



Commentary

Chemical carcinogenicity revisited 3: Risk assessment of carcinogenic potential based on the current state of knowledge of carcinogenesis in humans

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ABSTRACT

Over 50 years, we have learned a great deal about the biology that underpins cancer but our approach to testing chemicals for carcinogenic potential has not kept up. Only a small number of chemicals has been tested in animal-intensive, time consuming, and expensive long-term bioassays in rodents. We now recommend a transition from the bioassay to a decision-tree matrix that can be applied to a broader range of chemicals, with better predictivity, based on the premise that cancer is the consequence of DNA coding errors that arise either directly from mutagenic events or indirectly from sustained cell proliferation. The first step is *in silico* and *in vitro* assessment for mutagenic (DNA reactive) activity. If mutagenic, it is assumed to be carcinogenic unless evidence indicates otherwise. If the chemical does not show mutagenic potential, the next step is assessment of potential human exposure compared to the threshold for toxicological concern (TTC). If potential human exposure exceeds the TTC, then testing is done to look for effects associated with the key characteristics that are precursors to the carcinogenic process, such as increased cell proliferation, immunosuppression, or significant estrogenic activity. Protection of human health is achieved by limiting exposures to below NOEALs for these precursor effects. The decision tree matrix is animal-sparing, cost effective, and in step with our growing knowledge of the process of cancer formation.

1. Background

This paper is one of three: the first paper (Wolf et al., 2019) chronicled the history of carcinogenicity research and asserts that DNA coding errors that arise either through mutagenesis or cell proliferation lead to tumors, the second (Wolf et al 2019) explained why the two-year bioassay and associated classification is obsolete and unnecessary, and this paper describes an animal-sparing, cost-effective testing plan for determining carcinogenic potential and potency that would result in health protective risk management decisions.

We have previously suggested (Boobis et al., 2016) that there is no

longer scientific justification for evaluating the potential human carcinogenicity of a chemical based only on a hazard identification scheme, relying on a long term (18–24 month) bioassay in rodents. In a standard rodent cancer bioassay, two or three doses are examined in males and females of both species, and extensive blood chemistry, hematology, and histopathology evaluation is performed. Positive and negative determinations are based on a statistical evaluation of the tumor incidences. Numerous difficulties with the rodent cancer bioassay have been identified (Cohen, 2004; Boobis et al., 2016; Berry, 2018; Doe et al., 2019), including the high cost monetarily, the long time for it to be performed and the large number of animals required to perform such

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studies and their low reproducibility (Gottman et al., 2005). In addition, the high and variable background incidences of many tumors in each of the species and strain and sex used further complicate interpretation (Haseman, 1983). However, the most notable difficulty with this assay is the lack of useful information that it provides with regard to the relevance to humans of the mode of action or dose response, making hazard-based classifications scientifically indefensible (Boobis et al., 2016).

Any experiment performed in an animal model has two basic assumptions: 1) whatever occurs in the animal species tested will also occur in humans (interspecies extrapolation); and 2) whatever happens at the treatment dose used in the bioassay could occur at the doses to which humans are exposed (dose extrapolation). For many chemicals tested in the long term bioassay, one or both of these assumptions may be inappropriate.

When the two-year bioassay was developed in the 1960's, and associated hazard identification schemes such as those used by the IARC Monograph Program (IARC, 2015), relatively little was known about the detailed modes of action of how chemicals increase the risk of cancer. Furthermore, analytical chemistry techniques were relatively crude, with capabilities for routine measurement of substances at milligram levels, and occasionally at microgram levels. Since that time, we have gained considerable insight into the modes of action involved in carcinogenesis, and analytical chemistry has evolved with phenomenally more sensitive techniques, now able to measure chemicals at picogram or femtogram levels and lower. When mechanisms were not understood and miniscule amounts could not be detected of a chemical, a hazard-based system seemed reasonable, but this is no longer the case. For example, aflatoxin B1, a mycotoxin contaminating peanut products, is one of the most potent DNA reactive carcinogens known (Kensler et al., 2011). Aflatoxin can be measured at extremely low levels (< 1 pg), so that aflatoxin can be identified in all peanut products. In a hazard-based system, all peanut products need to be labeled as containing a carcinogen. For the public, this is misleading since the amounts present pose a negligible risk. The type of *reducto ab absurdum* approach was recently exemplified in the state of California with the debate over the requirement that brewed coffee has to be labeled as containing a carcinogen because of the miniscule amount of acrylamide present (California EPA, 2018). These type of judgements serve only to confuse people, and detract from legitimate concerns regarding carcinogenic exposures such as tobacco smoke. Such hazard-based rule-making also diverts valuable limited resources from other issues of greater public concern.

2. Human relevance of rodent bioassays

Initially, assays performed in rats and mice predominantly tested DNA reactive carcinogens, many of which were known to be human carcinogens based on environmental, often occupational, exposures (Klaunig and Kamendulis, 2008). The 2-year bioassay has been justified based on its ability to identify most known environmental carcinogens, and reasonable interspecies and dose extrapolations can be estimated. However, the reverse situation is not logical. Not all chemicals that produce tumors in rats and/or mice are human carcinogens. Numerous investigations on different modes of actions for different tissues and for many different chemical classes have shown a lack of human relevance including substances that produce urinary tract calculi and bladder tumors in rodents (IARC, 1999), α_{2u} -globulin-binding chemicals and male rat kidney tumors (IARC, 1999), PPAR α activation and rodent liver tumors (Corton et al., 2018), and numerous others. These are all non-DNA reactive substances, and the interspecies extrapolation assumption is inappropriate.

Various non-DNA reactive substances have been identified that induce tumors in rodents by inducing cytotoxicity (cell death) with regenerative proliferation (Cohen and Ellwein, 1990, 1991). Under such circumstances, the overall mode of action could be relevant to humans,

however, there needs to be a certain level of exposure and continued dosing that results in sufficient sustained cell killing which would lead to sustained regenerative proliferation. Without sustained cytotoxicity and proliferation an increased incidence of tumors does not occur. Thus, the exposure duration and dose extrapolation become critical such that linear extrapolation from high to low dose is inappropriate.

More rational screening systems based on mode of action and human relevance have been proposed (Cohen, 2004; Sistare et al., 2011; van der Laan et al., 2016). This type of information is essential in order to provide the general population with appropriate warnings regarding chemical exposures. Without such an approach essentially everything will end up being labeled as containing carcinogenic substances, since carcinogens, including naturally occurring carcinogenic substances are ubiquitous. However, these substances are usually present at extremely low exposure concentrations, but at detectable and quantifiable levels. To protect the human population against carcinogenic risk from these substances, a level has to be scientifically determined that is considered to pose negligible risk for cancer. For example, the current FDA standard for aflatoxin is < 20 ppb for any food product for a 1 in a million risk.

3. Carcinogenesis

A rational approach to evaluating carcinogenic potential requires a basic understanding of carcinogenesis, this has evolved over the last half century, and several conclusions have been established (Cohen and Arnold, 2011; Wolf et al., 2019):

- 1) Cancer is due to mistakes occurring in the DNA (usually in somatic cells, but can be inherited through germ cells).
- 2) More than one mistake in the DNA is necessary.
- 3) All of the mistakes need to accumulate in a single cell (clonal origin of cancer).
- 4) The cell population at risk are the tissue pluripotent (stem) cells.
- 5) Every time DNA replicates, permanent mistakes could occur.
- 6) Carcinogenesis is a stochastic process.

Given this understanding, there are two fundamental ways to increase the risk of cancer by any agent, chemical or otherwise. Damage DNA directly (Greenfield et al., 1984; Moolgavkar and Knudson, 1981) or increase the number of stem cell replications (Cohen, 1998a, 2010a, 1998b). Examples of chemicals that produce increased cell proliferation by direct mitogenesis include estrogen (Yager and Liehr, 1996; Yue et al., 2013) (in rodents and in humans), PPAR α activators (Corton et al., 2018) (rodents only) and CAR/PXR activators (Elcombe et al., 2014) (rodents only). Examples of cytotoxic chemicals are chloroform (Andersen et al., 2000; Meek et al., 2003) and inorganic arsenic (Cohen et al., 2013).

Since most tissues continue replicating throughout life, "spontaneous" errors in the DNA will occur. If the necessary mutations for cancer eventually accumulate in a single stem cell, cancer will arise. This is the basis for the background incidence of malignancies in tissues, which can vary by tissue depending on genetic background and normal tissue stem cell proliferation rates (Knudson, 1971; Moolgavkar and Knudson, 1981; Greenfield et al., 1984; Tomasetti and Vogelstein, 2015).

4. Mode of action/human relevance evaluation

Although there had been extensive research on various modes of action for chemical carcinogenesis for decades, a defined, disciplined, and transparent approach to evaluating mode of action was developed by committees supported by the US EPA, Health Canada, and International Programme on Chemical Safety (IPCS). A generalized framework for mode of action analysis for animal tumors was developed and published in 2001 (Sonich-Mullin et al., 2001). This was then

used as the basis for extending the framework to involve evaluation of potential human relevance of the animal tumors and other toxicities (Meek et al., 2003; Seed et al., 2005; Boobis et al., 2006, 2008). The mode of action was based on establishing specific key events that were necessary for the development of cancer or other toxicity. To evaluate the human relevance of the finding in animals, these key events were evaluated qualitatively and quantitatively both in the animal model and in humans and compared. The framework was initially developed for non-genotoxic carcinogens (Meek et al., 2003), but was then extended for all toxic endpoints (Seed et al., 2005; Boobis et al., 2006, 2008). This has further evolved into an overall risk assessment approach for the 21st century (Risk 21) based on starting with a problem formulation discussion and then performing an exposure evaluation rather than simply performing routine hazard identification assays (Pastoor et al., 2014; Embry et al., 2014; Simon et al., 2014). More recently, the mode of action analysis has been shown to intersect with and inform Adverse Outcome Pathway (AOP) analysis (Meek et al., 2013; Wittwehr et al., 2017). Utilizing these frameworks, one can identify the human relevance of a hazard found in animal models, and if relevant to humans, determine the quantitative extrapolation for humans.

Ames et al. (1990a; 1990b; Ames and Gold, 1990) described a large number of natural substances present in food that were established as rodent carcinogens. Most of these have a mode of action not relevant to humans or are only relevant to humans at extremely high exposures, considerably higher than most humans are exposed. Another example of the difficulty with hazard identification is that approximately 50% of the pharmaceuticals described in the Physicians' Desk Reference with a two year bioassay gave a positive result (Brambilla et al., 2012). An analysis of pharmaceuticals approved in Europe gave similar results (Friedrich and Olejniczak, 2011). Many of these drugs are widely used despite the tumor findings in animals, primarily because the modes of action have been established as not relevant to humans. These include the widely used statins (liver tumors in rats and mice), fibrates (liver tumors in rodents), and proton pump inhibitors (PPIs, stomach cancer in rodents). These drugs have been used for several decades in many millions of patients worldwide, and extensive epidemiology studies with these drugs have shown no evidence of an increased (or decreased) risk of cancer of any type.

5. Mode of action-based screen for carcinogenesis

Utilizing the basic concepts of carcinogenesis, our current scientific understanding (Wolf et al., 2019) and the mode of action/human relevance framework (Boobis et al., 2006) as a basis for an overall screening approach for carcinogenesis, a method has been described (Cohen, 2004), with additional detailed analyses for liver (Cohen, 2010b) and urinary bladder (Cohen, 2018). The initial step involves a short term screen for abnormalities in tissues that are potentially associated with tumorigenesis. This involves identification of proliferation such as hyperplasia or increased labeling index. For the liver, four markers were identified in a 13 week screen by the National Toxicology Program (NTP) that identified all eventual liver carcinogens in rats and mice that arose in 2-year bioassays (Allen et al., 2004; Boobis et al., 2009). There were numerous substances that increase proliferation at 13 weeks but did not result in tumors in the 2-year bioassay. Nevertheless, the important finding was that there were no false negatives (Allen et al., 2004; Boobis et al., 2009). If the substance does not produce any of these changes in a short term screen, it can be concluded that the substance will not produce liver tumors in rodents, and by implication, humans. However, if it is positive in the short term screen, one can quickly identify the mode of action for the toxic response, and determine whether it is relevant to humans. If it is relevant to humans, a specific dose response can be evaluated and an appropriate quantitative risk assessment can be estimated regarding human exposure. Even for DNA reactive substances, such as aflatoxin, a virtually negligible risk exposure level can be established. Importantly, for non-

genotoxic substances, the cancer risk is based on the toxicity endpoints. Since regulatory agencies are quite familiar with how to regulate non-cancer toxicity endpoints, this approach can be applied to cancer risk assessments for these non-genotoxic substances since the cancer response is just another outcome of chronic exposure. The toxicity endpoints and the non-genotoxic toxic effects will have a biologic threshold. An exposure with negligible cancer risk can be estimated based on the short term toxicity endpoint. Although for DNA reactive substances, such as aflatoxin, no threshold is assumed to be present, there is increasing evidence that thresholds might also be present for DNA reactive chemicals (Gollapudi et al., 2013).

6. Exposure evaluation and initial assessments

As discussed in detail in Risk 21 publications (Pastoor et al., 2014; Embry et al., 2014), the initial step before conducting a risk assessment is a well-conceived problem formulation statement that includes defining relevant exposure scenarios. For carcinogenicity, the problem formulation and risk hypothesis is focused on the potential of cancer to occur in humans, not rodents, and at levels, durations, and by routes to which humans are exposed. In the Risk21 process (Embry et al., 2014; Pastoor et al., 2014) once the problem statement is formulated, an exposure evaluation is performed which considers the relevant time-frame, magnitude, and routes for potential adverse outcomes to be exhibited. The anticipated toxicological profile is identified in the hazard assessment based on existing information, including mode of action, read across, and chemical structure. If a risk concern is identified, the problem formulation can be re-visited and the need for additional data gathering is determined.

For carcinogenesis, a major part of the overall assessment will be dependent on whether the chemical is DNA reactive or not. Therefore, the first step in evaluating mode of action for a chemical's carcinogenic potential is to evaluate genotoxicity. We prefer to focus on DNA reactivity or mutagenicity rather than the broader genotoxicity endpoints. If it is DNA reactive and mutagenic (can be assessed with Ames assay, structural alerts), a specific evaluation is required for metabolic activation to determine if the parent or a metabolite is the mutagenic moiety and species differences in metabolism, possibly including non-linear kinetics.

The next step is to evaluate whether the exposure is below the threshold for toxicologic concern (TTC) (Munro et al., 1996). If so, no further testing is required. This can be established for genotoxic substances as well as non-genotoxic substances based on the Cramer chemical class (Cramer et al., 1976). Few substances will be present at levels below the very stringent criteria needed for genotoxic substances to be below the TTC (Boobis et al., 2017), however, for non-genotoxic substances, this could readily occur depending on the Cramer class of the chemical (Cramer et al., 1976).

If exposure is greater than the TTC or if the agent is not evaluable by using the Cramer class framework, then the chemical is evaluated for possible dose-dependent effects on immunosuppression and estrogenic activity, both relevant modes of action for human carcinogenesis. Chemicals that are immunosuppressive at human exposures can also increase the risk of virally related cancers (lymphoma, squamous cell carcinomas, Kaposi's sarcoma, others), and chemicals with increased estrogenic activity at human exposures can increase the risk of certain tumors such as breast, endometrium, and liver (Cohen and Arnold, 2011; Cohen et al., 1991; Cohen, 1999). If positive for either immunosuppression or estrogenic activity, a detailed dose response assessment is needed.

If exposure is greater than the TTC, but the chemical is negative for DNA reactivity, immunosuppression and estrogenic activity, the chemical is then evaluated for proliferative activity in a short term screen. The duration of exposure necessary to define an appropriate dose response is related to the tissue and the mode of action but, in all cases, would be 13 weeks or less. For example some mitogenic tumorigenes can

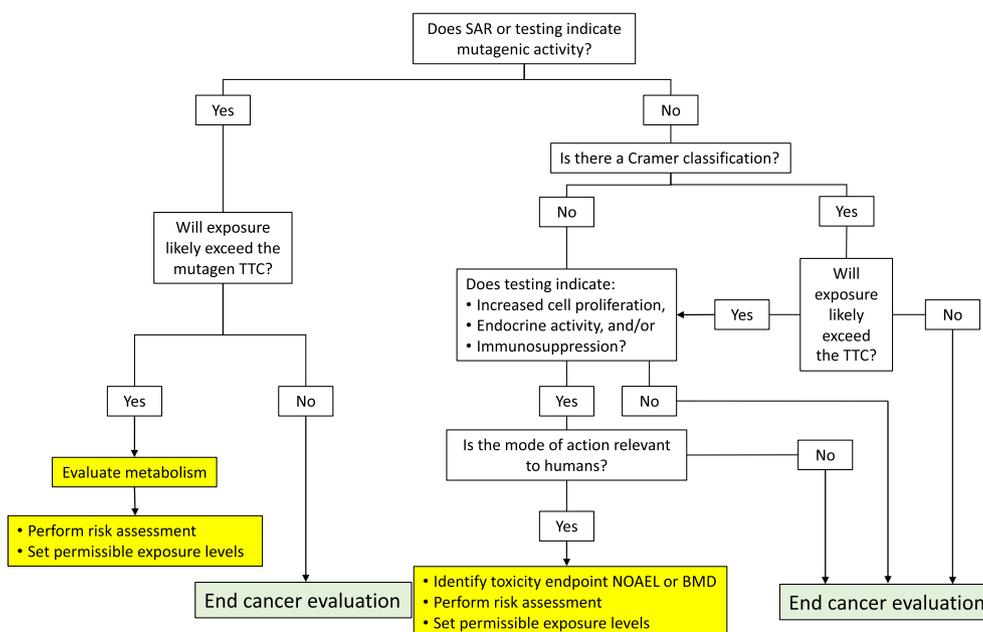


Fig. 1. Overview of suggested carcinogenicity assessment process.

be detected as soon as 2–4 days or, with some modes of action, *in vitro* assays should suffice.

7. Screen for increased cell proliferation

Some have indicated that the approach outlined would be impossible to implement for all tissues, since approximately forty tissues are examined in a two year bioassay. However, we have learned a great deal about carcinogenicity in rodent models using our understanding of mode of action to determine if a tumor response is relevant to humans. For example, thyroid follicular tumors that occur in rodents, particularly rats, are induced by several modes of action that are not relevant to humans (Hill et al., 1998; IARC, 1999; Dellarco et al., 2006). The same has been true for other endocrine tissues such as the adrenal medulla (Greim et al., 2009), testicular Leydig cells (Clegg et al., 1997), neuroendocrine cells of the stomach (Thoolen et al., 2002), and others. The rodent models can be used for toxicity evaluation, and the toxicity might be relevant to humans, but the cancer response identified in these rodent models is not relevant to humans. Thus, only a select number of tissues would need to be evaluated in this initial screen by evaluating for proliferative changes, such as can be easily evaluated in the liver, urinary bladder, kidney, and lung.

In addition, it may be that testing to 13 weeks is not necessary, as many times the relevant key events are identifiable as early as the first week of exposure. However, more realistically, a study with multiple time points and doses for 4 or up to 13 weeks will be adequate. For example, increased cell proliferation is identified in the liver and lungs with many chemicals within the first few days of exposure. Depending on the mode of action, the labeling index can return to control levels after the first week even with continued administration of the agent. However, the number of cells in these tissues is greatly increased so that there continues to be increased numbers of cells proliferating although the percent labeling index may be back to control levels. The number of cells at risk of mutation is related to the absolute numbers of cell that can replicate (Wood et al., 2015; Greenfield et al., 1984; Wolf et al., 1995). Evaluation for increased labeling index would miss these findings if only a 4 or 13 week study was performed.

For some tissues, routine histopathology does not have adequate sensitivity so that other techniques, such as immunohistochemistry for DNA replication or genomic analysis may be required. For example, in

the lung, histologic evidence of proliferation was not evident in a 13 week study for eventual lung carcinogens (Boobis et al., 2009) identified in NTP bioassays. However, short term (1–2 weeks) studies have detected rodent lung carcinogens if a proliferative index (Ki-67, BrdU) was evaluated (Strupp et al., 2016; Yamada et al., 2017).

An approach that can address these variations would include an evaluation after 1, 4, and 13 weeks. The evaluation would include weights for some tissues (such as liver, kidney) which can be a sensitive indicator of a proliferative effect. Histology is performed to evaluate tissues for hyperplasia and for cytotoxicity with regenerative proliferation. Ancillary studies, such as serum enzymes for liver toxicity, can also be performed. Histopathology will be adequate for detection of increased proliferation for many tissues, such as layered epithelia like the urinary bladder and for some endocrine tissues like thyroid follicles. A more sensitive assessment of increased cell proliferation involves an immunohistochemical evaluation of DNA replication, such as for bromodeoxyuridine (BrdU) or Ki-67 (which does not require administration of an exogenous labeling substance). Evaluation of a labeling index appears to be necessary for detection of increased proliferation within 13 weeks, such as for lung and some endocrine tissues.

If increased proliferation is detected in any tissue, evaluation of mode of action is undertaken. Mode of action analysis can be carried out using a variety of techniques, and as more sensitive and better understood transcriptomic and other -omic technologies are developed, these can also be applied to the evaluation of mode of action and human relevance.

In addition, a more detailed mode response can be evaluated. Thus, a qualitative and quantitative risk assessment becomes possible, including an assessment of human relevance. Using only the two-year bioassay provides only limited information regarding dose, and no information regarding mode of action or assessment of human relevance. Detailed examples of the approach described here have been reported for liver (Cohen, 2010b) and the urinary bladder (Cohen, 2018).

8. Summary and current status

The model that we are proposing is illustrated in Fig. 1 and builds on more than five decades of research on modes of action for chemicals and cancer in rodents and human relevance. Essentially, our proposal uses modern science and understanding of carcinogenesis through the

use of short term studies and accumulated knowledge for identifying chemicals for which dose response evaluation is necessary for informing a human risk assessment.

An approach similar to what we have described is already being investigated by various regulatory agencies. For example, in the pharmaceutical industry, it has been proposed to use a six month study to determine which substances would need to be evaluated in a full two year bioassay and a tiered decision strategy (Sistare et al., 2011; van der Laan et al., 2016). The lack of need of the two year bioassay is also being explored by the US EPA (Thomas et al., 2012, 2013) and by the European Chemical Agency (ECHA) (Braakhuis et al., 2018) through research and within their programs for granting waivers.

In conclusion, based on our increased understanding of chemical carcinogenesis, and experience developed from several hundred two year bioassays performed with follow-up mode of action analysis, and on the more sophisticated and sensitive analytical chemistry techniques now available, a hazard based classification scheme is no longer tenable nor rational. Utilizing mode of action analysis, a more direct and rational basis for human cancer risk assessment can be performed rather than simple hazard identification. This avoids the waste of money, time, and animals of the two year bioassay and would end up to be equally health protective to prevent adverse outcomes from chronic exposure including cancer.

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The authors have served as members of the following panels or committees and/or for the following organizations:

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