficient similarity will also be a critical contribution in interpreting data generated from other complex mixture projects. For example, sufficient similarity approaches allow for better definition for when we can extrapolate findings from a tested substance to related substances with variable composition, whereas bioassay-guided fractionation and polypharmacokinetics allow for identification of the bioactive constituent(s) and generation of relevant toxicokinetic data, respectively. These projects will improve our ability to translate animal study findings on complex mixtures to better understand their human relevance.

DNTP has the resources required to evaluate both individual components and whole mixtures using cutting-edge chemical characterization tools, in vitro assays, and in vivo studies. We have mixtures research experts within DNTP and at the NIEHS DIR and DERT in complementary disciplines (e.g., statistics, epidemiology) who share ideas and develop collaborations through quarterly meetings of the cross-divisional NIEHS Combined Exposures and Mixtures Working Group. We regularly engage with our federal partners and other stakeholders to design studies that address current mixtures of concern, provide data useful in the risk assessment of mixtures, and refine predictive methods for mixtures evaluation. We expect the projects outlined here to be critical contributions in providing the scientific basis for public health decision-making by informing:

- Selection of chemicals for inclusion in cumulative risk assessments using knowledge of biological pathways.
- Consideration of component-based versus whole-mixture approaches to minimize uncertainty in hazard evaluation.
- Application of methods for predicting mixture toxicity using data from constituents, determining sufficient similarity of whole mixtures, evaluating pharmacokinetics of multicomponent mixtures, and identifying active constituents.