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Using the Key Characteristics of Carcinogens to Develop Research on Chemical Mixtures and Cancer

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Background: People are exposed to numerous chemicals throughout their lifetimes. Many of these chemicals display one or more of the key characteristics of carcinogens or interact with processes described in the hallmarks of cancer. Therefore, evaluating the effects of chemical mixtures on cancer development is an important pursuit. Challenges involved in designing research studies to evaluate the joint action of chemicals on cancer risk include the time taken to perform the experiments because of the long latency and choosing an appropriate experimental design.

Objectives: The objectives of this work are to present the case for developing a research program on mixtures of environmental chemicals and cancer risk and describe recommended approaches.

Methods: A working group comprising the coauthors focused attention on the design of mixtures studies to inform cancer risk assessment as part of a larger effort to refine the key characteristics of carcinogens and explore their application. Working group members reviewed the key characteristics of carcinogens, hallmarks of cancer, and mixtures research for other disease end points. The group discussed options for developing tractable projects to evaluate the joint effects of environmental chemicals on cancer development.

Results and Discussion: Three approaches for developing a research program to evaluate the effects of mixtures on cancer development were proposed: a chemical screening approach, a transgenic model-based approach, and a disease-centered approach. Advantages and disadvantages of each are discussed. https://doi.org/10.1289/EHP8525

Introduction

Humans are exposed to numerous, dynamic environmental stressors (chemical, physical, biological, and social) over their lifetimes. Biomonitoring programs, such as the National Health and Nutrition Examination Survey (NHANES) in the United States and the Consortium to Perform Human Biomonitoring on a European Scale (COPHES), have increased awareness on the extent of exposure to diverse chemicals (Bocato et al. 2019). It follows that a single exposure approach to environmental health research is inadequate, and traditional research strategies (e.g., genome-wide association and Gene × Environment studies) have limited ability to assess complex exposures and their interactions with intrinsic factors to influence biology and health outcomes (McHale et al. 2018). Correspondingly, experts across multiple disciplines have acknowledged the need for research and regulatory frameworks that move beyond single chemicals and address the effects of combined exposures (encompassing both combinations of chemicals and chemical and nonchemical stressors) and complex mixtures [i.e., mixtures of unknown or variable composition, complex reaction products, or biological materials (UVCBs)] on disease (Carlin et al. 2013; Drakvik et al. 2020). Although nonchemical stressors can also play important roles in cancer development (Antoni et al. 2006) and many of the combined exposure concepts discussed can be applied to nonchemical stressors, the discussion herein centers on combined chemical exposures (commonly referred to as chemical mixtures). We distinguish between cumulative risk assessment, which examines risks posed by exposures to disparate stressors (e.g., chemical, biological, and physical stressors), and mixtures risk assessment, which examines risks associated with chemical mixtures (e.g., combinations ranging from different ratios of two chemicals to complex mixtures exceeding hundreds of individual chemicals).

In considering the effects of combined exposures on disease, cancer is both a particularly challenging and critical disease to study. Cancer is a complex disease with varied presentations (i.e., different etiologies and target tissues) (NCI 2021). The collection of pathologies classified as "cancer" involves dysregulation of multiple interconnected signaling cascades leading to acquisition of key features that in combination allow uncontrolled growth (Hanahan and Weinberg 2011). It is precisely because of the complexity and the latency observed in cancer development that the contribution from multiple diverse stressors should be investigated. Indeed, we believe this complexity—combined with recent advances in our understanding of cancer—brings a new perspective to mixtures research. Cancer is both well suited for research from a combined exposure perspective and a critical public health concern (Madia et al. 2019).

Considerable scientific debate has centered on attribution of risk factors contributing to cancer development. Although environmental exposures have been convincingly linked to certain cancer types, such as arsenic and cancers of the skin and lung (NTP 2016), some modeling efforts have suggested that most cancer incidences are attributable to random errors in DNA replication (Tomasetti and Vogelstein 2015; Tomasetti et al. 2017). The latter assertion has been vigorously contested, with competing modeling approaches suggesting that the contribution from

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intrinsic risk factors (e.g., DNA replication errors) has been greatly overstated (Wild et al. 2015; Wu et al. 2016). Furthermore, Wu et al. (2018) highlight the multifactorial nature of cancer causation and the possibility of interactions between intrinsic and extrinsic factors (e.g., environmental exposures that result in DNA mutations) in cancer development.

The history of cancer research provides a foundation for examining the effects of exposures to chemical mixtures on cancer development. Following identification of "initiation" and "promotion" as discernable stages of chemical carcinogenesis (Berenblum and Shubik 1947), subsequent observations of cancer development indicated more complex processes (Armitage and Doll 1954). Improved *in vitro* testing led to recognition of the importance of sequence of exposure to multiple carcinogens and the possibility that combinations of agents could increase carcinogenic responses, in comparison with responses associated with the individual agents (DiPaolo and Casto 1978). Based on these observations, there has been an explosion of research concerning the molecular mechanisms underlying carcinogenesis. Hanahan and Weinberg summarized these findings as the hallmarks of cancer (Hanahan and Weinberg 2000, 2011). The hallmarks include the underlying conditions of genetic instability and inflammation, along with the acquired capabilities of sustained proliferative signaling, evasion of growth suppressors, resistance to cell death, replicative immortality, induction of angiogenesis, activation of invasion and metastasis, reprogramming of energy metabolism, and elusion of immune surveillance (Hanahan and Weinberg 2011).

Although the hallmarks of cancer address the biological processes underlying cancer development, the key characteristics of carcinogens describe the properties of carcinogenic chemicals (Smith et al. 2016). The 10 key characteristics of carcinogens are that they: *a*) act as an electrophile either directly or after metabolic activation; *b*) are genotoxic; *c*) alter DNA repair or cause genomic instability; *d*) induce epigenetic alterations; *e*) induce oxidative stress; *f*) induce chronic inflammation; *g*) be immunosuppressive; *h*) modulate receptor-mediated effects; *i*) cause immortalization; and *j*) alter cell proliferation, cell death, or nutrient supply (Smith et al. 2016). The key characteristics of carcinogens have been used to evaluate the strength of mechanistic evidence of carcinogenicity of individual stressors (Temkin et al. 2020), and they hold promise for informing the development of research on combined exposures.

Consideration of the effects of combined exposures on cancer was the founding principle of the Halifax Project, in which cancer biologists and toxicologists investigated evidence for interactions between environmental chemicals and the hallmarks of cancer (Goodson et al. 2015). The group posited that chemicals present in the environment below levels considered to be harmful based on their individual dose–response relationships could act on different hallmark-related molecular targets and cumulatively contribute to the development of cancer (Goodson et al. 2015). Although there has been extensive work using initiation-promotion models that incorporate binary mixtures to explore mechanisms of carcinogenesis and evaluate interventions (DiGiovanni 1992; Hursting et al. 1999), few research efforts have attempted to elucidate how joint effects of combined exposures on cancer can quantitatively inform mixtures risk analysis (Arcos et al. 1988; Perez-Carreon et al. 2009; Siddens et al. 2012; Vial and Descotes 2003; Walker et al. 2005).

There are several examples of efforts to develop and refine predictive tools for estimating risk from combined exposures with noncancer diseases. Disruption of male reproductive tract development is a well-studied end point for combined exposures (Christiansen et al. 2009; Conley et al. 2018; Metzdorff et al. 2007; Rider et al. 2008). The premise is that chemicals act through different signaling pathways [e.g., phthalates that lower testosterone (Parks et al. 2000) and pesticides that block androgen receptor activity (Wilson et al. 2008)] to converge on a given adverse outcome or disease (e.g., malformations of the male reproductive tract) and exhibit effects at lower doses than if they were present alone. Numerous combinations of chemicals have been explored to interrogate the underlying hypothesis that responses to chemical mixtures occur at doses containing individual chemicals below their no observed effect levels (Howdeshell et al. 2017). In most cases, a model based on the concept of dose addition, where chemicals contribute cumulatively, appears to predict the observed chemical mixture responses (Kortenkamp 2020). The implication of these endocrine disruptor studies is that chemical mixtures risk assessments should not be limited to chemicals of a single class (e.g., phthalates) but instead also should include chemicals that target the same system or developmental process, such as male reproductive tract development (Kortenkamp 2020). However, it should be noted that the joint action of endocrine disruptors can be consistent with those predicted by dose additive models, or greater or less than those dose additive model predictions, depending on the dose–response relationships and underlying biology (Webster 2013).

More recent examples of newly developed tools to predict effects of mixtures can be found in the Horizon 20/20 EuroMix projects (Bopp et al. 2018; Lichtenstein et al. 2020; Rotter et al. 2018). Three different end points were selected for case study development: liver steatosis, adverse reproductive effects from endocrine disruption, and craniofacial malformations (Rotter et al. 2018). We contend that continuing to develop similar case studies on the use of mechanistic information to better understand the hazards posed by mixtures is necessary for increasing confidence in application of this disease-centric approach to grouping chemicals for risk assessment. Furthermore, we propose that cancer is an appropriate disease target for this type of mixtures research.

Considerations in Developing Research Approaches for Evaluating Chemical Mixtures and Cancer

A working group comprising the coauthors focused attention on the design of mixtures studies to inform cancer risk assessment as part of a larger effort to refine the key characteristics of carcinogens and explore their application. The underlying goal of the working group was to stimulate research in the area of mixtures and cancer by identifying and discussing potential approaches for researchers to consider. Toward this goal, working group members reviewed the key characteristics of carcinogens, hallmarks of cancer, mixtures research for other disease end points, and earlier efforts to highlight the need for research on mixtures and cancer (i.e., the Halifax Project). During two meetings at the University of California, Berkeley, and through subsequent correspondence, group participants discussed and debated research options. The approaches presented here represent consensus of the working group on promising paths for exploring the joint action of chemicals on cancer development. The considerations below and examples provided throughout this commentary are meant to provide a fresh perspective through the lens of the key characteristics approach and inform scoping activities, not to provide a definitive blueprint for execution of mixtures studies.

The low-dose hypothesis proposed by the Halifax Project and the mechanistic organization from the key characteristics of carcinogens provide a starting point to build a research program to elucidate possible joint action of chemicals on cancer (Goodson et al. 2015; Smith et al. 2016). We anticipate two goals for future work on the effects of combined exposures on cancer: *a*) to inform decisions on which chemicals to include in cancer-based chemical mixtures risk assessments, and *b*) to decrease

uncertainty in the quantitative evaluation of the cancer risks associated with those chemicals. With these goals as the foundation, several overarching considerations apply to research intended to inform decision-making on cancer and mixtures.

Traditionally, quantitative risk assessments rely on dose–response data from animal studies that are performed with relatively high doses of single chemicals. High doses facilitate detection of statistically significant adverse effects (e.g., tumors) while maintaining manageable sample sizes (Melnick et al. 2008). Significant debate has surrounded extrapolation from high doses to the lower doses that are more typical of human exposure to environmental contaminants (Rhomberg et al. 2011). The assumption of low-dose linearity based on the multistage somatic mutation theory (Armitage and Doll 1957) has been challenged based on examples of threshold-dependent mechanisms of action involved in cancer development such as inflammation (Bogen 2019). Such modeling issues are compounded in mixtures studies, which involve inputting data from dose–response analyses of multiple chemicals into additive models based on assumptions about their joint action (Rider et al. 2018). We propose that these challenges highlight the need for robust dose–response data for individual chemicals and recommend study designs that include careful dose selection to span the range of response levels as well as consideration of statistical power of the study.

Although a comprehensive review of the design options for mixtures studies is beyond the scope of this commentary and can be found elsewhere (Borgert et al. 2001; Simmons et al. 2018), some general principles bear emphasis. Two examples of mixture study designs that we recommend for consideration based on their utility in elucidating the joint action of chemicals include the isobolographic method for binary mixtures and fixed-ratio ray method for higher order mixtures (Figure 1) (Simmons et al. 2018). For example, the isobolographic method (Figure 1A) has been used in a high-throughput testing context to identify promising combination therapies that result in greater-than-additive effects (Griner et al. 2014). Alternatively, the fixed-ratio ray design (Figure 1B) has been used extensively to evaluate the joint action of chemical mixtures containing multiple chemicals (Conley et al. 2018; Crofton et al. 2005; Meadows et al. 2002).

Mutagenic or carcinogenic effects of environmentally relevant mixtures have been actively investigated (Benjamin et al. 1999; NTP 1993a, 1993b; Perez-Carreon et al. 2009; Shelby et al. 1990). We contend that there are significant challenges in interpreting studies performed exclusively in the low-dose region, particularly when expected mixture responses are not articulated. In general, such studies find a lack of clear carcinogenicity or potentiation with administration of environmentally relevant mixtures. However, it is important to note that these studies did not include modeling to establish expected outcomes based on knowledge of individual chemical dose–response relationships. Additionally, the chemical selection was based on environmental occurrence, not mechanistic information (NTP 1993a, 1993b). Another important factor in study design is selection of an appropriate sample size to allow for detection of statistically significant differences among treatment groups. An instructive example can be found in the prospective power calculations conducted to select sample sizes at each exposure concentration in a toxicological study on a complex mixture of drinking water disinfection byproducts (Dingus et al. 2011). In our opinion, the use of low exposure concentrations, lack of predicted effects, and inadequate sample sizes complicate extrapolation of findings to other mixture ratios, doses, or combinations of chemicals. Our recommendations include incorporating a range of mixture doses (from environmentally relevant to higher doses expected to elicit significant responses), selecting chemicals with available dose–response data or generating those data prior to mixture evaluation, using predictive additivity models to generate expected mixture outcomes for comparison to observed data, and incorporating power calculations to select sample sizes.

Toxicokinetic data are useful in understanding the behavior of mixtures within the model system and in translating data from the experimental model to the human experience (Thompson et al. 2008). For example, toxicokinetic and toxicodynamic models can be used to identify and characterize chemical interactions and as a basis for grouping chemicals for mixtures risk assessment according to common biological targets (Andersen and Dennison 2004). Although physiologically based toxicokinetic (PBTK) models have been used to evaluate some mixtures [e.g., benzene,

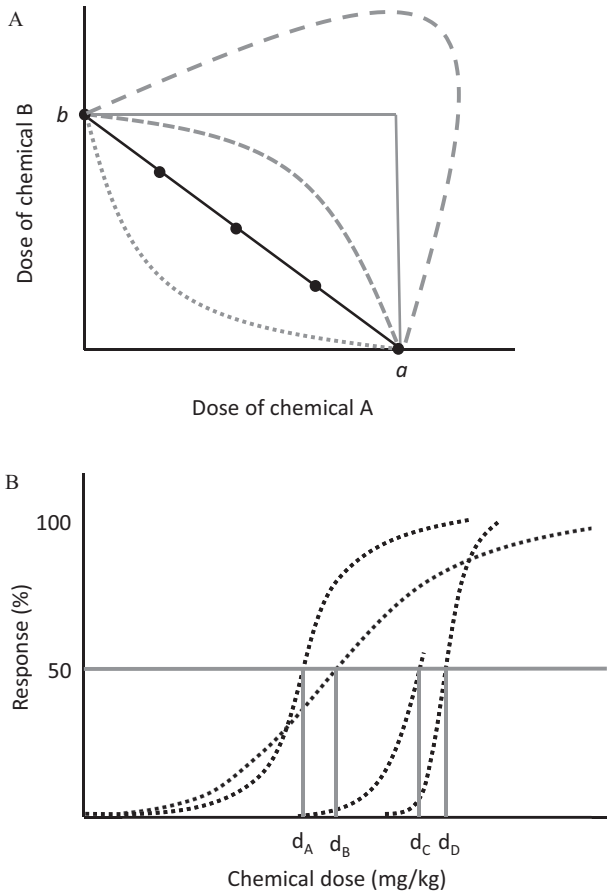

Figure 1. Mixture study designs. The isobolographic method (A) illustrates the possible effects of a binary combination of chemicals, where a and b represent the doses of chemicals A and B, respectively, that elicit equivalent effect levels [e.g., doses eliciting a response that is 50% of the maximum response (ED50)]. The solid black line connecting a and b is an isobole for two chemicals that are dose–additive. Selection of chemical ratios represented by the black dots along the isobole is recommended to provide multiple data points for comparison between observed and predicted responses. Experimental data could indicate the following types of joint action depending on the location of data points within the isobolograph: dose additivity (along the isobole), greater-than-additive interaction (e.g., dotted line), less-than-additive interactions (e.g., either of the dashed lines), or independent action (solid gray line). The fixed ratio ray method involves evaluation of the dose–response relationships of the individual chemicals (B) and a mixture containing each chemical in a set ratio. In this example, individual chemical dose–response relationships (dotted lines) are used to determine equipotent doses (i.e., d_A, d_B, d_C, d_D represent the ED50s for chemicals A, B, C, and D, respectively). Multiple doses (i.e., dilutions) of the mixture at the d_A:d_B:d_C:d_D ratio would then be evaluated, and mixture responses compared with predictions based on an assumption of dose additivity. Deviations of the experimental mixtures data from the predicted mixture responses could indicate less-than-additive or greater-than-additive interactions.

toluene, ethylbenzene, and xylene (Ruiz et al. 2020)], high data requirements preclude widespread adoption. However, we recommend including toxicokinetic measurements in mixtures study designs if at all possible.

Although the Halifax Project focused on promoting research on the combined effects of noncarcinogenic chemicals to induce cancer (Goodson et al. 2020), we propose that the initial goal should be to test the mixtures hypothesis itself. Thus, we propose that chemicals should be selected for inclusion in a mixtures research program based on the goal of targeting multiple molecular mechanisms involved in cancer (e.g., chemicals that display distinct key characteristics of carcinogens) in order to test hypotheses of joint action. At this time, we view focusing on chemicals that are likely to co-occur in the environment as important but secondary, and thus initial mixtures research might include known carcinogens to evaluate the underlying chemical interactions with biological targets. We contend that inclusion of a carcinogenic chemical(s) will allow quantitation of the change in potency of the carcinogen, or shift in the dose–response curve, when combined with noncarcinogenic chemicals.

The final consideration for a research program on mixtures and health outcomes pertains to selection of cancer as the disease of interest. As discussed in the "Introduction," cancer is a collection of pathologies that present in different tissues with varied etiologies (NCI 2021). Therefore, we contend that it is critical to consider whether a research program can be designed for a "generic" cancer, with principles applying globally to the disease, or whether a more targeted approach is required. Both scenarios are reflected among the options detailed below.

Approaches for Evaluating the Effects of Mixtures on Cancer

We developed three approaches (a chemical screening approach, a transgenic model-based approach, and a disease-centered approach) that consider the key elements of choice, namely chemicals, test model, and cancer type/site with different prioritization, which are reflected in the order of selection (Figure 2). Note, however, that the three approaches are not mutually exclusive and can be combined. For example, the screening approach to chemical selection can be used to identify chemicals that are then tested in either the transgenic model or disease-centered approach. Often, the selection of the first key element strongly influences the available options for the other element(s). All three approaches include chemicals expressing different key characteristics in mixtures studies.

Chemical Screening Approach

The first proposed approach for developing a research plan for evaluating chemical mixtures and cancer involves mining data from high-throughput screening (HTS) efforts and other databases to select chemicals that interact with key characteristic/hallmark-associated molecular targets (Figure 2A). In addition to being a stand-alone option for exploring combined effects of chemicals on cancer pathways, the screening approach could offer a complementary method for chemical selection to either the disease-centered or transgenic model-based approach.

This approach requires prioritization of chemical selection, followed by model selection. Whereas selected chemicals may target specific cancer sites, the chemical screening approach is cancer-site agnostic and relies on the common features of carcinogenesis. For example, Demetriou et al. (2018) analyzed biopsies from different cancer sites and proposed a temporal sequence for the acquisition of the hallmarks of cancer in which cancer unfolds in a generally common sequence with resisting cell death, insensitivity to antigrowth signals, and sustained proliferation occurring first (nearly simultaneously), followed by deregulated energetics, replicative immortality, and the activation of invasion and metastasis. They further noted that angiogenesis and avoiding immune destruction were hallmarks that could appear at varying steps in this common sequence (Demetriou et al. 2018).

Previous data mining efforts provide a model for identifying candidate chemicals. The U.S. Environmental Protection Agency proposed predicting the carcinogenic potential of chemicals in rodents by mining results from *in vitro* HTS assays, whose target genes mapped to pathways within the hallmarks of cancer framework (Kleinstreuer et al. 2013). Using a training set of 232 chemicals with data from 672 *in vitro* measurements, researchers identified *in vitro* end points that correlated with rodent carcinogenicity (e.g., mouse liver neoplasms). They mapped these measures to cancer hallmarks (e.g., angiogenesis, sustained proliferation), and in their model, the more activities induced at cancer-related endpoints by a chemical, the higher the probability that the chemical would be carcinogenic in rodents. Finally, the authors used the model to predict carcinogenicity of 33 chemicals not included in the training set and found that chemicals with higher scores (*in vitro* activity at more cancer-related end points) were more likely to be classified as "possible," "probable," or "likely" human carcinogens with few false negatives, i.e., 2 of the 33 chemicals with low *in vitro* scores were classified as "probable" or "likely" human carcinogens (Kleinstreuer et al. 2013).

This type of approach could be used to screen the library of chemicals evaluated in HTS programs such as ToxCast™ and Tox21 to identify environmental chemicals that act on molecular targets and indicate specific key characteristics of carcinogens. For example, chemicals that bind to the estrogen receptor would indicate potential for receptor-mediated activity. Other important factors to consider when using cell lines include: *a*) conclusions from research based solely on common cancer-derived cell lines may differ from results using cells derived from nonmalignant tissue, and thus key results should at least be confirmed in nonneoplastic cells; *b*) there are multiple examples in which exposure to a mixture of chemicals has effects that could not be anticipated from the results of exposure to the individual mixture components and, thus, there needs to be empirical study of mixture exposure in addition to estimates derived from the study of the individual chemicals within the mixture (Goodson et al. 2020).

Another example involves identification of the key characteristics expressed by carcinogens (listed by the International Agency for Research on Cancer based on literature review and ToxCast™ /Tox21 data) (Guyton et al. 2018). The authors concluded that most chemicals classified as Group 1 (carcinogenic to humans) and Group 2A (probably carcinogenic to humans) displayed multiple key characteristics of carcinogens, and some of the key characteristics appeared to be more prevalent than others. Although this analysis is limited by uneven data distribution (e.g., older, phased-out chemicals typically lack data on end points such as epigenetic changes) and other factors, the approach could be adapted to screen mechanistic data available for binary combinations of carcinogens to identify key characteristic combinations that result in greater-than-additive potency (Figure 2C). This principle is illustrated in a study of immunosuppressive drugs combined with carcinogens (Bugelski et al. 2010) that found no consistent potentiation of carcinogenic responses from known carcinogens in combination with immunosuppressive drugs. Instead, combinations resulted in either greater or lesser carcinogenic activity depending on the dose, cocarcinogen, and tissue (Bugelski et al. 2010). This finding likely reflects that immune modulation is a complex process that can be both pro- and anticarcinogenic, depending on the combination involved.

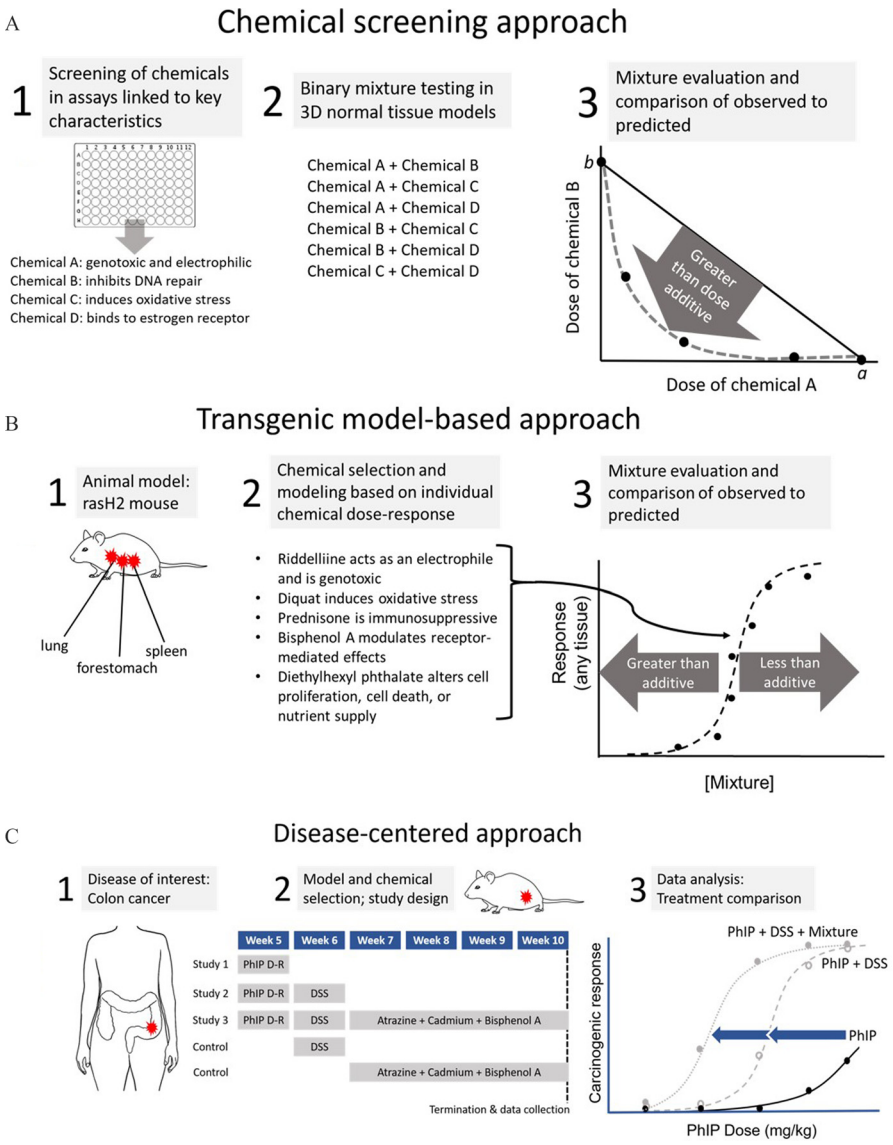

Figure 2. Three proposed approaches for designing studies to evaluate the combined action of chemicals on cancer. (A) An example of a chemical screening approach to study development and design. In this example, *in vitro* assays mapped to key characteristics of carcinogens are used to screen a library of chemicals. Chemicals that display specific activity at each of the key characteristics of carcinogens are selected. Binary combinations of chemicals are evaluated to elucidate the nature of joint action (e.g., dose addition, response addition, interaction). (B) An example of a transgenic model-based approach for study development and design is presented. In this example, the rasH2 mouse is the model and displays carcinogenicity at multiple sites. Next, chemicals are selected based on their expression of key characteristics of carcinogens. Dose–response data are generated for individual chemicals and additivity models are used to predict mixture responses (dashed dose–response curve). Finally, predicted responses are compared to observed mixture data (dots). (C) An example of a disease-centered approach for study development and design. First, colon cancer is selected as the disease of interest. Next, a PhIP/DSS mouse model (i.e., chemically induced model of colon cancer) and additional target chemicals (atrazine, cadmium, and bisphenol A) that exhibit different key characteristics of carcinogens are selected. Finally, a series of studies with the progressive addition of chemicals is conducted and data are analyzed to evaluate additivity.

The goals of this screening approach are to identify candidate chemicals for inclusion in mixture assessments and evaluate a number of combinations for joint action using *in vitro* models (Figure 2A). Recent efforts have sought molecular biomarkers and *in silico*/*in vitro* assays that correspond to key characteristics (Smith et al. 2020). The tools created through this work could be used to screen candidate chemicals for efficacy and potency. Based on the resulting list of candidate chemicals, research could then test binary combinations of chemicals in models with higher human relevance (but likely lower throughput), such as 3D tissue models to identify potential interactions (Figure 2A). For example, Williams et al. (2016) developed a mouse mammary gland organoid and investigated proteomic signatures following exposure to three chemical classes [phthalate, bisphenol A (BPA), and polychlorinated biphenyl] or to estrogen as a comparator. We view such models as useful to explore chemical combinations because they start with normal tissue and measure signatures indicative of processes involved in carcinogenesis (e.g., apoptosis, cell adhesion, and proliferation) (Williams et al. 2016). Higher order cell culture systems are improving our ability to mimic complex biological interactions *in vitro*, but challenges remain (Duval et al. 2017).

In the approach outlined in Figure 2A, individual chemicals are first screened for activity in *in vitro* assays mapped to the key characteristics of carcinogens. Chemicals that display significant activity for different key characteristics can then be evaluated

Table 1. Advantages and limitations of the three proposed approaches for evaluating mixtures and cancer.

Approach	Advantages	Limitations
Chemical screening	• Can generate rapid and cost-effective information on activity and potency of many chemicals— good for identifying unknowns • Incorporation of both screening assays and 3D tissue assays increases confidence in findings	• Lack of complexity in test systems complicates translation to whole animal models and humans • Limited ability to observe interactions among chemicals that require higher order systems
Transgenic model-based	• Can use a model with a large historical database to leverage existing data • Generalizable across cancer types • Robust design facilitates interpretation and extrapolation	• Requires significant investment due to need for individual chemical and mixture dose–response data • Translation complicated by a lack of one-to-one relationship with human disease (e.g., some cancer sites less relevant than others)
Disease-based	• Targeting specific cancer types can allow for greater translational context (focus on cancers with high human relevance and confidence in model) • Streamlined design minimizes dose groups required while providing data on potential chemical interactions • Flexibility to add chemicals (with unique key characteristics of carcinogens) in progressive studies • Biology of these cancers is well understood; system changes due to chemical insults can be compared to historical data	• Can only address cancers for which models are available • Models may have limited generalizability to other types of cancer • Should only include chemicals with key characteristics of carcinogens relevant to cancer of interest • Single dose of "additional" chemicals complicates extrapolation of findings to other exposure scenarios (doses, chemical ratios)

systematically in binary combinations using an isobolographic method to identify dose additivity and greater-than-dose–additive interactions. We anticipate that this type of investigation could provide important information on which key characteristics of carcinogens are more likely to additively contribute to carcinogenicity.

Transgenic Model-Based Approach

A second approach for designing studies on cancer and mixtures is to select a well-established transgenic rodent model and use a chemical mixture with appropriate key characteristics to target a combination of hallmarks critical to cancer development in that model. In other words, selection of the transgenic model is the priority, and key elements of the research program (e.g., cancer type, chemicals) are dependent on which transgenic model is selected. For study feasibility, both the p53+/– mouse and rasH2 mouse (e.g., CByB6F1-Tg(HRAS)2Jic) are generally accepted as a second-species short-term alternative to the lengthy and expensive conventional 2-y, 2-rodent test for cancer hazard identification/carcinogenicity by the International Conference on Harmonization (ICH) and the U.S. Food and Drug Administration (U.S. FDA) (Nambiar et al. 2012). For the purpose of building a research program on mixtures and cancer, the transgenic rasH2 mouse (Tg rasH2) 26-wk bioassay may be useful for several reasons. First, the presence of HRAS isolated from human cancer, which encodes oncogenic p21ras protein, makes Tg rasH2 mice highly susceptible to tumor development when exposed to chemicals known to cause cancer in humans. Overexpression of HRAS (defined as 2- to 3-fold higher expression levels compared with wild-type mice) rather than induction of point mutations is thought to be responsible for accelerated tumor development (Tamaoki 2001), although point mutations may play a role in some Tg rasH2 tumors (Mitsumori et al. 1998). Second, the incidence of spontaneous tumors across target tissues is generally low until 6 months of age, i.e., the duration of the Tg rasH2 bioassay (Mitsumori et al. 1998). Based on extensive historical control data, the model has a very stable tumor incidence range (Paranjpe et al. 2019). These features allow for the examination of multiple target sites of malignancies. The lung, spleen, and forestomach are considered informative target organs in the Tg rasH2 model, in addition to the target organs identified in standard 2-y carcinogenicity assays in rodents (e.g., colon, hematopoietic organs, skin, urinary bladder) reported in previous long-term carcinogenicity bioassays in rats and mice (Mitsumori et al. 1998). Third, the Tg rasH2 26-wk (6-month) bioassay is accepted by the ICH and the U.S. FDA for testing both nongenotoxic and genotoxic agents, whereas p53+/– mice are generally accepted only for genotoxic agents (Jacobs and Brown 2015). Results from a series of comprehensive studies showed that Tg rasH2 mice were more sensitive (with more rapid onset and/or higher incidence of more malignant tumors) to both genotoxic and nongenotoxic human carcinogens than non-Tg mice and unresponsive to noncarcinogens (Mitsumori et al. 1998; Morton et al. 2002; Usui et al. 2001; Yamamoto et al. 1998). Further, the Tg rasH2 bioassay predicts neoplastic findings relevant to human cancer risk assessment on par with 2-y rodent models and produces fewer human-irrelevant neoplastic outcomes (Morton et al. 2002).

The Tg rasH2 model has been used to test multiple chemicals, including known human and rodent carcinogens and noncarcinogens, as well as other chemicals, such as immunosuppressive agents, hormones, and peroxisome proliferators (Mitsumori et al. 1998; Morton et al. 2002; Usui et al. 2001; Yamamoto et al. 1998). From these reviews and additional studies we identified in PubMed, we gathered information on Tg rasH2 bioassay testing of 59 individual agents, 2 mixtures (tobacco smoke, mixed xylenes), and 17 chemical combinations (mainly initiator-promoter studies) (Excel Table S1). We propose leveraging the experimental details (e.g., route of exposure, dose) and outcome information (e.g., tumors, preneoplastic lesions) from these studies to inform study design of chemical mixtures in the Tg rasH2 assay.

We propose to test whether specific combinations of chemicals *collectively* possess sufficient critical key characteristics relevant to cancer development such that together they induce tumors in Tg rasH2 mice, even if the chemicals are unable to do so individually. Important considerations are the selection of the most informative and relevant: *a*) key characteristics (i.e., characteristics that drive cancer development and are not markers of late-stage tumorigenesis), and *b*) chemicals (i.e., chemicals that are

linked to specific key characteristics and have high exposure potential) to include in mixtures. The study design proposed for the transgenic model approach differs from that proposed for the disease-centered approach in two important ways: *a*) It considers multiple tumor types in a single model, and *b*) It involves a classic mixtures fixed-ratio ray design (Figure 2B). In contrast to the disease-centered approach, which has a preselected cancer site of interest, the transgenic model approach includes all tumor sites for analysis. Lung adenoma and carcinoma, forestomach papilloma and carcinoma, skin papilloma, and spleen hemangiosarcoma are all relevant tumor end points in Tg rasH2 mice. We assert that HRAS2 overexpression leads to the cancer hallmark of "sustained proliferative signaling" and overlaps with the key characteristic of carcinogens of "alters cell proliferation, cell death, or nutrient supply." Additional chemicals to be considered for inclusion in mixtures studies should act through other mechanisms to incorporate different key characteristics of carcinogens and hallmarks of cancer. The fixed-ratio ray design is commonly employed in mixtures experiments (Meadows et al. 2002). According to this method, a fixed ratio of chemicals is evaluated at multiple dilutions to generate observed dose–response data, which is then compared to responses predicted using individual chemical data and assumptions about the type of joint action (e.g., dose additivity, independent action). Concordance between predicted and observed data indicates support for the underlying additivity assumption, whereas deviation indicates a potential interaction among mixture components (Figure 2B). Methods for statistically comparing predicted to observed dose–response curves have been described previously (Gennings 2018; Jonker et al. 2005).

Chemicals identified through either the Halifax Project or review of publications using the Tg rasH2 model (Excel Table S1) are candidate mixture components. For example, chemicals that were negative or equivocal when tested individually in the Tg rasH2 bioassay but possess some key characteristics of carcinogens would be candidate mixture components. For such chemicals, the highest negative individual dose previously tested could be used in the mixture. Another important consideration in study design involves power to detect treatment-related changes, as discussed in the "Introduction" section above. Based on power calculations, 20–25 mice per sex per group were recommended for carcinogenicity assessment studies in Tg rasH2 mice (Morton et al. 2002). Because power calculation results are derived from expected magnitude and frequency of change, testing of chemicals with less carcinogenic potential may require more animals, longer study duration, and/or other modifications to assure adequate power to detect differences in tumor incidence (Maronpot et al. 2000). Preneoplastic proliferative lesions could be informative intermediate outcomes. For example, proliferative lesions (e.g., hyperplasia, polyp) mark an important step in the continuum from healthy tissue to metastatic tumor in some cancer types (Cardiff et al. 2006).

Although the transgenic model approach described here is focused on simultaneous chemical exposures, the study design could easily be adapted to assess more complex mixture scenarios, such as sequential exposures, exposure during critical developmental windows, and addition of nonchemical stressors (e.g., physiological stress, chronic infection, obesity) (McHale et al. 2018). For example, obesity increases the risk of arsenic-associated lung and bladder cancer in humans by several-fold compared with risks for nonobese individuals (Steinmaus et al. 2015). Human cancer has been associated with early-life environmental conditions (Grandjean et al. 2015; Moore 2016; Walker and Ho 2012) and is sometimes mediated by persistent epigenetic modifications (a key characteristic of carcinogens) (El Hajj et al. 2014). Examples of animal studies that have assessed cumulative risk include early-life environmental tobacco smoke exposure, later exposure to asbestos, and increased risk of lung disease via immune effects in mice (Brown et al. 2016); prenatal dioxin exposure, later high-fat feeding, and elevated risk of mammary cancer in mice (La Merrill et al. 2010).

Disease-Centered Approach

In a disease-centered research program, key elements of the program are decided sequentially, in the order of cancer type, model system(s) that reflects the selected cancer type, and chemical selection (Figure 2C). Among other features, the ideal cancer type for study has relatively well-defined etiology with multiple contributing mechanisms, widespread occurrence in diverse populations, and evidence for environmental contributions. A well-defined etiology and knowledge of key events in development of the selected cancer can help discern which key characteristics are likely to contribute meaningfully to early stages of disease. Colorectal cancer is an example where many important molecular targets have been identified (Marmol et al. 2017), and therefore it constitutes an instructive example for how to build a research plan using the disease-centered approach. The working group also considered cancers of the breast and liver.

Following cancer type selection, the next steps are to select a model system that reflects that cancer type and the molecular targets of interest (Figure 2C). Selecting a cancer model with human relevance is critical. Although human cell-based systems can aid in mechanistic understanding of discrete molecular events in colorectal carcinogenesis, more complex systems that provide integration across multiple signaling pathways are recommended for evaluating the joint effects of chemicals that act at different targets. Multiple animal models of colorectal cancer are available (Johnson and Fleet 2013). Because large intestine tumors are rare in rodents, chemical or genetic interventions might help increase the background occurrence of human-relevant tumors in animal models of colorectal cancer (Johnson and Fleet 2013). Recently developed organoid models (Lau et al. 2020) may also be useful for studying the joint action of chemicals that act at multiple molecular targets.

Selection of an animal model is linked to identification of molecular targets or key characteristics of carcinogens because the chemical treatment or genetic manipulation used to induce the cancer will dictate one or more molecular target(s). Among various animal models for colorectal cancer (Johnson and Fleet 2013), treatment with 2-amino-1-methyl-6-phenylimidozo [4,5-*b*] pyridine (PhIP) is a particularly attractive choice because this heterocyclic amine is prevalent in cooked meat and is linked to colon cancer in humans (Góngora et al. 2019). One variation of the PhIP model uses a transgenic mouse with human cytochrome P450 1A2 (CYP1A2) enzyme, which unlike the endogenous mouse CYP1A2, converts PhIP to its active metabolite (Cheung et al. 2005). In a further refinement of this model, addition of dextran sodium sulfate (DSS) promoted inflammation in the form of colitis (Chen et al. 2017) and produced colon tumors that displayed molecular and histological features observed in human colon cancer. Treating rodents with PhIP caused mutations in *Cnntb1* and *Apc* genes (Dashwood et al. 1998). PhIP is both electrophilic and genotoxic (Peng et al. 2012), encompassing two key characteristics of carcinogens, and inclusion of DSS adds the key characteristic of chronic inflammation. Based on colon cancer etiology and known risk factors, other key characteristics might include genetic instability and altering DNA repair [microsatellite instability pathway (Marmol et al. 2017)], inducing epigenetic alterations [DNA methylation and histone modification (Jung et al. 2020)], oxidative stress [lipid peroxidation (Bastide et al.

2011)], and receptor-mediated effects altering cell proliferation [epidermal growth factor receptor (EGFR) and transforming growth factor-beta (TGF-β) receptor pathways (Marmol et al. 2017)].

Finally, chemical selection for a mixture study could consider the chemical's established key characteristics that will produce the hallmarks observed in the specific cancer under study, in addition to the chemical's relevance to human exposure. For colorectal cancer, PhIP would be a clear candidate for inclusion in a mixtures research program, whereas DSS is an experimental tool for inducing colitis and not an environmental chemical of concern for human exposure. A decision to use DSS must balance its proven effectiveness in inducing inflammation in the target tissue against the use of a different, more human exposure-relevant inflammatory chemical in mixture assessment, e.g., glycation end products such as imidazole that are found in the diet and have inflammatory properties (Băbţan et al. 2019). Other candidates to consider include atrazine for potential effects on the epigenome (Sanchez et al. 2020), cadmium for its capacity to induce oxidative stress (Mezynska and Brzóska 2018), and estrogenic chemicals such as BPA for EGFR activation (Sauer et al. 2017). Ideally, dose–response data would be available for each candidate chemical in the animal model selected for study. In the case of PhIP, it is both intrinsic to the model for colon cancer and one of the mixture components. Therefore, analysis would involve measurement of the shift in the PhIP dose–response curve when it is present along with the additional chemicals. We recommend selecting doses for the remaining chemicals based on existing toxicity data with a goal of providing a challenging dose (i.e., inducing significant changes in select measured parameters) at the top dose of the mixture and serial dilutions of that dose. It is important to recall that environmental chemicals are not necessarily specific-acting, i.e., many environmental chemicals can act via multiple mechanisms [e.g., benzo[*a*]pyrene is metabolized to a diol epoxide electrophile that interacts with DNA to form adducts and also binds to the aryl hydrocarbon receptor (AhR) (van Delft et al. 2012)]. Therefore, many environmental chemicals will likely induce a cascade of molecular events that touch upon multiple mechanisms as a result of expressing different key characteristics.

Other considerations in the disease-centered approach include timing and dose selection for individual mixture constituents. Sequential dosing is used in the PhIP/DSS model described above, and this design could be adapted for a more quantitative evaluation of mixture effects by combining elements from the mixtures literature. An example of a disease-centered design using the PhIP/DSS model is presented in Figure 2C. This design has been used previously to quantify the shift in the dose–response curve of a chemical when a second chemical is added at a dose at or around its no observed adverse effect level (Blystone et al. 2009). In the PhIP/DSS example, the first study consists of a series of doses of PhIP alone, aimed at generating a dose–response relationship. The second study builds on this by adding a single dose of DSS during the window established as being effective at promoting the effects of PhIP according to model development (Chen et al. 2017). Additional treatments, such as an individual chemical or a combination of chemicals (e.g., atrazine, cadmium, and BPA; Figure 1C), are included in the third study. Finally, two control groups, consisting of DSS alone and the chemical combination, are also included to confirm a lack of tumor development with each of the non-PhIP treatments alone at the dose level tested in the mixture studies (studies 2 and 3). In this example, results from studies 1, 2, and 3 are compared to quantify the shift in the PhIP dose–response curve. A shift to the left would indicate an additive or greater-than-additive mixture effect, whereas a shift to the right would indicate a less-than-additive interaction (Figure 1C). Differentiating between additivity and a greater-than-additive interaction would require knowledge of the dose–response curves for the secondary treatments (DSS and chemical combination), which could be explored in subsequent studies if a notable leftward shift is observed.

Conclusions

Humans are exposed to numerous chemicals over their lifetimes, and more work is needed to understand how combined exposures to chemicals in the form of mixtures influence disease, particularly cancer, because the accumulation of insults over time contributes to cancer development and progression. Here, we offer three different options for investigating the joint effects of chemicals on cancer (a chemical-based approach, a model-based approach, and a disease-based approach) to stimulate discussion and research efforts around this critical topic. Some advantages and limitations of each approach are provided in Table 1. These approaches represent a distinct shift in the current paradigm for risk evaluation in which there is limited consideration of cumulative impacts on a disease. Further, findings from research studies based on these approaches could help to refine the existing concepts, frameworks, resources, and data on which the approaches are built.

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