CC/2019/16

COMMITTEE ON CARCINOGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT (COC)

Guidance Statement on the risk assessment of the effects of combined exposure to multiple chemicals on carcinogenicity – first draft

1. In the COC Guidance Statement series, G08 is entitled *Risk assessment of mixtures of chemical carcinogen,* which was produced following a literature review during 2008-2009. Since that time there have been developments in the risk assessment of mixtures and an increased understanding of the carcinogenic process. These developments have led COC to explore whether a cancer endpoint-specific approach could be derived to allow the risk assessment of combined exposure to multiple potential carcinogens.

2. The COC previously considered a scoping paper outlining the potential for a novel carcinogen-specific risk assessment paradigm for combined exposures to possible carcinogenic chemicals in November 2018 (CC/2018/09).

3. The potential approach discussed was based on a multistage model of cancer as an adverse outcome pathway. The utility of such an approach was explored using two examples of known synergistic chemicals (alcohol and tobacco smoking; asbestos and tobacco smoking) that have previously been considered by COC.

4. A revised discussion paper was presented in March 2019 (CC/2019/03) and following discussions, it was agreed that a Guidance Statement should be prepared reflecting the COC's thinking on new approaches to the risk assessment of the effects of combined exposures on carcinogenicity.

5. The paper presented in Annex A is a first draft of the Guidance Statement.

Questions for the Committee

6. Members are asked to consider this draft and in particular:

   i. Whether they have any comments on the structure and contents of the draft document

   ii. Whether this paper can be published as a COC Guidance Statement

NCET at WRc/IEH-C under contract supporting the PHE COC Secretariat
November 2019

CC/2019/16 Annex A

COMMITTEE ON CARCINOGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT (COC)

Guidance Statement on the risk assessment of the effects of combined exposure to multiple chemicals on carcinogenicity – first draft

First draft statement

This annex is attached.

NCET at WRc/IEH-C under contract supporting the PHE COC Secretariat
November 2019

Committee on CARCINOGENICITY

Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment (COC)

COC Guidance Statement G08 –version 2.0 Draft v0.1

Risk assessment of the effects of combined exposure to multiple chemicals on carcinogenicity

https://www.gov.uk/government/groups/committee-on-carcinogenicity-of-chemicals-in-food-consumer-products-and-the-environment-coc

COC Secretariat

c/o Public Health England

Centre for Radiation, Chemical and Environmental Hazards

Chilton, Didcot, Oxfordshire OX11 0RQ

Committee on CARCINOGENICITY

COMMITTEE ON CARCINOGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

Risk assessment of the effects of combined exposure to multiple chemicals on carcinogenicity

Introduction

1. The principles of risk assessment for chemical carcinogenicity have traditionally been based on cancer endpoints, or sometimes pre-cancer endpoints, particularly in laboratory animals. For many years, the development of chemically-induced cancer in experimental models was based on the simple paradigm of "initiation and promotion". These traditional approaches to the assessment of carcinogens have recently been reviewed and challenged in a series of papers (Cohen et al., 2019; Doe et al., 2019; Woolf et al., 2019) in which the authors concluded that the two-year rodent cancer assay was neither appropriate nor efficient in evaluating carcinogenic potential in humans. They suggested a decision-tree matrix based on the premise that cancer is a consequence of DNA coding errors arising either by direct mutagenic events or indirectly from sustained cell proliferation. Higher considerations might include other key effects which are precursors in the carcinogenic process such as increased cellular proliferation, immunosuppression or significant oestrogenic activity. Moreover, the discovery of mutations in (proto)oncogenes and tumour suppressor genes in many human cancers has led to a number of more complex and detailed multistage models being derived with a subsequent improvement in our understanding of the molecular and pathological changes of cancer development in humans.

2. These advances in understanding have been accompanied by improvements in methodology for occupational and environmental epidemiology. These have led to the demonstration of statistically significant smaller increases in relative risk from a greater number of substances in individuals exposed through the environment and occupationally. Therefore, there is an increased interest in the potential adverse effects following low-dose exposures, which are more relevant to the human situation.

3. These scientific advances have also enabled realistic consideration of the risk of combined exposure to multiple chemicals and how mixtures of chemicals might interact during the multiple stages of cancer aetiology. This is important as one-third of the chemicals classified by IARC as Group 1 carcinogens are mixtures, including

mineral oils, coal tar, alcoholic beverages and mixtures of aflatoxins, which have been classified mainly on epidemiological evidence. This new approach should enable the consideration, not simply of a single exposure to a mixture of chemicals (albeit that this could be for a length of time), but towards assessing combined exposure to chemicals that might take place at different times, for varying periods of time and at different life stages. Such risk assessment currently remains problematic owing to the complexity of cancer development and the lack of information on the effects of multiple chemicals on stages in this development; however, this Guidance Statement summarises recent advances in approaches and identifies future research directions that will contribute to the knowledge needed.

Harmonised risk assessment for mixtures

4. The basic principles of risk assessment, driven by the policy needs of various regulatory requirements, have been developed over many decades for individual chemicals and more recent interest and advances in the assessment of combined exposure to chemicals have built on this paradigm. In 2019, the European Food Safety Authority (EFSA) provided detailed guidance on harmonised methodologies for assessing the risk of combined exposure to multiple chemicals (see Figure 1), which is based on the same paradigm (EFSA, 2019a). Such details are beyond the scope of this Guidance Statement and the EFSA document should be consulted for further guidance.

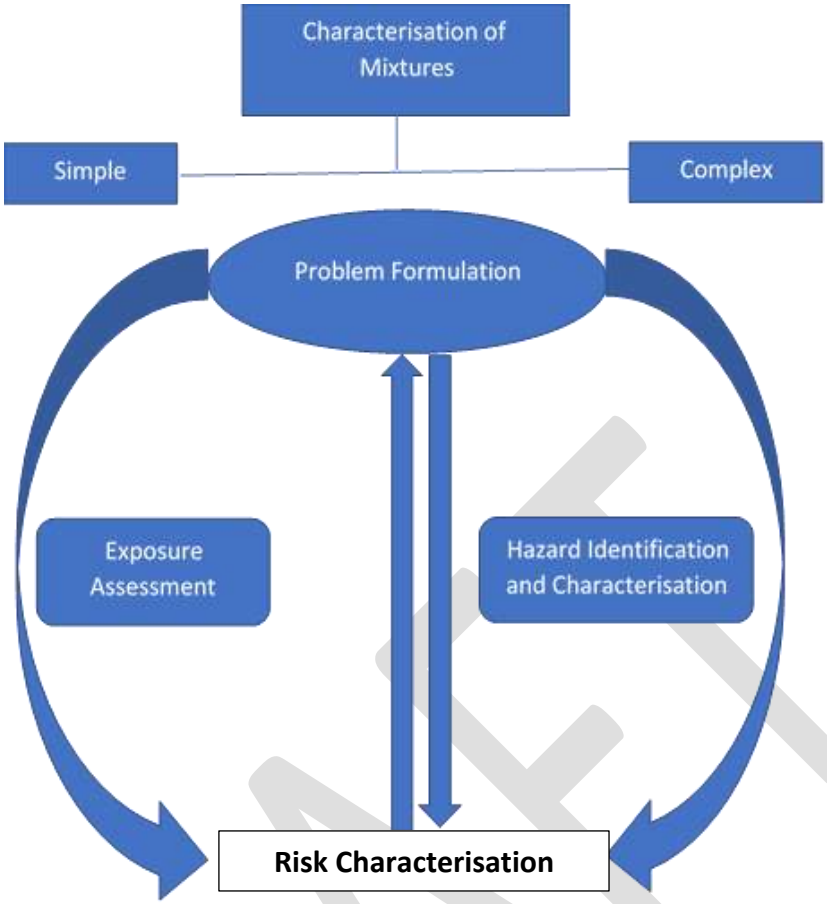

Figure 1. Paradigm for the risk assessment of chemical mixtures including carcinogens and consideration of chemical mixtures (Adapted from EFSA (2019a)).

Characterisation of simple and complex mixtures

5. When the components of the combined exposure are largely known, as in a fixed composition (e.g. a registered and authorised agrochemical), this is regarded as a *simple mixture* and the risk assessment can be based on the individual components.

6. Risk assessment can also be based on a "whole mixture approach" which considers the mixture (combined exposure) as a single entity equivalent to a single chemical. When the composition of a mixture is unknown or difficult to characterise, it is considered to be a *complex mixture*. Examples of *complex mixtures* are chemical residues in food and drinking water and soil contaminants in old industrial sites (where component data may be available) and mixtures produced as reaction products, from refining processes, process emissions, welding fumes and air pollution (IGHRC, 2008).

Problem formulation

7. Problem formulation is the initial step in an iterative process involving consideration of the need for, and extent of, a risk assessment (EFSA, 2019a). For a mixture, this would include the generation of a conceptual model describing the source of the combined exposure, characterisation of the mixture (*simple* or *complex*), exposure pathways, populations and life stages involved, endpoints to be considered and their relationships, using models such as Mode of action (MoA) and Adverse Outcome Pathways (AOP) which are more fully described later(EFSA, 2019a). This could be very complex in any risk assessment of the carcinogenic potential of combination effects of multiple chemicals where, for example, length of exposure, lifetime versus limited exposure and spatial considerations of endpoints also need to be assessed and considered.

Exposure assessment

8. For any chemical, exposure assessment requires consideration of the exposure pathway, length and time of exposure, the exposed population, variation of levels in the exposed population and uncertainty in exposure estimates. The assessment of combined exposure to multiple chemicals can use the same general methods and concepts as for individual chemicals, but additional considerations are required (more detail is given in EFSA, 2019a).

9. In instances of formulated products, exposure to the mixture components are likely to occur at the same time and via the same route(s) of exposure. In other instances, complex pathways of exposure may mean that the population will not be equally exposed to all components, and exposures may not occur at the same time. As a result, the hazard exposure characteristics of individual components may differ from that of the whole mixture. This is particularly important for the assessment of carcinogenic potential of combined exposure to multiple chemicals where exposure to different chemicals may affect different stages in the development of cancer at different sites. Therefore, pathways of exposure both by route and time (limited and lifetime exposure) may be of vital importance in their risk assessment.

Low dose exposures

10. Concern over effects of ‘low-dose’ exposure to chemicals is often described in the literature and particularly also linked to mixtures or combined exposure. In considering such literature, it is important to be clear on the definition used for ‘low dose’ in each instance.

11. The term is often used to mean relevant or typical environmental (general population or occupational) exposure, such as in the US National Toxicology Program. In other cases, e.g. the Halifax Project on risk assessment of combined exposure to multiple potential carcinogens outlined below used the EFSA definition, which is when responses that occur at doses well below the traditional lowest dose of 1 mg/kg body weight that is used in animal toxicology studies. Other low-dose

effects could be defined as being below the No Observed Adverse Effect Levels (NOAEL) or Benchmark Dose (BMD) for individual chemicals, although such use could be difficult when multiple chemicals are being considered, or in the future when long-term animal studies may not be conducted.

12. It has been shown that endogenous hormones have biological effects at low concentrations with the consequence that endocrine disrupting chemicals have dominated most discussions of low dose effects. At present, there is no generally accepted definition of 'low-dose' in risk assessment paradigms but if used it should be defined.

Hazard identification

13. *Hazard identification is a qualitative process which plays an important role in the* assessment of mixtures in determining the grouping of chemicals with similar adverse effects (assessment group), e.g. liver toxicity, thyroid toxicity, neurotoxicity. It may be possible to identify a single component in an assessment group which has the highest toxic potency and so be the driver in risk assessment. However, this may be difficult in the assessment of carcinogenic potential when the adverse effects of different chemicals in the mixture may act at different stages in the aetiology of the carcinogenic process leading to cancer.

Hazard characterisation

14. Hazard characterisation is a quantitative process which results in the identification of a point of departure for the whole mixture or for each of its component chemicals based on the health identification. This hazard characterisation of potential carcinogenicity from combined exposures is difficult, as dose-response information is unlikely to be available on how a mixture of chemicals act on the many different processes, or failures of processes, that occur during cancer development. Where chemical exposures occur at different times, the inter-play of individual dose-responses is unlikely to be investigated in sufficient detail to make an assessment.

15. As an example, the conclusions of the EFSA statement on genotoxic potential of chemical mixtures (EFSA, 2019b) give some indications of the difficulties of assessing the adverse effects of exposure to multiple chemicals by just one potential mechanism, genotoxicity, of the many involved in cancer development. These conclusions are:

- The mixture should be chemically characterised as far as possible;
- If the mixture contains one or more substances assessed to be genotoxic *in vivo* via a relevant route of administration, the mixture is considered genotoxic;
- If the assessment of all components of a fully characterised mixture results in the conclusion that none of these raises a concern for genotoxicity, the mixture should also be considered of no concern;

- If a fraction of substances in the mixture have not been chemically identified, experimental testing of this unidentified fraction should be considered as a first option, or if not feasible, testing of the whole mixture should be undertaken.

Risk characterisation

16. Risk characterisation of chemical mixtures aims to:
    a. Calculate the ratio of exposure to hazard to determine whether there is a potential health concern to the exposed population (if the exposure concentration is greater than the hazard there is a potential health concern); and
    b. Identify the components in an assessment group that represent particularly important risk drivers for the component-based approach (EFSA, 2019a).

17. A number of methods (detailed further in EFSA, 2019a) have been developed to characterise this risk in terms of a comparison between exposure and toxic effects. In the whole *complex mixture* approach, the mixture is essentially treated as a single substance, and so requires dose-response information for the mixture of concern (or a sufficiently similar mixture). Therefore, if taking into account uncertainties, the estimated exposure exceeds any reference value derived from the hazard data, there is a potential for risk.

18. In a component-based (*simple mixture*) approach, there are several methodologies for assessing dose addition. One example is the Hazard Index (HI) which is the sum of the hazard quotients of the individual components of an assessment group. Each of these hazard quotients is calculated as the ratio between exposure to a chemical and the respective health-based guidance value (such as ADI, TDI). The problem with this simple additive approach when applied to mixtures is that differing uncertainty factors (e.g. intra-and inter-species variability), inherent in each component, are also combined when calculating the HI. In addition, the health-based guidance values may have been derived from different types of study with differing endpoints and quality. It would therefore need to be agreed how to express levels of concern for HI values; often a HI of 1 or less is not considered of concern, however values above 1 would need interpretation and possibly investigation of any hazard quotients making the greatest contribution to the HI. These factors combined mean that there could be a lack of consistency between different assessors, depending of their interpretation of studies.

19. When a mixture contains substances that act by independent actions or mechanisms the response addition approach can be used to determine the effects of exposure. The approach uses “response points” (reference values derived from hazard data) or, ideally, the full dose-response curve for all, or at least two, of the substances within the mixture. A probability for adverse effects occurring in an individual following exposure to each of the mixture components is calculated and multiplied together using the formula for statistical independence, to give a risk

estimate for adverse effects to arise from the mixture as a whole (IGHRC, 2008; EFSA, 2019a).

New approaches to cancer research and risk assessment

Adverse Outcome Pathways (AOPs)

20. Traditional methods of risk assessment, including the use of animal studies, have been developed and used over many decades, but new approaches are required to meet ethical and cost concerns, particularly in the assessment of carcinogenic potential where long-term rodent studies have been the gold-standard test. There is also a need to consider improved knowledge of cancer development as well as doubts about the validity of findings from animal studies when extrapolated to humans.

21. New approaches have focused on *in vitro* screening assays and knowledge of the biological pathways leading to the adverse outcome of the chemical exposure. An illustration of a generalised AOP is shown in Figure 2.

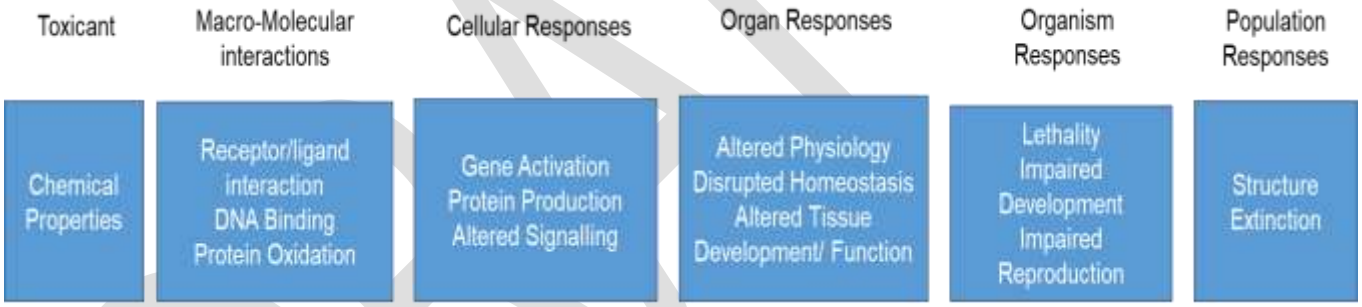

Figure 2 Generalised Adverse Outcome Pathway[1]

22. Both MoA and AOP are frameworks for relating these new types of non-animal mechanistically-based data for risk assessment and are currently being widely investigated for use in chemical risk assessment and, more recently, for mixtures (EFSA, 2019a) including carcinogens (Goodson et al., 2015). Unlike MoA, AOP does not consider toxicokinetics as part of the framework. In an AOP, a pathway causally links a chemically-induced molecular initiating event (MIE) leading to key events and an adverse outcome (AO). The confidence in the data underpinning the formation of an AOP has been investigated using an adaption of the Bradford-Hill (1965) criteria of causality used in epidemiological investigations and, a complementary approach focusing on scientific confidence in the assays used and the development of predictive models. As an example, Perkins and colleagues examined AOPs for four case studies with different degrees of completeness and scientific confidence (Perkins et al., 2015). This included the AOP for hepatocellular proliferation leading to cancer with 1,4-dioxane being an example chemical that may

[1] Taken from OECD website at http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm [permission not required for inclusion of this Figure]

act through this pathway. The mechanistic and causal understanding of the events leading to the adverse outcome was considered to have a moderate level of confidence, while the MIE is unknown. The MoA is believed to be one of the two pathways shown in Figure 3.

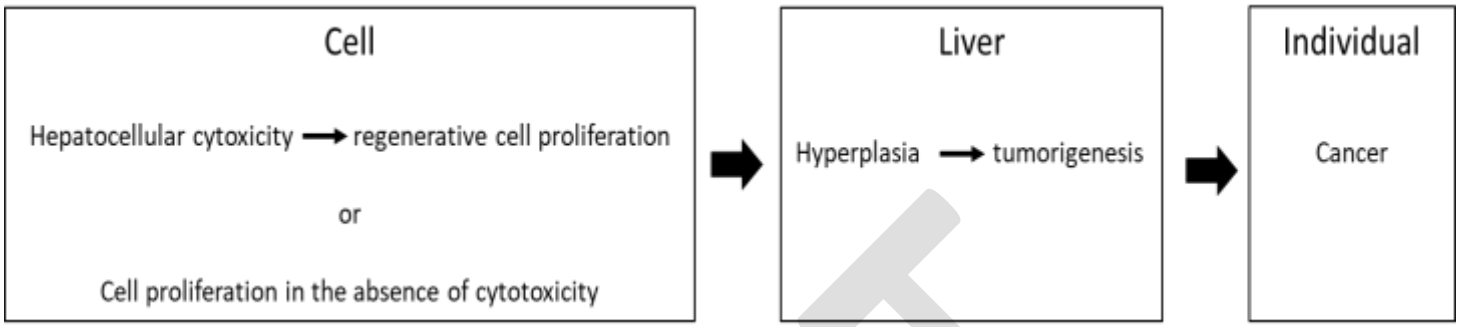

Figure 3. AOP for hepatocellular proliferation leading to cancer (modified from Perkins *et al.,* 2015).

23. The toxicological evidence for the AOP outlined in Figure 3 is sufficient to establish key events of either liver cytotoxicity followed by regenerative hyperplasia and tumour formation or cell proliferation, in the absence of liver cytotoxicity, leading to hyperplasia and tumour formation. The MoA involving sustained proliferation of spontaneously transformed liver cells is supported by evidence that 1,4-dioxane is a tumour promotor in mouse skin and rat liver bioassays.

24. More recent advances in technology can be used to gain further confidence in the AOP framework. Targeted gene arrays can be used to investigate the expression of genes specific for certain cellular pathways. For 1,4-dioxane, gene expression datasets are available for 3, 14 and 28-day time courses following gavage exposure. Investigation of the gene expression of growth factors, signalling pathways and transcription factors support regenerative cell proliferation and cell proliferation in the absence of cytotoxicity. Other gene expression including NF-κB suggests a role for the 'inflammation-fibrosis-cancer axis'. These observations suggest that 1,4-dioxane could lead to tumour initiation and cellular proliferation. There is no evidence of epigenetic effects for 1,4-dioxane, but it is metabolised in rat liver by P4502E1, suggesting that prolonged exposure could generate free radical species.

25. Perkins et al. (2015) suggest that this incomplete AOP has sufficient scientific data to support categorisation of 1,4-dioxane as a likely carcinogen to humans, given appropriate exposure and dose conditions. This example indicates how an AOP can be used to assess a potential carcinogenic chemical. Although this is for a single chemical, it demonstrates the complexities that would be encountered when assessing combined exposure to multiple chemicals.

Hallmarks of cancer

26. Since Armitage and Doll first outlined a multistage theory of cancer in the 1950s, molecular and pathological studies have greatly advanced our knowledge of the carcinogenic process. This led Hanahan et al. (2000, 2011) to propose the 'Hallmarks of Cancer' which outline essential alterations in cell physiology that define malignant growth (defined as '….. acquired capabilities common to most cancers that incipient cancer cells … [must acquire to] enable them to become tumorigenic and ultimately malignant'. These Hallmarks are outlined in Figure 4.

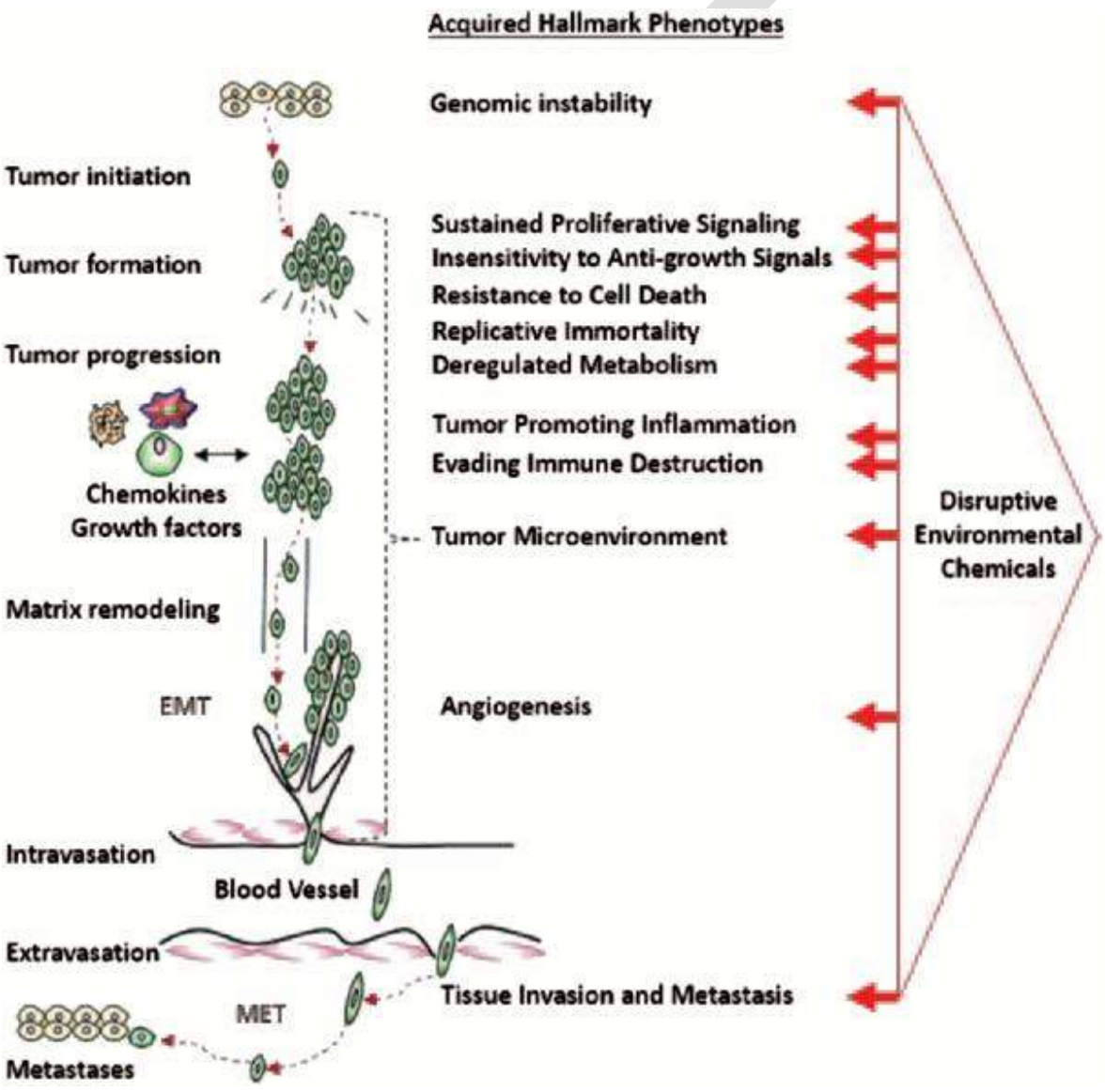

Figure 4. Potential disruption of hallmarks of cancer by environmental chemicals (Goodson *et al.*, 2015)[2]

[2] This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited.

The Ten Hallmarks of Cancer

- *Genetic instability and mutation* – allowing changes in one cell to pass to daughter cells through mutation or epigenetic changes in the parent cell DNA.
- *Tumour-promoting inflammation* – helping cancer cells grow using the same growth signals which normal cells provide to each other during wound healing and embryonic growth; inflammation further contributes to the survival of malignant cells, angiogenesis, metastasis and the subversion of adaptive immunity.
- *Sustained proliferative signalling* – cancer cells appear to grow at an unlimited rate.
- *Insensitivity to anti-growth signals* – cancer cells are insensitive to anti-growth signals or withdrawal of normal growth signals.
- *Resistance to cell death* – cancer cells avoid the processes by which abnormal or redundant cells trigger apoptosis.
- *Replicative immortality* – cancer cells do not senesce or die after a limited number of cell divisions.
- *Dysregulated metabolism* – disrupting metabolism is needed to support the increased demands of rapid proliferation, thus enabling the development of cancer.
- *Angiogenesis* – eliciting new blood vessels to sustain growth.
- *Tissue invasion and metastasis* – invasive tumours creating a space to expand into normal tissue, while in situ or non-invasive cancers (e.g. breast ductal carcinoma in situ; carcinoma in situ in colon polyps) grow into pre-existing spaces.
- *Avoiding immune destruction* – tumour cells avoiding immune surveillance that would otherwise mark them out for destruction.

27. Although there is now a considerable body of research underpinning these multiple stage Hallmarks in cancer development, little of this has been translated into the assessment of the carcinogenic potential of chemicals. A number of the Hallmarks, such as the effects of chemicals on metabolism and the immune system, would not traditionally be considered indicative of carcinogenic potential. For example, a chemical that disrupts DNA repair may prove to be non-carcinogenic at any level of exposure when tested alone but could contribute to carcinogenesis in the presence of other exogenous chemicals such as mutagens which directly damage DNA. A further example is that a chemical or pharmaceutical that suppresses immune responses might well prove negative in standard carcinogenicity assays, but may play a part in the development of cancer when other chemicals are present. Such considerations are essential when assessing the risk of combined exposure to multiple chemicals and it is clear that the Hallmarks of Cancer may be an important tool in the risk assessment of such mixtures. Indeed, in 2012, participants at two workshops convened by IARC (Miller et al., 2017) concluded that *human carcinogens* (Group 1) frequently exhibit one or more of these 10 key (Hallmark) characteristics.

Concept of grouping of chemicals according to Hallmark effects

28. The Halifax Project was a large-scale, multi-centre project with the aim of examining the challenge of assessing the carcinogenic potential of low-dose exposure to chemical mixtures in the environment (Goodson et al., 2015). The underlying concept of this project suggests that if individual chemicals can induce some, but not all, of the Hallmarks of Cancer, then combinations of chemicals at low doses may be able to act through different MoAs in concert to induce carcinogenesis.

29. The Halifax Project reviewed toxicological data on 85 environmental chemicals not considered to be carcinogens, including pesticides, metals, plasticisers, etc. These chemicals were all judged to have Hallmark-inducing actions for key pathways and mechanisms relating to carcinogenesis and were divided into groups according to their Hallmark effect, with some chemicals appearing in more than one group. Of these, 15% showed evidence of a dose-response threshold, 59% had evidence of effects at low dose, with the remaining 26% having no dose-response data.

30. The authors concluded that there are a significant number of environmental chemicals exerting non-genotoxic, low-dose effects through Hallmark mechanisms for which there is evidence for a contributory role in carcinogenesis. Therefore, there is a possibility that low-dose exposure to a chemical mixture may contribute to cancer development. For example, a mixture might contain several chemicals, none of which are “complete” carcinogens; however, one chemical might support one Hallmark while another results in a different Hallmark and so forth until the result may be a carcinogenic potential, similar to an exposure to a single “complete” carcinogen (Goodson et al., 2015).

31. There is a danger, however, in following this or similar approaches that individual chemicals resulting in single specific effects, could become classifiable as a carcinogen, which would be undesirable.

IARC cancer markers

32. IARC has also considered possible mechanisms by which agents may cause cancer in humans (Smith et al., 2016) and also identified 10 characteristics. These mechanisms are similar to the Hallmarks of cancer except for emphasising the electrophilic nature of many carcinogens and detailing effects on DNA to a greater extent.

- Is electrophilic or can be metabolically activated to electrophiles
- Is genotoxic
- Alters DNA repair or causes genomic instability
- Induces epigenetic alterations
- Induces oxidative stress

- Induces chronic inflammation
- Is immunosuppressive
- Modulates receptor-mediated effects
- Causes immortalization
- Alters cell proliferation, cell death, or nutrient supply.

Individual susceptibility, synergism and additive effects

33. Taking account of individual susceptibility to cancer has been considered in traditional risk assessment paradigms by addition of an uncertainty factor to account for variability within a human population. New approaches such as AOP and Hallmarks of Cancer do not directly address metabolism of chemicals but polymorphic variance in the genes for metabolic enzymes (such as the CYP genes for the P450 family) can affect the potential carcinogenicity of chemicals. Differences in susceptibility due to variation in metabolism can further add to the complexity when assessing the risk of combined exposure to multiple chemicals. It is possible that certain chemicals may induce enzymes which affect the metabolism of other chemicals in the mixture which could either activate or inactivate effects on stages in cancer development. An example of this is the induction of CYP2E1 by ethanol which may potentiate the effects of chemicals present in tobacco smoke (Smith et al., 1998).

34. Although asbestos is negative in the Ames test, both asbestos and tobacco smoking are complete carcinogens (COC, 2008). An assessment based on AOP and Hallmarks of Cancer of the combined exposure suggests increase (and supra-additivity in some cases) *genetic instability* (both directly and via oxidative damage) which potentially leads to increased mutations in the key regulatory genes, such as the oncogene, Ki-ras and the tumour suppressor genes, FHIT and p53. These mutations in key genes affect other Hallmarks, such as *insensitivity to anti-growth signals* and *sustained proliferative signalling*. Observed synergistic inflammation and proliferative cell growth from exposure to asbestos and tobacco smoking also meets the Hallmark of t*umour-promoting inflammation*.

35. Other individual susceptibilities may also be present in other stages in the development of cancer including other genetic and epigenetic mechanisms (e.g. epigenetic mechanisms have been observed in exposure to benzene as well as genetic changes (Chappell, 2016; Fenga et al., 2016). At present, little is known of these effects; however, new sequencing and molecular techniques are now available to examine these.

Present status of carcinogenic risk assessment of combined exposure to multiple chemicals

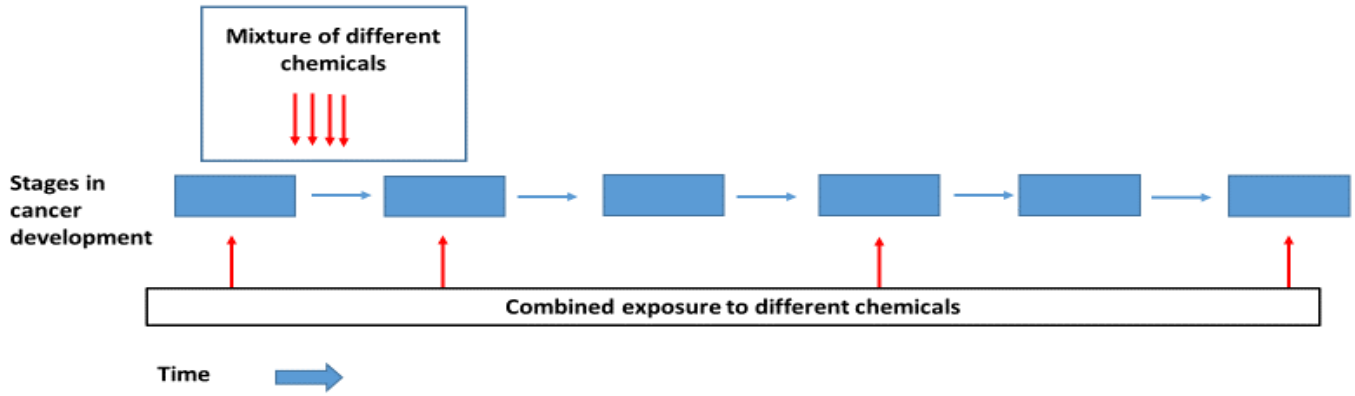

Figure 5. Illustration of chemical mixtures and combined exposure to multiple chemicals for potential carcinogenicity

36. The regulatory assessment of the carcinogenic potential of single chemicals has developed based mainly on long-term, high exposure doses in animals, together with in vitro and in vivo assays that investigate genotoxicity and/or mutagenicity. More recently there has been greater consideration of the potential toxicity of chemical mixtures; however, for carcinogenic potential, this has mainly consisted of accumulative effects of exposure to individual carcinogens identified in a mixture. For ethical and resource reasons, as well as reduced confidence in their relevance, long-term animal studies are much less likely to be undertaken in the future. The concept of AOP has been developed to provide a framework to better use results from newly developed *in vitro assays*, tissue models and computational methods for risk assessment. For assessment of potential carcinogens, it is also desirable to consider combined exposure to multiple chemicals over potentially a long period of time as well as mixtures of chemicals over a defined period (see Figure 5.)

37. Advances in cancer research have led to the better understanding and establishment of defined cellular alterations in the development of cancer, i.e. the Hallmarks of Cancer. The Halifax Project has studied a large number of non-carcinogenic chemicals and found that many affect one or more of these Hallmark stages. This has led the authors to suggest that combined exposure to multiple chemicals (which may individually be non-carcinogenic) might potentially lead to cancer (Goodson et al., 2015). The use of grouping of chemicals having similar Hallmark effects could also be used for assessment of additive effects of chemicals in combined exposure.

38. This concept could form the basis of new paradigms for carcinogenic risk assessment in the future, particularly for assessing the potential effects of exposure to low doses of multiple chemicals. However, at present, the basic cellular and pathological data for the chemicals tested have come from traditional animal studies. At the present time, it appears unlikely that sufficiently robust data can be derived

from *in vitro* cell and tissue models, computational modelling and other approaches which are have not yet been validated.

39. A further potential problem is that the Hallmarks of Cancer refers to different molecular/pathological events occurring over an extended period. It is not clear how the combined exposure of multiple chemicals may affect this development when different chemicals in the mixture may affect different temporal stages. For example, whether is sequential exposure to chemicals necessary for progression to cancer.

Future development and research

40. It is acknowledged that there are clear limitations to high-dose, animal-based models in predicting human responses to potential carcinogens. Therefore, it is necessary to establish principles and guidelines for the future testing of combined exposure to multiple chemicals, so they are relevant to human exposures. This includes increased understanding of the mechanism underlying the initiation and development of cancer and identifying biomarkers that can distinguish genetic and epigenetic alterations. Human-based 3-D tissue models, pathway-based approaches and toxicokinetic and computational models (integrative and targeted quantitative structure-activity (QSAR) predictions) need to be further developed to give validated, robust results and increase our knowledge of the carcinogenic process (Cohen et al., 2019).

41. Following publication of the Halifax Project (Goodson et al., 2015), recommendations from subsequent workshops were also published (Miller et al., 2017). These authors included proposals for future research to fill current gaps in knowledge and underpin the risk assessment of combined exposure to multiple chemicals and low-dose mixture hypotheses of carcinogenesis.

42. Although the Hallmarks of Cancer have been described (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011), there is a need to better understand the mechanisms and their relationships and the temporal and spatial relevance of the different hallmarks. This includes understanding the biology of early stages of carcinogenesis including: DNA repair, tumour suppressor genes, circulating tumour cells, tumour microenvironment, tumour promotion and associated inflammation and immune system evasion. Increased use of techniques such as ‘omics’, whole exome sequencing (WES) and microRNA sequencing has allowed detailed knowledge of these mechanisms in the aetiology of different cancers.

43. A mutation-based risk assessment process may not include epigenetic modulation, which has been increasingly shown to play a role in cancer progression (e.g. epigenetic changes in exposure to benzene including changes in methylation, modification of histones, regulation of microRNAs and other non-coding RNAs; Chappell, 2016; Fenga et al., 2016); some chemicals, in combined exposure with other chemicals, may affect epigenetic mechanisms. Therefore, increased research

is needed into epigenetic mechanisms in carcinogenesis and the effect of chemicals on these.

44. Previous chemical mixture studies, conducted in rodents, observed a dose-additive effect for a defined mixture in which the chemicals affect the same MoA, e.g. dioxin-like compounds (Walker et al., 2005). The Halifax Project suggests that if individual chemicals can induce some, but not all, Hallmarks of Cancer, then combinations of chemicals may be able to act through different MoAs in concert to induce carcinogenesis. Therefore, common environmental chemicals with known effects on different Hallmarks may require detailed investigation to test effects, both spatially and temporally, in experimental systems to investigate combined effects.

Summary

45. Current risk assessment methods including those for potential carcinogens are often based on one endpoint from experimental studies using high-dose exposures for a single chemical. Recent advances in our knowledge of the aetiology of cancer, interest in relevant ("real-life") environmental exposure and combined exposure to multiple chemicals means that new assessment paradigms need to be developed. As a response to ethical and resource considerations, the concept of AOP has been developed as a framework for developing new non-animal tests for investigating the multiple steps in the development of toxicity.

46. For potential carcinogens, 10 key Hallmarks of Cancer have been proposed for outlining essential temporal and spatial alterations in cell physiology that define malignant growth. These Hallmarks could be integrated into a new paradigm for assessing the risk of chemical carcinogens in mixtures. This would include chemicals affecting different Hallmarks (stages) in the development of malignancy. However as individual chemicals may not be complete carcinogens, care needs to be taken to avoid inappropriate classification of chemicals as carcinogens.

47. At present, this approach, based on multiple potential endpoints tested by non-animal assays, is at the development stage and is a challenge for the future. Detailed research is required, including further knowledge of basic stages in cancer, improved *in vitro* techniques and assays, epidemiology and the testing of the new paradigm using combined exposure to known multiple chemicals. The first step in this new approach, the Halifax Project, was a collaboration of world-wide experts to consider the theoretical grouping of chemicals according to the Hallmarks of Cancer. The next stage could be further collaboration with groups involved in the development of tests within the AOP framework (under authoritative bodies such as OECD and the US National Toxicology Program) to consider assays to represent each pathological/molecular stage in cancer development. Known mixtures of chemicals could then be assessed through the multiple stages of cancer.

References

Bradford-Hill, A. (1965) The environment and disease: association or causation. *Proc. R. Soc Med*., 58, 295-300.

Chappell, G., Pogribny, I.P., Guyton, K.Z. and Rusyn, I. (2016) Epigenetic alterations induced by genotoxic occupational and environmental human chemical carcinogens: A systematic literature review. *Mutation Research - Reviews in Mutation Research,* 768, 27-45.

COC (2008) Mecahnisms contributing to the synergism of asbestos and tobacco in human cancers, CC/08/10 Draft paper for discussion, Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment.

Cohen, S.M., Boobis, A.R., Dellarco, V.L., Doe, J.E., Fenner-Crisp, P.A., Moretti, A., Pastoor, T.P., Schoeny, R.S., Seed, J.G. & Wolf, D.C. (2019) Chemical carcinogenicity revisited 3: Risk assessment of carcinogenic potential based on the current state of knowledge of carcinogennesis in humans. *Regul. Toxicol. Pharmacol.*, 103, 100-105.

Doe, J.E., Boobis, A.R., Dellarco, V., Fenner-Crisp, P.A., Moretti, A., Pastoor, T.P., Schoeny, R.S., Seed, J.G. &, D.C. (2019) Chemical carcinogenicity revisited 2: Currnt knowledge of carcinogenesis shows that categorization as a carcinogen or non-carcinogen is not scientifically credible. *Regul. Toxicol. Pharmacol.*, 103, 124-129.

EFSA 2013. International Frameworks Dealing with Human Risk Assessment of Combined Exposure to Multiple Chemicals. *EFSA Journal,* 11**,** 3313.

EFSA 2019a. Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals. *EFSA Journal*, 17, 5634.

EFSA 2019b. Genotoxicity assessment of chemical mixtures. *EFSA Journal*, 17, 5519.

Fenga, C., Gangemi, S. & Costa, C. (2016) Benzene exposure is associated with epigenetic changes (Review) *Molecular Medicine Reports,* 13, 3401-3405.oodson, W. H., 3rd, Lowe, L., Carpenter, D. O., et al. 2015. Assessing the carcinogenic potential of low-dose exposures to chemical mixtures in the environment: the challenge ahead. *Carcinogenesis,* 36 Suppl 1**,** S254-96.

Hanahan, D. & Weinberg, R. A. 2000. The hallmarks of cancer. *Cell,* 100**,** 57-70.

Hanahan, D. & Weinberg, R. A. 2011. Hallmarks of cancer: the next generation. *Cell,* 144**,** 646-74.

IGHRC 2008. Chemical mixtures: a framework for assessing risks to health. Report cr14. The Interdepartmental Group on Health Risks from Chemicals. .

Miller, M. F., Goodson, W. H., Manjili, M. H., Kleinstreuer, N., Bisson, W. H. & Lowe, L. 2017. Low-Dose Mixture Hypothesis of Carcinogenesis Workshop: Scientific Underpinnings and Research Recommendations. *Environ Health Perspect.,* 125**,** 163-169.

NTP (2019) 14th Report on Carcinogens 2016. US Dept. of Health and Human Services, National Toxicology Program at https://ntp.niehs.nih.gov/ntp/roc/

OECD (2018) Test No. 451: Carcinogenicity Studies. OECD Guidelines foir the Testing of Chemicals, Section 4: Health Effects at http://www.oecd.org/env/test-no-451-carcinogenicity-studies-9789264071186-en.htm

Perkins, E. J., Antczak, P., Burgoon, L., Falciani, F., Garcia-Reyero, N., Gutsell, S., Hodges, G., Kienzler, A., Knapen, D., Mcbride, M. & Willett, C. 2015. Adverse Outcome Pathways for Regulatory Applications: Examination of Four Case Studies With Different Degrees of Completeness and Scientific Confidence. *Toxicol Sci,* 148**,** 14-25.

Smith, T.J., Liao, A., Wang, L-D. et al. (1998) Characterization of xenobiotic-metabolizing enzymes and nitrosamine metabolism in human esophagus. *Carcinogenesis*, 19, 667-672.

Smith, M.T., Guyton, K.Z., Gibbons, C.F., Fritz, J.M., Portier, C.J., Rusyn, I., DeMarini, D.M., Caldwell, J.C., Kavlock, R.J., Lambert, P., Hecht, S.S., Bucher, J.R., Stewart, B.W., Baan, R., Cogliani, V.J. & Straif, K. (2016) Key characteristics of carcinogens as a basis for organizing data on mechanisms of carcinogenesis. *Environ Health Perspect.*, 124, 713-721.

Walker, N. J., Crockett, P. W., Nyska, A., Brix, A. E., Jokinen, M. P., Sells, D. M., Hailey, J. R., Easterling, M., Haseman, J. K., Yin, M., Wyde, M. E., Bucher, J. R. & Portier, C. J. 2005. Dose-additive carcinogenicity of a defined mixture of "dioxin-like compounds". *Environ Health Perspect,* 113**,** 43-8.7

Woolf, D.C., Cohen, S.M., Boobis, A.R., Dellarco, V.L., Fenner-Crisp, P.A., Moretto, A., Pastoor, T.P., Schoeny, R.S., Seed, J.G. & Doe, J.E. (2019) Chemical carcinogenicity revisited 1: A unified theory of carcinogenicity based on contemporary knowledge. *Regul. Toxicol. Pharmacol*., 103, 86-92.