

Key Characteristics of Carcinogens as a Basis for Organizing Data on Mechanisms of Carcinogenesis

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Characteristic Properties of Human Carcinogens

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Abstract

Background: A recent review by the International Agency for Research on Cancer (IARC) updated the assessments of the more than 100 agents classified as Group 1, carcinogenic to humans (IARC Monographs Volume 100, parts A-F). This exercise was complicated by the absence of a broadly accepted, systematic method for evaluating mechanistic data to support conclusions regarding human hazard from exposure to carcinogens. Objectives and Methods: IARC therefore convened two workshops in which an international Working Group of experts identified 10 key characteristics, one or more of which are commonly exhibited by established human carcinogens. Discussion: These characteristics provide the basis for an objective approach to identifying and organizing results from pertinent mechanistic studies. The ten characteristics are the abilities of an agent to: (1) act as an electrophile either directly or after metabolic activation; (2) be genotoxic; (3) alter DNA repair or cause genomic instability; (4) induce epigenetic alterations; (5) induce oxidative stress; (6) induce chronic inflammation; (7) be immunosuppressive; (8) modulate receptor-mediated effects; (9) cause immortalization; and (10) alter cell proliferation, cell death, or nutrient supply. Conclusion: We describe the use of the 10 key characteristics to conduct a systematic literature search focused on relevant endpoints and construct a graphical representation of the identified mechanistic information. Next, we use benzene and polychlorinated biphenyls as examples to illustrate how this approach may work in practice. The approach described is similar in many respects to those currently being implemented by the U.S. EPA's IRIS Program and the U.S. National Toxicology Program.

250 words

Introduction

Recently, the International Agency for Research on Cancer (IARC) completed a review of all its Group 1 human carcinogens and updated information on tumor sites and mechanisms of carcinogenesis (IARC Monograph Volume 100A-F). About half of the agents classified in Group 1 had been last reviewed more than 25 years ago, before mechanistic studies became prominent in evaluations of carcinogenicity. In addition, more recent studies have demonstrated that many cancer hazards reported in earlier studies were later observed to also cause cancer in other organs or through different exposure scenarios (Cogliano et al. 2011).

In compiling and updating the information for Volume 100A-F, two overarching issues became apparent. First, no broadly accepted systematic method for identifying, organizing, and summarizing mechanistic data for the purpose of decision-making in cancer hazard identification was readily available. Second, the agents documented and listed as human carcinogens showed a number of characteristics that are shared among many carcinogenic agents. Many human carcinogens act via multiple mechanisms causing various biological changes in the multistage process of carcinogenesis. Indeed, cancer was once described by reference to causative agents, with multistage development of tumors being characterized through the impact of particular chemicals described as initiators and promoters of cancer. Subsequently, multistage development of cancer was identified with morphological change being correlated with genetic alterations. The more recent description by Hanahan and Weinberg of hallmarks of cancer is not predicated on morphology or the impact of carcinogens, but on changes in gene expression and cell signaling (Hanahan and Weinberg 2011). These hallmarks are the properties of cancer cells and neoplasms, and are not characteristic of the agents that cause cancer. Tumors attributable to chemical carcinogens may be distinct by mutational analysis (Westcott et al, 2015), but all neoplasms exhibit the hallmarks. A recent computational toxicology study has shown that chemicals that alter the targets or pathways among the hallmarks of cancer are likely to be carcinogenic (Kleinstreuer et al. 2013). In addition, a series of reviews in *Carcinogenesis* by members of the Halifax Project Task Force utilized the hallmarks framework to identify the carcinogenic potential of low doses and mixtures of chemicals (Harris, 2015).

In 2012, participants at two workshops convened by the IARC in Lyon, France extensively debated the mechanisms by which agents identified as human carcinogens (Group 1) produce cancer. The participants concluded that these carcinogens frequently exhibit one or more of 10 key characteristics (Figure 1). Herein we describe these 10 key characteristics and discuss their importance in carcinogenesis. These characteristics are properties that human carcinogens commonly show and can encompass many different types of mechanistic endpoints. They are not mechanisms in and of themselves nor are they adverse outcome pathways.

Further, we describe how the 10 key characteristics can provide a basis for systematically identifying, organizing, and summarizing mechanistic information as part of the carcinogen evaluation process. The U.S. Environmental Protection Agency (EPA) and the National Toxicology Program (NTP) in the U.S., as well as the IARC internationally, have recognized a need for such an approach (Rooney et al. 2014). The U.S. National Research Council emphasized the need for consistent, transparent, systematic approaches for the identification, evaluation, and integration of data in EPA's IRIS assessments of carcinogens and elsewhere in

human health hazard assessments (NRC 2014).

Progress in the systematic evaluation of published evidence on the adverse health effects of environmental agents has been made through application of methods developed by evidence-based medicine (Koustas et al. 2014). However, mechanistic study databases present a challenge to systematic reviews in that the studies are typically both numerous and diverse, reporting on a multitude of endpoints and toxicity pathways. One recent example of a systematic approach searched for studies on endpoints relevant to nine cancer-related mechanistic categories in identifying and presenting mechanistic evidence on di(2-ethylhexyl)phthalate, a chemical with a complex database of over 3000 research papers (Kushman et al. 2013). In this publication, the categories of mechanistic evidence were identified from a compendium of published reviews. This approach may be difficult to translate to agents with controversial or limited mechanistic evidence. It also would not permit comparisons across agents, including attempts to understand similarities or differences with human carcinogens. Further, it may be biased against the most recent mechanistic and molecular epidemiology studies that have not been the subject of a prior expert review.

To facilitate a systematic and uniform approach to organizing mechanistic data relevant to carcinogens, we propose the use of 10 key characteristics of human carcinogens as a basis for identifying and categorizing scientific findings relevant to cancer mechanisms when assessing whether an agent is a potential human carcinogen. A significant advantage of this approach is that it would encompass a wide range of endpoints of known relevance to carcinogenesis as identified through examination of the IARC Monographs on Group 1 carcinogens. Mechanistic topics can be included regardless of whether they have been the subject of prior expert reviews of any particular chemical. This should introduce objectivity that could reduce reliance on expert opinion, as well as facilitate comparisons across agents. Moreover, at its essence, the approach may afford a broad consideration of the mechanistic evidence rather than focusing narrowly on independent mechanistic hypotheses or pathways in isolation.

Herein, we demonstrate the applicability of this proposed systematic strategy for searching and organizing the literature using benzene and polychlorinated biphenyls (PCBs) as examples. The mechanistic study database for both of these chemicals is large, comprising over 1,800 studies for benzene and almost 3,900 for PCBs, many with multiple mechanistic endpoints. We conducted systematic literature searches for endpoints pertinent to the 10 key characteristics of human carcinogens, utilizing literature trees to indicate the human and experimental animal studies that reported endpoints relevant to each characteristic. To further indicate their potential contribution to benzene and PCB carcinogenesis, we organized the characteristics into a graphical network representative of an overall mechanistic pathway.

Two recent IARC Monographs (Guyton et al. 2015; Loomis et al. 2015) have applied the 10 key characteristics described here for a variety of agents and also organized the results into graphical networks. Overall, this categorization facilitated objective consideration of the relevant mechanistic information, thereby advancing analyses of hypothesized mechanisms and toxicity pathways. Because mechanistic data may provide evidence of carcinogenicity, and can play a role in up- or downgrading an evaluation based on cancer findings in animals, we suggest that this systematic approach to organizing the available data will assist future IARC Working

Groups and other agencies in evaluating agents as potential human carcinogens especially in the absence of convincing epidemiological data on cancer in humans.

Description of the Key Characteristics of Carcinogens

The number of ways by which agents contribute to carcinogenesis can be extensive if all biochemical or molecular endpoints are considered. However, these mechanisms can be grouped into a limited number of categories (e.g., genotoxicity, immunosuppression, etc.). Guyton and coworkers described 15 types of “key events” associated with human carcinogens that collectively represented many carcinogenic mechanisms (Guyton et al. 2009). The experts present at the first of the IARC meetings in 2012 originally identified 24 mechanistic endpoints with several subcategories in each. This number of endpoints was considered too impractical as a guide for categorizing the literature, and the Working Group merged these categories into 10 at the second meeting in 2012, concluding that human carcinogens commonly show one or more of the 10 key characteristic properties listed in Figure 1. These represent the majority of established properties of human carcinogens as described below.

Characteristic 1: Is Electrophilic or Can Be Metabolically Activated to Electrophiles

Electrophiles are electron-seeking molecules that commonly form addition products, commonly referred to as adducts, with cellular macromolecules including DNA, RNA, lipids and proteins. Some chemical carcinogens are direct-acting electrophiles, whereas others require chemical conversion within the body (Salnikow and Zhitkovich 2008), or biotransformation by enzymes in a process termed metabolic activation (Miller 1970). Examples of direct-acting electrophilic carcinogens include sulfur mustards and ethylene oxide (Batal et al. 2014; Grosse et al. 2007; IARC 2008; Rusyn et al. 2005). The classic examples of chemical agents that require metabolic activation to become carcinogenic include polycyclic aromatic hydrocarbons, aromatic amines, *N*-nitrosamines, aflatoxins and benzene, which by themselves are relatively inert (Slaga et al. 1980; Smith 1996). A number of enzymes, including cytochrome P450s, flavin mono-oxygenase, prostaglandin synthase and various peroxidases, can biotransform relatively inert chemical compounds to potent toxic and carcinogenic metabolites or reactive intermediates (Hecht 2012; O'Brien 2000). The ability to form adducts on nucleic acids and proteins is a common property of these inherently electrophilic and/or metabolically activated human carcinogens (Ehrenberg 1984).

Characteristic 2: Is Genotoxic

The term genotoxic (Ehrenberg 1973) refers to an agent that induces DNA damage, mutation, or both. DNA damage can be spontaneous in origin through errors of nucleic acid metabolism or can be induced by endogenous or exogenous agents. In some cases the exogenous agents may also be generated endogenously, such as formaldehyde and acetaldehyde, producing a background level of DNA damage. Examples of DNA damage include DNA adducts (a molecule bound covalently to DNA), DNA strand breaks (breaks in the phosphodiester bonds), DNA crosslinks, and DNA alkylation. DNA damage by itself is not a mutation and generally does not alter the linear sequence of nucleotides (or bases) in the DNA, whereas a mutation is a change in the DNA sequence and usually arises as the cell attempts to repair the DNA damage

(Shaughnessy and DeMarini 2009).

Mutations can be classified into three groups based on their location or involvement in the genome. Gene or point mutations are changes in nucleotide sequence within a gene (e.g., base substitutions, frameshifts, and small deletions/duplications). Chromosomal mutations are changes in nucleotide sequence that extend over multiple genes (e.g., chromosome aberrations, translocations, large deletions, duplications, insertions, inversions, or micronuclei due to chromosome breakage). Genomic mutations involve the duplication or deletion of nucleotide sequences of an entire chromosome, an example of which is aneuploidy or formation of micronuclei that contain a centromere. A large proportion of Group 1 carcinogens are genotoxic, as documented in IARC Monograph Volume 100 A-F.

Characteristic 3: Alters DNA Repair or Causes Genomic Instability

Normal cells avoid deleterious mutations by replicating their genomes with high accuracy. However, the fidelity of DNA replication can vary widely depending on the DNA polymerase involved, introducing the possibility of error. Indeed, most spontaneous mutations are caused by polymerase error (Preston et al. 2010). The nature of the error, the flanking sequence, the presence of DNA damage and the ability to correct errors, all impact on the outcome of this process (Arana and Kunkel 2010). As a consequence, defects in processes that determine DNA-replication fidelity can confer strong mutator phenotypes that result in genomic instability. Thus, carcinogens may act not only by producing DNA damage directly, but also by altering the processes that control normal DNA replication or repair of DNA damage. Examples include the inhibition of DNA repair by cadmium (Candeias et al. 2010) and formaldehyde (Luch et al. 2014).

Genomic instability is a well-recognized feature of many cancers (Bielas et al. 2006) and considered to be one of the enabling characteristics of cancer (Hanahan and Weinberg 2011). Cells exposed to ionizing radiation have genetic instability that is a relatively late-occurring event that appears several cell generations after irradiation and results in a reduced ability to replicate the genotype faithfully (Kadhim et al. 2013). The events indicating genomic instability include chromosome aberrations, gene mutations, microsatellite instability, and apoptosis. These events are observed after exposure to arsenic (Bhattacharjee et al. 2013) and cadmium (Filipic 2012).

Characteristic 4: Induces Epigenetic Alterations

The term “epigenetic” refers to stable changes in gene expression and chromatin organization that are not caused by changes in the DNA sequence itself and can be inherited over cell divisions (Herceg et al. 2013). Epigenetic phenomena, including changes to the DNA methylome and chromatin compaction states, along with histone modification can impact the carcinogenic process by affecting gene expression and DNA repair dynamics (Herceg et al. 2013). A wide range of carcinogens have been shown to deregulate the epigenome, and it has been suggested that their mechanism may involve disruption of epigenetic mechanisms (Pogribny and Rusyn 2013). However, evidence for a causal role of epigenetic changes in cancer caused by Group 1

agents was considered to be limited in Volume 100, and for many agents, their impact on the epigenome was considered to be a secondary mechanism of carcinogenesis. Herceg and others (Herceg et al. 2013) have described a wealth of studies demonstrating the impact of carcinogens on epigenetic mechanisms. They note, however, that most carcinogens (even those reviewed for Volume 100 in 2008 and 2009) were evaluated by IARC Working Groups before new data on their epigenetic effects became available. This evolving area will generate new mechanistic data in the years to come.

Characteristic 5: Induces Oxidative stress

Many carcinogens are capable of influencing redox balance within target cells. If an imbalance occurs, favoring formation of reactive oxygen and/or nitrogen species at the expense of their detoxification, this is referred to as oxidative stress. Reactive oxygen species and other free radicals arising from tissue inflammation, xenobiotic metabolism, interruption of mitochondrial oxidative phosphorylation (Figueira et al. 2013), or reduced turnover of oxidized cellular components may play key roles in many of the processes necessary for the conversion of normal cells to cancer cells. However, oxidative stress is not unique to cancer induction and is associated with a number of chronic diseases and pathological conditions, e.g., cardiovascular disease, neurodegenerative disease, and chronic inflammation. Oxidative stress is also a common occurrence in neoplastic tissue and can be part of the tumor environment.

Oxidative damage is considered a major factor in the generation of mutations in DNA and over 100 different types of oxidative DNA damage have been identified (Klaunig et al. 2011). At least 24 base modifications are produced by reactive oxygen species, as well as DNA-protein crosslinks and other lesions (Berquist and Wilson 2012), all potentially leading to genomic instability. Oxidative damage to DNA can lead to point mutations, deletions, insertions, or chromosomal translocations, which may cause oncogene activation and tumor suppressor gene inactivation, and potentially initiate or promote carcinogenesis (Berquist and Wilson 2012; Klaunig et al. 2011). Thus, the induction of oxygen radical-induced cellular injury is a characteristic of a set of diverse carcinogens, including radiation, asbestos, and carcinogenic infectious agents.

Characteristic 6: Induces Chronic Inflammation

Chronic inflammation from persistent infections, such as that caused by *H. pylori*, as well as that produced by chemical agents including silica or asbestos fibers, has been associated with several forms of cancer (Grivennikov et al. 2010). Indeed, inflammation has been hypothesized to contribute to multiple aspects of cancer development and progression (Trinchieri 2012) and is an enabling hallmark of cancer (Hanahan and Weinberg 2011). Inflammation acts by both intrinsic and extrinsic pathways. Persistent infection and chronic inflammation disrupt local tissue homeostasis and alter cell signaling, leading to the recruitment and activation of inflammatory cells. These constitute extrinsic pathways linking inflammation to cancer (Multhoff and Radons 2012). On the other hand, intrinsic pathways driven by activation of proto-oncogenes in pre-neoplastic and neoplastic cells recruit host-derived inflammatory cells that accelerate tumor promotion and progression (Grivennikov et al. 2010). Because strong links exist between inflammation and the induction of oxidative stress and genomic instability, it may be difficult to

separate out the importance of each of these mechanisms.

Characteristic 7: Is Immunosuppressive

Immunosuppression is a reduction in the capacity of the immune system to respond effectively to foreign antigens, including antigens on tumor cells. Persistent immunosuppression presents a risk of cancer, especially excess risk for lymphoma. For example, immunosuppression poses a significant risk when it is accompanied by continuing exposure to foreign antigens, such as in people with organ transplants, or when it occurs in individuals who are latently infected with a carcinogenic virus (Hartge and Smith 2007; Smith et al. 2004). Immune suppression differs from other mechanisms of carcinogenesis in that agents that cause immunosuppression may not directly transform normal cells into potential tumor cells. Potentially neoplastic cells that arise naturally, or that have been transformed by other carcinogens acting by a mechanism such as genotoxicity or by the various mechanisms of action associated with carcinogenic viruses, escape immune surveillance in immunosuppressed individuals. As a result, survival of these cells and their replication to form tumors is greatly facilitated by immune suppression. Several carcinogens act entirely or largely by immunosuppression, often in concert with other Group 1 agents, especially oncogenic infectious agents. The Group 1 agents that act by immunosuppression include Human Immunodeficiency Virus (HIV-1) and the immunosuppressive drug cyclosporin (Rafferty et al. 2012).

Characteristic 8: Modulates Receptor-mediated effects

Numerous carcinogens act as ligands to receptor proteins, including menopausal hormone therapy, 2,3,7,8-tetrachlorodibenzo-para-dioxin and PCBs. Receptor-mediated activation broadly falls into two categories: (a) intracellular activation, mediated by nuclear receptors that translocate into the nucleus and act on DNA as transcription factors (Aranda and Pascual 2001); and (b) activation of cell surface receptors that induce signal-transduction pathways resulting in biological responses that involve a variety of protein kinases (Griner and Kazanietz 2007). Most exogenous agents act as agonists by competing for binding with an endogenous ligand; however, there are also receptors for which few or no endogenous ligands have been identified, such as the aryl-hydrocarbon (Ah) receptor (Baek and Kim 2014; Ma 2011). Receptor-mediated activation most often results in changes in gene transcription. Molecular pathways that are regulated through ligand-receptor interaction and are most relevant to carcinogenesis include cell proliferation (e.g., stimulation of the normal proliferative pathways as is the case for estrogen-dependent tissues and hormone therapy), xenobiotic metabolism, apoptosis, as well as modulation of the bioavailability of endogenous ligands by affecting biosynthesis, bioactivation, and degradation.

Characteristic 9: Causes Immortalization

Several human DNA and RNA viruses, including various human papillomaviruses, Epstein-Barr virus, Kaposi's sarcoma-associated herpesvirus, hepatitis B virus, hepatitis C virus, and human immunodeficiency virus, are carcinogenic to humans. These viruses have evolved multiple molecular mechanisms to disrupt specific cellular pathways to facilitate aberrant replication. Although oncogenic viruses belong to different families, their strategies in human

cancer development show many similarities and involve viral-encoded oncoproteins targeting the key cellular proteins that regulate cell growth (Saha et al. 2010). Recent studies show that virus and host interactions also occur at the epigenetic level (Allday 2013). The result of these viral effects is to immortalize the target tissue cells such that they are not subject to the Hayflick limit, the point at which cells can no longer divide due to DNA damage or shortened telomeres. For example, the Human Papillomavirus type-16 (HPV-16) *E6* and *E7* oncogenes are selectively retained and expressed in cervical carcinomas, and expression of *E6* and *E7* is sufficient to immortalize human cervical epithelial cells.

Characteristic 10: Alters Cell Proliferation, Cell Death or Nutrient Supply

There are at least three scenarios related to carcinogenesis in which alterations in cellular replication and/or cell-cycle control have been described. One invokes the predisposition for unrepaired DNA damage leading to cancer-initiating mutations in replicating cells, another has attempted to identify sustained replication as a key mechanistic event, and a third describes the ability of a transformed cell to escape normal cell-cycle control and to continue replication. A component common to all three scenarios is the evasion of apoptosis or other terminal programming, including autophagy, in at least a proportion of the cell population (Ryder et al. 2014).

Necrotic cell death releases pro-inflammatory signals into the surrounding tissue microenvironment, recruiting inflammatory immune cells to the site of trauma, which can enhance cancer-cell proliferation and promote cancer metastasis (Coussens and Pollard 2011; Coussens et al. 2013; Pollard 2008). In contrast, various forms of apoptosis and autophagy (Galluzzi et al. 2015) have the opposite effect by removing potentially cancerous cells from a population before they acquire the changes permitting malignancy. Many agents affect necrosis, apoptosis and/or autophagy and can have profoundly divergent effects on cancer induction in different tissues.

In addition to cell death caused directly by agent toxicity, cells may die within a tumor as a result of an impaired nutrient supply. Neoplastic cell numbers can increase exponentially, quickly outstripping the supply capabilities of the existing tissue vasculature. Neoangiogenesis, in which new blood vessels grow into a tumor, is key to providing this supply of nutrients. Thus, agents that promote or inhibit angiogenesis, such as arsenic, will promote or delay tumor growth (Wang et al. 2013; Yang et al. 2014).

Cancer cells also usually show quite different cellular energetics, relying on glycolysis for energy even under aerobic conditions (Rajendran et al. 2004). Although a likely consequence of mutation and altered gene expression rather than a cancer-inducing mechanism, any modification of cellular energetics may reflect an important cancer-relevant switch in the cell or tissue's metabolic state.

Using the key characteristics to systematically identify, organize, and summarize mechanistic information

Step 1: Identifying the relevant information

The starting point for systematic evaluation is to conduct comprehensive searches of the peer-reviewed literature aimed at identifying mechanistic data (Kushman et al. 2013). The searches can be constructed to address a series of study questions in the PECO (population, exposure, comparator, and outcomes) framework (Higgins and Green 2011) wherein endpoints associated with the key characteristics are identified. Specifically, the questions to be answered by the searches are, “Does exposure to the agent induce endpoints associated with one or more specific key characteristic properties of carcinogens”? The population (humans and any relevant experimental systems) and exposure and comparator (the agent and relevant metabolites compared to unexposed) may be sufficiently broad to identify a range of available mechanistic data informative of the overall evaluation of carcinogenic hazard. This approach thus entails comprehensive, targeted literature searches using appropriate Medical Search Heading (MeSH) terms and key words to identify evidence on the 10 key characteristics for the agent(s) or exposure(s) under evaluation.

Additional complementary literature searches may incorporate terms for the agent and its metabolites, alone or in combination with broad terms for carcinogenicity or related effects. For instance, because US EPA Integrated Risk Information System (IRIS) toxicological reviews also encompass a range of non-cancer toxicities, “top-down” broad literature searches aimed at comprehensively identifying studies on all potential toxic effects of an agent are employed (EPA 2014; NRC 2014). These comprehensive searches of peer-reviewed literature are supplemented by examining past IARC Monographs or other authoritative reviews; databases (e.g., PubChem); and, peer-reviewed government reports can also be systematically searched. The search terms used and literature retrieved can be documented (e.g., using MyNCBI, which saves searches of the National Center for Biotechnology database, or <https://hawcproject.org>).

Step 2: Screening and organizing the results

Based on title and abstract review, studies identified initially are excluded if no toxicological endpoints are reported or if no data on the chemical or a metabolite are reported. Included studies are then organized by the population (human or experimental systems) and by the endpoints associated with the 10 key characteristics (see Figure 1). Studies relevant to toxicokinetics (covering absorption, distribution, metabolism and excretion) are also identified. Additionally, authoritative, comprehensive review articles are identified, as are studies reporting toxicological endpoints in cancer target and non-target tissues. These may include morphological evaluations pertaining to the dysfunction of organs, tissues, and cells. Importantly, studies reporting endpoints that are relevant to multiple characteristics may fall under several categories.

To illustrate these two steps, targeted literature searches were conducted to identify endpoints for the effects of benzene pertinent to the 10 key characteristics, in populations comprising humans or experimental systems. The literature searches were conducted using the Health Assessment Workplace Collaborative (HAWC) Literature Search tool (<https://hawcproject.org/>), documenting the search terms, sources, and articles retrieved. Following title and abstract review, studies were excluded if they were not about benzene or its metabolites, or if they reported no data on toxicological endpoints. Included studies were further sorted into categories representing the 10 key characteristics based on the mechanistic endpoints and species evaluated

(see Figure 2). The figure also identifies reviews, gene expression studies, and articles relevant to toxicokinetics, toxicity, or susceptibility.

Step 3: Using the key characteristics to synthesize mechanistic information and to develop adverse-outcome networks

It is increasingly evident that multiple biological alterations or sets of different perturbations are necessary to convert a normal cell to a transformed cell and ultimately a tumor (Hanahan and Weinberg 2011). Carcinogens appear to impact this complex process in various ways and can act through multiple mechanisms to induce cancer and other adverse health outcomes. Using the 10 key characteristics as a basis, the collected information can be organized to form hypotheses and evaluate the evidentiary support for mechanistic events as a function of relevant aspects (e.g. dose, species, temporality, etc). The diverse and complex mechanistic endpoints elicited by benzene can then be organized into an overview inclusive of multiple alterations and any linkages thereof (Figure 3). The resulting overview can provide guidance for further assessments of the literature, including dose relevance, species relevance, and temporality of events. This additional detailed information can then be used to produce proposed mechanisms or adverse outcome pathway networks as described in (McHale et al. 2012) and the EPA's NexGen Risk Assessment Report (EPA 2014). We note that there is evidence that benzene is associated with 8 of the 10 key characteristics we have described.

Figure 4 presents a similar overview for PCBs based on data from IARC Monograph Volume 107 (IARC 2015). In summarizing the mechanistic evidence, this Monograph Working Group indicated that PCBs may induce up to 7 of the 10 key characteristics in producing carcinogenicity (IARC 2015). We note that the less chlorinated PCBs are associated with key characteristics similar to benzene (metabolic activation, DNA damage, cellular proliferation), whereas the dioxin-like PCBs are associated primarily with receptor-mediated activities.

Recently, using this same approach, the Working Groups of IARC Monograph Volume 112 and Volume 113 concluded that strong mechanistic evidence exists for 5 key characteristics being involved in malathion carcinogenicity, 3 in DDT carcinogenicity and 2 each for diazinon and glyphosate, providing evidence to support their classification as probable human carcinogens in Group 2A (Guyton et al. 2015; Loomis et al. 2015).

Discussion and Conclusions

Identification and incorporation of important, novel scientific findings providing insights into cancer mechanisms is an increasingly essential aspect of carcinogen hazard identification and risk assessment. Systematic approaches are needed to organize the available mechanistic data relevant to the overall evaluation of the carcinogenic hazard of an agent. Information to support the identification of 10 key characteristics of human carcinogens was obtained during the Volume 100 Monographs and two subsequent expert workshops. These characteristics, although not necessarily representing mechanisms themselves, provide the rationale for an objective approach to identifying and organizing relevant mechanistic data. Using literature collected previously by others as well as by us, we have categorized the literature data according to the 10 characteristics for benzene and PCBs. This approach identified pertinent positive literature for 8 of the 10 key characteristics on benzene and 7 for PCBs, thereby providing a practical, objective

method for organizing the large mechanistic literature associated with these chemicals.

This approach also lays the groundwork for a structured evaluation of the strength of the mechanistic evidence base, and therefore its utility in supporting hazard classifications. In the IARC Monographs the strength of the evidence that any carcinogenic effect observed is due to a particular mechanism is evaluated using the terms 'weak', 'moderate' or 'strong' (<http://monographs.iarc.fr/ENG/Preamble/index.php>). In general, the strongest indications that a particular mechanism operates in humans derive from data on humans or by measuring intermediate biomarkers in biospecimens obtained from exposed humans. Data from experimental animals can support a mechanism by findings of consistent results and from studies that challenge the hypothesized mechanism experimentally. Other considerations include whether multiple mechanisms might contribute to tumor development, whether different mechanisms might operate in different dose ranges, whether separate mechanisms might operate in humans and experimental animals and whether a unique mechanism might operate in a susceptible group. The possible contribution of alternative mechanisms must be considered before concluding that tumors observed in experimental animals are not relevant to humans. An uneven level of experimental support for different mechanisms may reflect that disproportionate resources have been focused on investigating a favored mechanism. All of these factors make assignment of descriptors such as 'strong' to the mechanistic evidence challenging, but recent experience with two IARC Monograph meetings suggest that the weighing of the evidence on the basis of the 10 key characteristics focuses the group discussion on the available science and allows rapid consensus to be reached regardless of the strength of the evidence base (Guyton et al. 2015; Loomis et al. 2015).

Because the literature search and categorization approach described herein is comprehensive, it may aid consideration of the overall strength of the mechanistic database according to these principles. In particular, it is inclusive of diverse mechanistic evidence, enabling support for divergent or related mechanisms from human and experimental systems to be identified. Moreover, the literature support for endpoints relevant to specific mechanisms can be evaluated in an integrated fashion when the mechanism is complex. Additionally, comparisons across agents will be facilitated, including evaluation of any similarities or differences in the pattern of key characteristics with agents that are currently classified.

As this approach is carried forward, we hope it will facilitate the objective identification of mechanistic data for consideration in the context of epidemiology, animal bioassay, or other types of evidence (e.g., studies in model organisms or *in vitro* assays) when classifying agents with regard to carcinogenic hazard. Equally important is to consider whether key characteristics of carcinogens are apparent upon exposures that are relevant to human health (Thomas et al. 2013). Overall, these developments will aid advancement of future evaluations of newly introduced chemicals, including those for which mechanistic data provide the primary evidence of carcinogenicity.

REFERENCES

- Allday MJ. 2013. Ebv finds a polycomb-mediated, epigenetic solution to the problem of oncogenic stress responses triggered by infection. *Frontiers in genetics* 4:212.
- Arana ME, Kunkel TA. 2010. Mutator phenotypes due to DNA replication infidelity. *Semin Cancer Biol* 20:304-311.
- Aranda A, Pascual A. 2001. Nuclear hormone receptors and gene expression. *Physiological reviews* 81:1269-1304.
- Baek SH, Kim KI. 2014. Emerging roles of orphan nuclear receptors in cancer. *Annual review of physiology* 76:177-195.
- Batal M, Boudry I, Mouret S, Clery-Barraud C, Wartelle J, Berard I, et al. 2014. DNA damage in internal organs after cutaneous exposure to sulphur mustard. *Toxicology and applied pharmacology* 278:39-44.
- Berquist BR, Wilson DM, 3rd. 2012. Pathways for repairing and tolerating the spectrum of oxidative DNA lesions. *Cancer Lett* 327:61-72.
- Bhattacharjee P, Banerjee M, Giri AK. 2013. Role of genomic instability in arsenic-induced carcinogenicity. A review. *Environment international* 53:29-40.
- Bielas JH, Loeb KR, Rubin BP, True LD, Loeb LA. 2006. Human cancers express a mutator phenotype. *Proc Natl Acad Sci U S A* 103:18238-18242.
- Candeias S, Pons B, Viau M, Caillat S, Sauvaigo S. 2010. Direct inhibition of excision/synthesis DNA repair activities by cadmium: Analysis on dedicated biochips. *Mutat Res* 694:53-59.
- Cogliano VJ, Baan R, Straif K, Grosse Y, Lauby-Secretan B, El Ghissassi F, et al. 2011. Preventable exposures associated with human cancers. *Journal of the National Cancer Institute* 103:1827-1839.
- Coussens LM, Pollard JW. 2011. Leukocytes in mammary development and cancer. *Cold Spring Harbor perspectives in biology* 3.
- Coussens LM, Zitvogel L, Palucka AK. 2013. Neutralizing tumor-promoting chronic inflammation: A magic bullet? *Science* 339:286-291.
- Ehrenberg L. 1984. Covalent binding of genotoxic agents to proteins and nucleic acids. *IARC scientific publications*:107-114.
- Ehrenberg L, P. Brooks, H. Druckrey, B. Langerlöf, J. Litwin, G. Williams 1973. Report of group 3. Vol. 3:AMBio Special Report, 15-16.
- EPA US. 2014. Next generation risk assessment: Incorporation of recent advances in molecular, computational, and systems biology. EPA/600/R-14/004. Washington, DC:U.S. Environmental Protection Agency.
- Figueira TR, Barros MH, Camargo AA, Castilho RF, Ferreira JC, Kowaltowski AJ, et al. 2013. Mitochondria as a source of reactive oxygen and nitrogen species: From molecular mechanisms to human health. *Antioxidants & redox signaling* 18:2029-2074.
- Filipic M. 2012. Mechanisms of cadmium induced genomic instability. *Mutat Res* 733:69-77.
- Galluzzi L, Bravo-San Pedro JM, Vitale I, Aaronson SA, Abrams JM, Adam D, et al. 2015. Essential versus accessory aspects of cell death: Recommendations of the nccd 2015. *Cell death and differentiation* 22:58-73.
- Griner EM, Kazanietz MG. 2007. Protein kinase c and other diacylglycerol effectors in cancer. *Nature reviews Cancer* 7:281-294.
- Grivennikov SI, Greten FR, Karin M. 2010. Immunity, inflammation, and cancer. *Cell* 140:883-899.

Grosse Y, Baan R, Straif K, Secretan B, El Ghissassi F, Bouvard V, et al. 2007. Carcinogenicity of 1,3-butadiene, ethylene oxide, vinyl chloride, vinyl fluoride, and vinyl bromide. *The Lancet Oncology* 8:679-680.

Guyton KZ, Kyle AD, Aubrecht J, Coglianò VJ, Eastmond DA, Jackson M, et al. 2009. Improving prediction of chemical carcinogenicity by considering multiple mechanisms and applying toxicogenomic approaches. *Mutat Res* 681:230-240.

Guyton KZ, Loomis D, Grosse Y, El Ghissassi F, Benbrahim-Tallaa L, Guha N, et al. 2015. Carcinogenicity of tetrachlorvinphos, parathion, malathion, diazinon, and glyphosate. *The Lancet Oncology* 16:490-491.

Hanahan D, Weinberg RA. 2011. Hallmarks of cancer: The next generation. *Cell* 144:646-674.

Harris CS. 2015. Cause and prevention of human cancer. *Carcinogenesis* 36: S1.

Hartge P, Smith MT. 2007. Environmental and behavioral factors and the risk of non-hodgkin lymphoma. *Cancer Epidemiol Biomarkers Prev* 16:367-368.

Hecht SS. 2012. Lung carcinogenesis by tobacco smoke. *Int J Cancer* 131:2724-2732.

Herceg Z, Lambert MP, van Veldhoven K, Demetriou C, Vineis P, Smith MT, et al. 2013. Towards incorporating epigenetic mechanisms into carcinogen identification and evaluation. *Carcinogenesis* 34:1955-1967.

Higgins JPT, Green S. 2011. *Cochrane handbook for systematic reviews of interventions*, version 5.1.0. .

IARC. 2008. IARC monographs on the evaluation of carcinogenic risks to humans. Volume 97. 1,3-butadiene, ethylene oxide and vinyl halides (vinyl fluoride, vinyl chloride and vinyl bromide). IARC monographs on the evaluation of carcinogenic risks to humans / World Health Organization, International Agency for Research on Cancer 97:3-471.

IARC. 2015. IARC Monograph Volume 107: Polychlorinated Biphenyls and Polybrominated Biphenyls. <http://monographs.iarc.fr/ENG/Monographs/vol107/mono107.pdf>

Kadhim M, Salomaa S, Wright E, Hildebrandt G, Belyakov OV, Prise KM, et al. 2013. Non-targeted effects of ionising radiation-implications for low dose risk. *Mutat Res* 752:84-98.

Klaunig JE, Wang Z, Pu X, Zhou S. 2011. Oxidative stress and oxidative damage in chemical carcinogenesis. *Toxicology and applied pharmacology* 254:86-99.

Kleinstreuer NC, Dix DJ, Houck KA, Kavlock RJ, Knudsen TB, Martin MT, et al. 2013. In vitro perturbations of targets in cancer hallmark processes predict rodent chemical carcinogenesis. *Toxicol Sci* 131:40-55.

Koustaş E, Lam J, Sutton P, Johnson PI, Atchley DS, Sen S, et al. 2014. The navigation guide - evidence-based medicine meets environmental health: Systematic review of nonhuman evidence for pfoa effects on fetal growth. *Environ Health Perspect* 122:1015-1027.

Kushman ME, Kraft AD, Guyton KZ, Chiu WA, Makris SL, Rusyn I. 2013. A systematic approach for identifying and presenting mechanistic evidence in human health assessments. *Regulatory toxicology and pharmacology* : RTP 67:266-277.

Loomis D, Guyton K, Grosse Y, El Ghissassi F, Bouvard V, Benbrahim-Tallaa L, et al. 2015. Carcinogenicity of lindane, ddt, and 2,4-dichlorophenoxyacetic acid. *The Lancet Oncology*.

Luch A, Frey FC, Meier R, Fei J, Naegeli H. 2014. Low-dose formaldehyde delays DNA damage recognition and DNA excision repair in human cells. *PloS one* 9:e94149.

Ma Q. 2011. Influence of light on aryl hydrocarbon receptor signaling and consequences in drug metabolism, physiology and disease. *Expert opinion on drug metabolism & toxicology* 7:1267-1293.

McHale CM, Zhang L, Smith MT. 2012. Current understanding of the mechanism of benzene-

induced leukemia in humans: Implications for risk assessment. *Carcinogenesis* 33:240-252.

Miller JA. 1970. Carcinogenesis by chemicals: An overview--g. H. A. Clowes memorial lecture. *Cancer Res* 30:559-576.

Multhoff G, Radons J. 2012. Radiation, inflammation, and immune responses in cancer. *Front Oncol* 2:58.

NRC. 2014. Review of epa's integrated risk information system (iris) process. Washington, DC:National Research Council.

O'Brien PJ. 2000. Peroxidases. *Chem Biol Interact* 129:113-139.

Pogribny IP, Rusyn I. 2013. Environmental toxicants, epigenetics, and cancer. *Advances in experimental medicine and biology* 754:215-232.

Pollard JW. 2008. Macrophages define the invasive microenvironment in breast cancer. *Journal of leukocyte biology* 84:623-630.

Preston BD, Albertson TM, Herr AJ. 2010. DNA replication fidelity and cancer. *Semin Cancer Biol* 20:281-293.

Rafferty P, Egenolf D, Brosnan K, Makropoulos D, Jordan J, Meshaw K, et al. 2012. Immunotoxicologic effects of cyclosporine on tumor progression in models of squamous cell carcinoma and b-cell lymphoma in c3h mice. *Journal of immunotoxicology* 9:43-55.

Rajendran JG, Mankoff DA, O'Sullivan F, Peterson LM, Schwartz DL, Conrad EU, et al. 2004. Hypoxia and glucose metabolism in malignant tumors: Evaluation by [18f]fluoromisonidazole and [18f]fluorodeoxyglucose positron emission tomography imaging. *Clinical cancer research : an official journal of the American Association for Cancer Research* 10:2245-2252.

Rooney AA, Boyles AL, Wolfe MS, Bucher JR, Thayer KA. 2014. Systematic review and evidence integration for literature-based environmental health science assessments. *Environ Health Perspect* 122:711-718.

Rusyn I, Asakura S, Li Y, Kosyk O, Koc H, Nakamura J, et al. 2005. Effects of ethylene oxide and ethylene inhalation on DNA adducts, apurinic/apyrimidinic sites and expression of base excision DNA repair genes in rat brain, spleen, and liver. *DNA repair* 4:1099-1110.

Ryter SW, Mizumura K, Choi AM. 2014. The impact of autophagy on cell death modalities. *International journal of cell biology* 2014:502676.

Saha A, Kaul R, Murakami M, Robertson ES. 2010. Tumor viruses and cancer biology: Modulating signaling pathways for therapeutic intervention. *Cancer biology & therapy* 10:961-978.

Salnikow K, Zhitkovich A. 2008. Genetic and epigenetic mechanisms in metal carcinogenesis and cocarcinogenesis: Nickel, arsenic, and chromium. *Chemical research in toxicology* 21:28-44.

Shaughnessy DT, DeMarini, D. M. 2009. Types and consequences of DNA damage. In: *Chemoprevention of cancer and DNA damage by dietary factors*, (S. Knasmüller DMD, I. Johnson and C. Gerhäuser, ed). Weinheim, Germany:Wiley-VCH Verlag GmbH & Co. KGaA.

Slaga TJ, Fischer SM, Weeks CE, Klein-Szanto AJ. 1980. Multistage chemical carcinogenesis in mouse skin. *Current problems in dermatology* 10:193-218.

Smith MT. 1996. The mechanism of benzene-induced leukemia: A hypothesis and speculations on the causes of leukemia. *Environ Health Perspect* 104 Suppl 6:1219-1225.

Smith MT, Skibola CF, Allan JM, Morgan GJ. 2004. Causal models of leukaemia and lymphoma. *IARC scientific publications*:373-392.

Thomas RS, Philbert MA, Auerbach SS, Wetmore BA, Devito MJ, Cote I, et al. 2013. Incorporating new technologies into toxicity testing and risk assessment: Moving from 21st century vision to a data-driven framework. *Toxicological sciences : an official journal of the*

Society of Toxicology 136:4-18.

Trinchieri G. 2012. Cancer and inflammation: An old intuition with rapidly evolving new concepts. *Annu Rev Immunol* 30:677-706.

Wang F, Liu S, Xi S, Yan L, Wang H, Song Y, et al. 2013. Arsenic induces the expressions of angiogenesis-related factors through pi3k and mapk pathways in sv-huc-1 human uroepithelial cells. *Toxicology letters* 222:303-311.

Yang MH, Zang YS, Huang H, Chen K, Li B, Sun GY, et al. 2014. Arsenic trioxide exerts anti-lung cancer activity by inhibiting angiogenesis. *Current cancer drug targets* 14:557-566.

Figure 1: Key Characteristics of Carcinogens

Characteristic¹	Examples of relevant evidence²
1. Is Electrophilic or Can Be Metabolically Activated	Parent compound or metabolite with an electrophilic structure (e.g., epoxide, quinone, etc), formation of DNA and protein adducts.
2. Is Genotoxic	DNA damage (DNA strand breaks, DNA-protein cross-links, unscheduled DNA synthesis), intercalation, gene mutations, cytogenetic changes (e.g., chromosome aberrations, micronuclei).
3. Alters DNA repair or causes genomic instability	Alterations of DNA replication or repair (e.g., topoisomerase II, base-excision or double-strand break repair)
4. Induces Epigenetic Alterations	DNA methylation, histone modification, microRNA expression
5. Induces Oxidative Stress	Oxygen radicals, oxidative stress, oxidative damage to macromolecules (e.g., DNA, lipids)
6. Induces chronic inflammation	Elevated white blood cells, myeloperoxidase activity, altered cytokine and/or chemokine production
7. Is Immunosuppressive	Decreased immunosurveillance, immune system dysfunction
8. Modulates receptor-mediated effects	Receptor in/activation (e.g., ER, PPAR, AhR) or modulation of exogenous ligands (including hormones)
9. Causes Immortalization	Inhibition of senescence, cell transformation
10. Alters cell proliferation, cell death or nutrient supply	Increased proliferation, decreased apoptosis, changes in growth factors, energetics and signaling pathways related to cellular replication or cell cycle control, angiogenesis

¹ Colors in this column indicate characteristics for which an individual working group or group of members work together to identify data and draft the initial language.

²Any of the 10 characteristics in this table could interact with any other (e.g. oxidative stress, DNA damage and chronic inflammation, which when combined provides stronger evidence for a cancer mechanism than would oxidative stress alone)

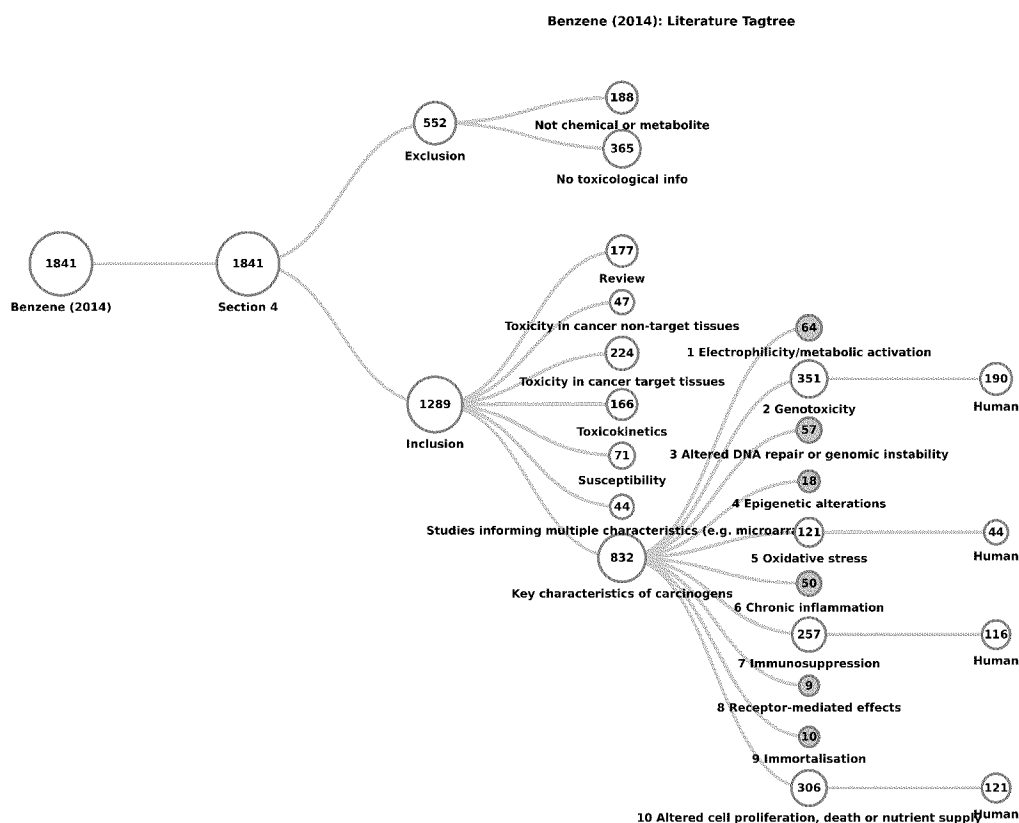


Figure 2: Literature flow diagram, illustrating the systematic identification and categorization process for benzene mechanistic studies. Using appropriate MeSH terms and key words, targeted literature searches were conducted for the 10 key characteristics using online tools available from the HAWC Project (<https://hawcproject.org/>). Section 4 refers to the location of the discussion of mechanistic data within the IARC Monograph structure. All inclusion categories were expanded to document the number of studies attributed to each, down to the individual key characteristic level, which were expanded to illustrate human information when >100 total studies were identified. Less frequently encountered key characteristic categories (grey circles) were left unexpanded for clarity.

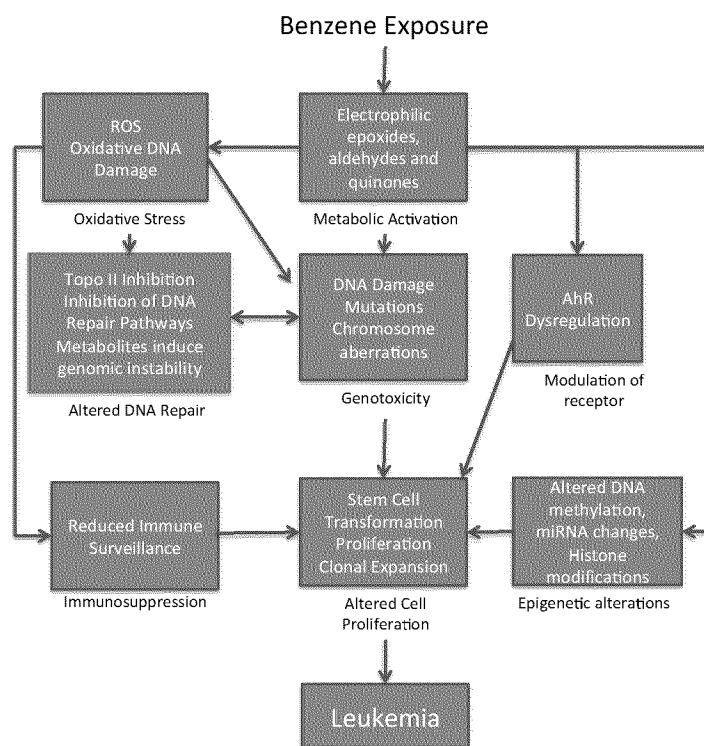


Figure 3: An overview of how benzene induces 8 of the key characteristics in a probable mechanism of carcinogenicity. A full review of these mechanistic data is given in (McHale et al. 2012), from which this Figure was adapted.

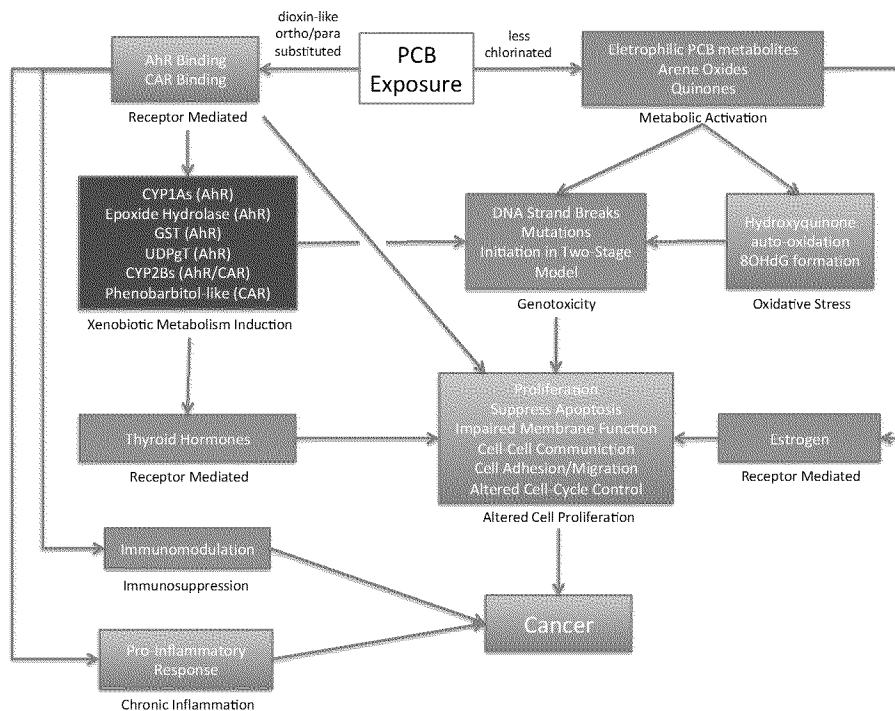


Figure 4: An overview of how polychlorinated biphenyls (PCBs) may induce 7 key characteristics in their carcinogenicity. Highly chlorinated PCBs act as ligands for the aryl hydrocarbon receptor (AhR) and other receptors activating a large number of genes in a tissue- and cell-specific manner that can lead to cell proliferation, apoptosis and other effects that influence cancer risk. Less chlorinated PCBs can be activated to electrophilic metabolites, such as arene oxides and quinones, which can cause genotoxic effects and induce oxidative stress.

