CC/2018/09

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

Risk assessment of the effects of combined exposures to chemicals on carcinogenicity

Background

1. In the COC Guidance statement series, G08 is entitled 'Risk assessment of mixtures of chemical carcinogens', produced following a review by COC of a number of papers on the topic during 2008-2009. Since its publication, there have been many developments in the field of the risk assessment of chemical mixtures. This, combined with an increased knowledge of the development of cancer, has led to COC wishing to explore whether a cancer endpoint-specific approach could be derived to allow the risk assessment of combined exposures to chemicals on carcinogenicity. This review is based on improved knowledge of mechanisms of action and, as such, epidemiological studies have not been considered.

2. The principles of risk assessment of chemical carcinogenicity have traditionally been based on cancer endpoints, particularly in laboratory animals. For many years, the development of cancer in such animal models was based on the simple paradigm of "initiation and promotion", while the discovery of mutations in (proto)oncogenes and tumour suppressor genes in many human cancers has led to a number of more detailed multistage models. These discoveries and improvement in the understanding of the pathology of cancer development enabled Hanahan and Weinberg (2000) to describe phenotypical 'Hallmarks of Cancer', and these have been further refined (Hanahan and Weinberg, 2011). These advances have been accompanied by increasing interest in low-dose exposures which encompass the more realistic concentrations of chemicals present in human environmental scenarios, rather than the high doses which have historically been used in long-term rodent studies and have generally been used for the purpose of hazard identification rather than risk assessment. These scientific advances have enabled real consideration of the risk assessment of both individual mixtures of chemicals and how such mixtures might interact during the multistages of cancer aetiology.

3. A number of publications have been published, or are currently out for comment, that have furthered the development of the concepts and subsequent frameworks necessary for such risk assessments. For example, relevant documents have been produced by EFSA, currently in draft form for consultation: one comprises draft guidance for the harmonised risk assessment of combined exposure to multiple chemicals (EFSA, 2018a); the other, relates more specifically to the genotoxic

assessment of chemical mixtures (EFSA, 2018b). In addition, a world-wide collaboration known as the 'Halifax Project' has also published a review on the carcinogenic potential of low-dose exposures to chemical mixtures in the environment (Goodson et al., 2015).

4. In July, 2018, COC discussed an initial paper (CC/2018/03) on assessment of combined exposure to chemicals including the two EFSA consultation documents. COC noted that the frameworks and approaches were generally not appropriate for considering potential carcinogens, as tumour development is a series of events and not a single toxicological effect. COC considered it more appropriate to reflect on the process of carcinogenesis rather than use numerical calculations based on chemical exposure analyses. This would enable assessment of the potential for chemicals with different modes of action (MoA) to act together to induce carcinogenesis. COC noted that the initiation-promotion model for carcinogenesis was outdated and that new models of toxicology and cancer development, such as Adverse Outcome Pathways (AOPs) and the Hallmarks of Cancer, respectively, may provide a more realistic approach when considering multiple exposures to chemicals including potential carcinogens, e.g. exposure to an immunosuppressive drug in combination with a genotoxic chemical.

5. As requested at the July 2018 COC meeting, this document considers which principles can be applied in a novel way to the risk assessment of exposure to mixtures containing potential carcinogens. Evaluation of an AOP approach in conjunction with the principles of the Hallmarks of Cancer that takes into account the role played by individual susceptibility is evaluated. This includes consideration of the findings of the Halifax Project (Goodson et al., 2015), which assessed how non-carcinogens may affect stages in cancer development and how a combined exposure might lead to the development of cancer. Two examples of combined exposure with known synergism that have previously been assessed by COC include alcohol and tobacco smoking (CC/08/10) and asbestos and tobacco smoking (CC/08/20). These are considered here using an AOP/Hallmarks of Cancer approach. Future development of these new approaches including the development of tissue and cellular models, 'omics' technology and QSAR based on biological effects are also suggested.

Harmonised risk assessment for mixtures

6. The basic principles of risk assessment have been developed over many decades for individual chemicals and so, recent advances in the assessment of combined exposure to chemicals have been built on this paradigm. The recent EFSA draft document (EFSA, 2018a) provides detailed guidance on harmonised methodologies for combined exposure to multiple chemicals. This guidance on the risk assessment of mixtures is fundamentally based on the same paradigm. This EFSA document does not consider carcinogens as a separate category, but the EFSA guidance is briefly considered in this review below under the basic headings.

For further information on these topics the document should be consulted (EFSA, 2018a).

Characterisation of simple and complex mixtures

7. A “whole mixture approach” is defined as a risk assessment in which the mixture (combined exposure) is treated as a single entity equivalent to a single chemical (such as pesticide/biocide formulations usually restricted to single dose toxicity, irritancy and skin sensitisation (IGHRC, 2008)). This approach requires dose-response data either for the mixture of interest or as a ‘read-across’ from a similar mixture. This approach may be particularly required when the composition of the mixture is unknown or difficult to characterise. These are also called complex mixtures. Examples of these 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 and air pollution (IGHRC, 2008).

8. When the components of the combined exposure (mixture) are largely known, as in a fixed composition (e.g. a registered and authorised agrochemical) including exposure levels, then this can be regarded as a simple mixture and a risk assessment based on individual components can be made. This requires exposure and effects data on individual components. One way in which this can be practically considered is by organising the components into chemical assessment groups. Such groupings reduce the potential for over-estimating risk by combining the risks from independent chemicals and enables the combination of dose-response effects. Examples of criteria for such groupings include physicochemical properties, hazard characteristics or exposure considerations. Toxicological mechanistic concepts such as mode of action (MoA), mechanism of action and AOP can play an important role when grouping chemicals. The MoA identifies key events such as cytological or biochemical changes that are both measurable and necessary for the observed toxic effect (EFSA, 2013). Related to this concept is the AOP, which traces the mechanistic pathway between the initial chemical interaction and the subsequent perturbations to cellular function; finally leading to the adverse toxic outcome. The EFSA document (EFSA, 2018a) suggests that although the AOP approach has potential applications in defining assessment groups, as yet it has found little practical application in mixture risk assessment. The potential use and shortcomings of the use of AOP and models of cancer aetiology in the assessment of carcinogenic potential of combined exposure to chemicals is discussed further in paragraphs 17-22.

Problem formulation

9. Problem formulation is the initial step in an iterative process involving consideration of the need for, and extent of, a risk assessment (EFSA, 2018a). 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, MoA and AOP (EFSA, 2018a). This would be very complex in any risk assessment of the carcinogenic potential of mixtures where, for example, length of exposure, lifetime versus limited exposure and spatial considerations of endpoints need to be assessed and considered.

Exposure assessment

10. The exposure assessment requires consideration of exposure pathway, exposed population, variation of dose 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 single chemicals. However, there are additional considerations to be taken into account for both mixtures and the carcinogenic potential of combined exposure. Whole mixture approaches are usually limited to cases (such as pesticides, biocides, and other formulated products, (IGHRC, 2008)) in which the combined exposure is restricted to a single route of exposure, as complex pathways of exposure may mean that the population will not be equally exposed to all components. 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 temporal (limited and lifetime exposure) may be of vital importance. Therefore, knowledge of MoA is also necessary for this assessment.

Hazard identification and characterisation

11. Hazard identification and characterisation (hazard assessment) of combined exposure to multiple chemicals aims to derive quantitative metrics reflecting the combined toxicity of the mixture (EFSA, 2018a). Hazard identification is a qualitative process that plays an important role in the assessment of mixtures in determining the grouping of chemicals with similar adverse effects. 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. For example, genotoxicity, an important adverse effect in cancer risk assessment is not presently used in quantitative risk characterisation as there is no agreed dose-response, even for single chemicals. The complexity of the assessment of the genotoxic potential of chemical mixtures has been addressed by the second EFSA draft guidance document described earlier (EFSA, 2018b).

12. The conclusions of the draft EFSA document (EFSA, 2018b) give some indications of the difficulties of assessing the adverse effects of exposure to multiple chemicals by just one potential mechanism, genotoxicity, in multistage cancer development as suggested by the hallmarks of cancer (paragraphs 23-25):

- 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.

    - If adequate *in vitro* testing is clearly negative, the mixture should be considered of no concern for genotoxicity.

    - If adequate *in vitro* testing provides one or more positive results, a follow-up *in vivo* study should be considered.

    - If these follow-up *in vivo* tests are negative, the possible limitations of these tests should be considered in an uncertainty analysis before a conclusion is reached on concern for genotoxicity.

    - For positive results in these *in vivo* tests, the mixture does raise a concern for genotoxicity.

Risk characterisation

13. The risk characterisation of chemical mixtures aims to:

a. Calculate the ratio of exposure to hazard to determine whether there is a possible concern to the identified population; and

b. Identify the components in an assessment group that represent particularly important risk drivers for the component-based approach (EFSA, 2018a).

14. In the whole 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 the estimated exposure exceeds the reference value derived from the hazard data, there is a potential risk. The margin of exposure (MoE) represents the uncertainty of the data and the nature of the toxic effect. In the case of the assessment of carcinogenic potential in a whole mixture approach, the mixture would be required to be considered as a carcinogen.

15. In a component-based approach, there are several methodologies for dose addition, such as the reference values, Hazard Index (HI) and Margin of Exposure (MOE) to sum the effects of individual components. The problem with this simple approach with mixtures is that uncertainty factors for each component are combined

when calculating the HI. In addition, such reference values may have been derived from different types of study of differing endpoints and quality.

16. There is also an option for response addition when substances are likely to act by independent actions or mechanisms; here, no interaction between the substances is expected, either in the exposure medium or in the exposed population, and "response points" (reference value derived from hazard data) and ideally the full dose-response is available for all, or at least two, substances in the mixture. A combined response can then be calculated using the equation for independent random events.

New Approaches to cancer research and risk assessment

Adverse Outcome Pathways (AOPs)

17. Traditional methods of risk assessment including the results of animal testing, 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 are standard. These 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 an AOP is shown in Figure 1.

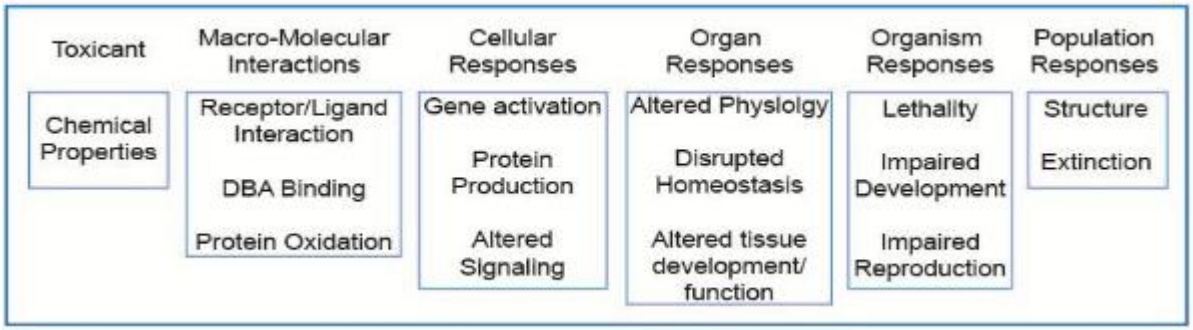

Figure 1: Generalised Adverse Outcome Pathway[1]

18. Both MoA and AOP are frameworks for relating these new types of data for risk assessment and are currently being widely investigated for chemical risk assessment and more recently for mixtures (EFSA, 2018a) 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 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

1 Taken from OECD website at http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm

et al., 2015) examined AOPs for four case studies with different degrees of completeness and scientific confidence. This study included the AOP for 1,4-dioxane where hepatocellular proliferation leads to cancer. The mechanistic and causal understanding of the events leading to the adverse 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 2.

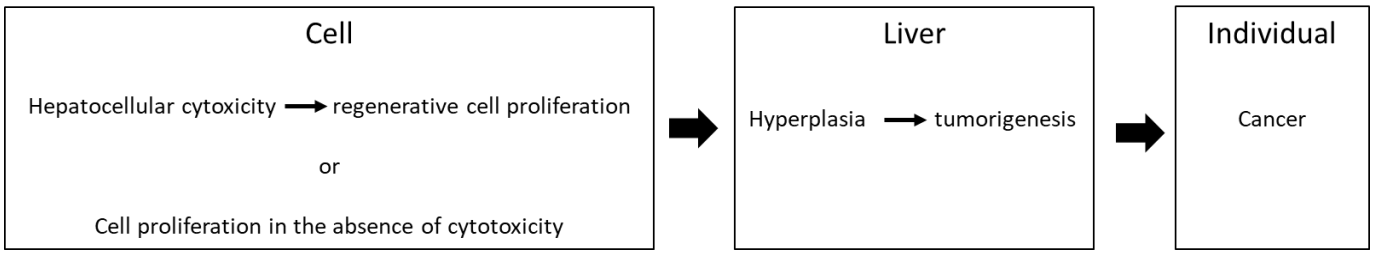

Figure 2: AOP for 1,4-dioxane leading through hepatocellular proliferation leading to cancer (modified from Perkins et al., 2015).

19. The toxicological evidence for the AOP outlined in Figure 2 is sufficient to establish key events of either cell proliferation in the absence of liver cytotoxicity leading to hyperplasia and tumour formation or, liver cytotoxicity followed by regenerative 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.

20. More recent advances in technology can be used to gain further confidence in the AOP. 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 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.

21. 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 is for a single chemical exposure, but indicates how an AOP/cancer development approach 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

22. Since Armitage and Doll first outlined a multistage theory of cancer in the 1950s and initiation and promotion were established as distinct steps in cancer aetiology, 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' for outlining essential alterations in cell physiology that define malignant growth (see Figure 2; defined as '….. acquired capabilities common to most cancers that incipient cancer cells … [must acquire to] enable them to become tumorigenic and ultimately malignant'.

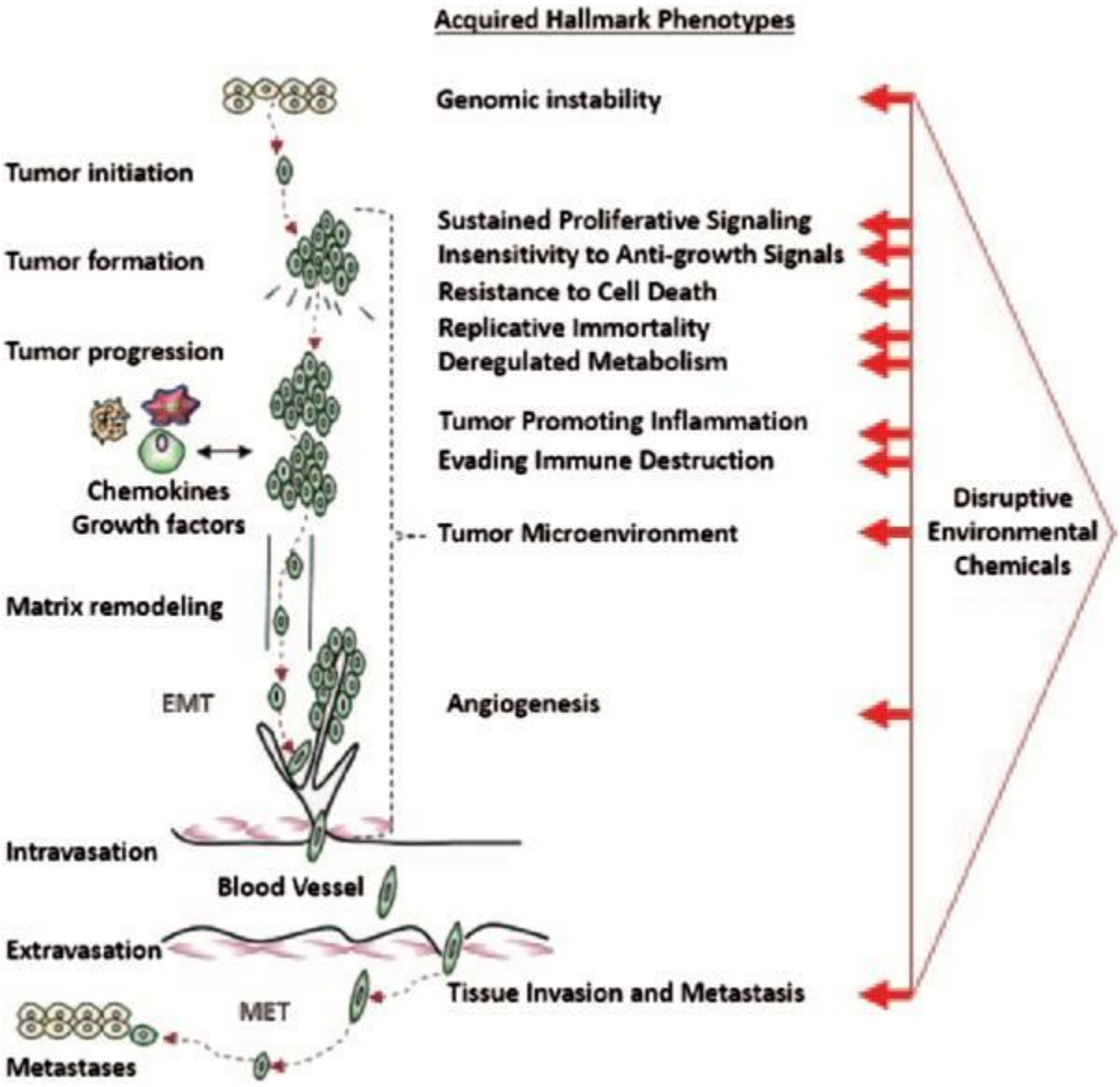

Figure 3: Potential disruption of hallmarks of cancer by environmental chemicals (Goodson et al., 2015) reproduced under Creative Commons Attribution Non-Commercial License

The Ten Hallmarks of Cancer

- *Genetic instability and mutation* – allowing changes in one cell to pass to a daughter cells through mutation or epigenetic changes in the parent cell DNA.

- *Tumour-promoting inflammation* – helping cancer cells grow using the same growth signals that 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 (cell death using internal mechanisms).

- *Replicative immortality* – cancer cells do not senesce (or age) 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.

23. Although there is now much research underpinning these Hallmark multiple stages in cancer development, little of this has been translated into the assessment of the carcinogenic potential of chemicals. A number of these Hallmarks, such as effects of chemicals on metabolism and the immune system, are not what would 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 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, plays a part in the development of cancer when other chemicals are present. It is clear that such considerations are important when considering the risk assessment of combined exposure to multiple chemicals and that Hallmarks of Cancer may prove to be useful in the risk assessment of such mixtures.

24. 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

25. The Halifax Project was a large-scale 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.

26. In the Halifax Project, the toxicological data on 85 environmental chemicals not considered to be carcinogens was reviewed, including pesticides, metals, plasticisers, etc. These chemicals were all considered 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 (see paragraph 30), with the remaining 26% having no dose-response data.

27. The paper (Goodson et al., 2015) 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 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 classified as carcinogens; however, one chemical might support one Hallmark while another two Hallmarks and so forth until the result may be a carcinogenic potential, similar to an exposure to a classified carcinogen.

28. There is a danger, however, that an individual chemical that affects only a single stage may thus be considered to be a carcinogen, rather than part of a potentially carcinogenic mixture. The COC indicated at the July 2018 meeting, that classification of chemicals as carcinogens on this basis would be undesirable.

Low-dose exposure

29. An important consideration when assessing evidence such as that described above, is the definition of ‘low-dose’ exposure. The term is often used to mean

relevant environmental (general population or occupational) exposure. However, the Halifax Project used the EFSA definition, i.e. 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 effect definitions could be based on as being below the No Observed Adverse Effect Levels (NOAEL) or Benchmark Dose (BMD), although such use could be difficult when multiple chemicals are being considered, or in the future when the results of long-term animal studies are not available. Low dose exposures have been defined by the US National Toxicology Program as those occurring within the range of typical human exposures. It has been shown that endogenous hormones have effects at low concentrations and so endocrine disrupters have dominated most discussions of low dose effects. At present, there is no accepted definition of 'low-dose' in risk assessment paradigms.

Individual susceptibility

30. Individual susceptibility to cancer has only 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 given in paragraphs 38 and 39 when induction of CYP2E1 by ethanol may potentiate the effects of chemicals present in tobacco smoke. However, other individual susceptibilities may also be present in other stages in the development of cancer including other genetic and epigenetic mechanisms. At present, little is known of these effects; however, new sequencing and molecular techniques are available to examine these.

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

31. The regulatory assessment of the carcinogenic potential of single chemicals has developed based mainly on long-term, high dose exposure in animals, together with *in vitro* and *in vivo* assays considering genotoxicity and/or mutagenicity. More recently there has been greater consideration of the potential toxicity of chemical mixtures,

32. however, for carcinogenic potential, this has mainly consisted of accumulative effects of exposure to individual carcinogens identified in the mixture. For ethical and financial reasons, 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 use results from newly developed *in vitro assays*, tissue models and computational methods for risk assessment.

33. 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.

34. This concept could form the basis of new paradigms for carcinogenic risk assessment in the future, particularly for 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. It appears unlikely that sufficiently robust data can be derived from *in vitro* cell and tissue models, computational modelling and other approaches which are not yet validated.

35. A further potential problem is that the Hallmarks of Cancer approach refers to a stepped development 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.

36. With the conclusion that this concept may not yet be applicable to carcinogenic risk assessment of the combined exposure to multiple chemicals, the following sections examine two examples of substances acting synergistically and how they might be studied using AOP/Hallmarks of cancer methods and then possible future developments and research are suggested.

Examples of Synergistic chemicals

37. The combined exposures to alcohol and tobacco smoke and asbestos and tobacco smoke have both been previously considered by COC (2008a, 2008b). Although these examples are not typical of the combined exposure of multiple chemicals addressed so far in this paper, it is well-documented from human studies that the two components behave in a synergistic manner in the development of malignancy. The COC previously considered these examples in terms of their MoA; however, they are used here to assess whether the synergy can be predicted using an AOP/Hallmarks of Cancer approach.

Alcohol and tobacco smoking

38. Tobacco smoking and alcohol are well-established risk factors for cancers of the head and neck, larynx and squamous-cell carcinoma of the oesophagus. Studies indicate that the combined use of alcohol and tobacco interact to induce these cancers in a greater than additive frequency. The mechanisms by which this synergism occurs are not well-understood but the following have been proposed (COC, 2008a).

a) *Induction of CYP enzymes by ethanol* – Alcohol is not considered to be a carcinogen in experimental animals. Although alcohol is metabolised mainly by alcohol dehydrogenase, CYP2E1 is thought to be responsible for 20% of metabolism at low blood levels. Alcohol is also known to induce CYP2E1 via stabilisation of the protein. The potential significance of this in the carcinogenic synergism with tobacco smoke is apparent as the most carcinogenic nitrosamines present in tobacco smoke are metabolically activated by CYP enzymes. This has been shown when rats were given N-nitrosomethylbenzylamine (NMBA) together with ethanol for 10 weeks. There was an increased number of oesophageal polyps, together with increased CYP2E1 expression, in the oesophageal mucosa. It should be noted that alcohol has been observed to reduce nitrosamine metabolism in the liver; possibly by competitive inhibition. Other metabolising enzymes have also been proposed as having a role in the synergistic effects of alcohol and tobacco.

b) *Increased permeability of epithelial cells* – Another plausible mechanism for the synergism is that alcohol increases the permeability of the oral mucosa to carcinogens present in tobacco smoke. This has been shown using *in vitro* porcine oral mucosal cells where the presence of ethanol enhanced the penetration of nitronornicotine. This was also reported with benzo(a)pyrene (B(a)P).

39. As noted above, alcohol is not considered a carcinogen in experimental animals although it is a human carcinogen when taken as a beverage. In an assessment based on AOP and Hallmarks of Cancer, metabolism is not considered and so these synergistic mechanisms based on induction of CYP2E1 would have to be considered separately. The more physicochemical enhancement of permeability would also not be assessed in this process. Therefore, this example highlights the potential shortcoming of these novel paradigms for carcinogenic risk assessment of combined exposure of multiple chemicals. It should be noted that, in general, metabolism and physicochemical effects are not well addressed in risk assessment methodologies. However, risk assessments based on the key Hallmarks of Cancer would identify further potential synergies and gaps in knowledge which need to be filled.

Asbestos and tobacco smoking

40. Cigarette smoking and exposure to asbestos can both individually cause lung cancer in humans. Epidemiological studies have shown that combined exposure results in a synergistic effect on lung cancer induction. Despite extensive research, the precise mechanism involved in the interaction at the cellular and molecular level has not been established. Both asbestos and cigarette smoking are complex carcinogens and affect more than one stage of cancer development. Research into the mechanisms behind this synergism has revealed a number of mechanisms. In

many of these studies, B(a)P has been used as a representative of tobacco smoking (COC, 2008b).

a) *Cytotoxic, genotoxic and clastogenicity* – Although asbestos is not positive in an Ames test, it is mutagenic in tests which detect large mutations. Combined exposure with B(a)P leads to a synergistic (supra-additive) increase in mutation frequency in rat lung. Increased genotoxicity measured by a number of parameters (including micronuclei and sister chromatid exchange) has also been observed with combined exposure in asbestos workers.

b) *Generation of oxidative damage, reactive oxygen species (ROS) and DNA damage* – A number of studies have indicated that the generation of ROS by both asbestos and tobacco is an important mechanism in synergistic lung damage. Tobacco smoke can produce superoxide anion, hydrogen peroxide and hydroxyl radicals, and ferrous iron in asbestos forms hydroxyl radicals by a Fenton reaction with hydrogen peroxide. This may lead to both oxidative DNA damage and lipid peroxidation.

   Fibres can also cause inflammation which results in chronic proliferation of epithelial cells. Smoking also induces cell proliferation, inflammation and the production of ROS. A combination of amosite asbestos and cigarette smoke has been shown to cause a synergistic increase in the number of proliferating cells in the small airways.

c) *Somatic mutations in Ki-ras, FHIT and p53 genes* – The synergistic increased mutation rates outlined in a) may be important in the frequency of mutations in key genes in the development of lung cancer. In lung tumours, the loss of a fragile region, FRA3B on the short arm of chromosome 3 (3p14) containing the tumour suppressor gene, *FHIT*, has been associated with both asbestos exposure and tobacco smoking. Both asbestos exposure and polyaromatic hydrocarbons present in cigarette smoke are associated with G to T transversions at codon 12 of the *Ki-ras* oncogene. Mutations in the *p53* tumour suppressor gene are frequent events in carcinogenesis and are found in the lungs of about a third of smokers with lung cancer, particularly those who were older and had smoked longer. Those smokers with *p53* mutation were also more likely to be occupationally-exposed to asbestos. However, such mutations have not been observed in all studies on asbestos-induced lung cancer.

d) *Tobacco smoking enhances the penetration and accumulation of asbestos in the lung* – Another proposed mechanism for the observed synergistic effect is the demonstrated ability of tobacco smoke to facilitate the increased penetration of asbestos into bronchial walls. In a further study, this enhanced effect is abolished by catalase and superoxide dismutase, suggesting that this is an oxygen radical mediated mechanism. There is also evidence that cigarette smoke increases the pulmonary retention of asbestos in human subjects.

e) *Asbestos serves as a vehicle for delivery of tobacco carcinogens into the lung and enhances the metabolism of tobacco carcinogens* – A further hypothesis is that asbestos enhances the delivery of mutagenic polyaromatic compounds in tobacco smoke to the respiratory epithelium, possibly by holding them *in situ.* However, the liberation of bound polyaromatic compounds is extremely fast from most alveolar or bronchial surfaces. There is also evidence that asbestos increases uptake of xenobiotics and inhibits glucuronide conjugation and these events could increase the levels of toxic xenobiotic metabolites in asbestos-damaged tissue and increase the probability of malignancy.

41. Although asbestos is negative in the Ames test, both asbestos and tobacco smoking are classfied carcinogens. Therefore, an assessment based on AOP and Hallmarks of Cancer would not yield much extra information for hazard assessment. An assessment based on Hallmarks of Cancer, combined exposure gives increased (and supra-additive in some cases) *genetic instability* (both directly and via oxidative damage) and when this potentially leads to increased mutations in the key regulatory genes, *FHIT*, Ki-*ras* and *p53,* this affects other Hallmarks, such as *insensitivity to anti-growth signals* and *sustained proliferative signalling*. Further studies based on the Hallmarks of Cancer might also identify additional events leading to malignancy. Observed synergistic inflammation and proliferative cell growth from exposure to asbestos and tobacco smoking also meets the Hallmark of *tumour-promoting inflammation*.

42. However, the more physicochemical synergistic events of tobacco smoking enhancing the penetration and accumulation of asbestos in the lung and asbestos serving as a vehicle for delivery of tobacco carcinogens into the lung may not be properly addressed in a risk assessment based on AOP and Hallmarks of Cancer. This is also true of changes in metabolism of a carcinogen (which may lead to an enhanced or reduced effect) induced by another chemical in a mixture. This remains a problem with all methods of carcinogen risk assessment of combined exposure to multiple chemicals, as seen in the previous example.

Future development and research

43. It is acknowledged that there are clear limitations to high-dose, animal-based models in predicting human responses to potential carcinogens and therefore, it is necessary to establish principles and guidelines for 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, reliable results and increase our knowledge of the carcinogenic process.

44. Following publication of the Halifax Project (Goodson et al., 2015), recommendations from subsequent workshops were published (Miller et al., 2017).

The 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.

45. 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.

46. A mutation-based risk assessment process may not include epigenetic modulation which has been increasingly shown to play a role in cancer progression; 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.

47. 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 reference environmental chemicals with known effects on defined Hallmarks require detailed investigation to test effects both spatially and temporally on newly developed experimental systems. Currently, there is no agreed definition of low-dose and so this needs further research and discussion to settle whether doses particularly used *in vitro* are relevant to human exposure.

Summary

48. Current risk assessment methods including those for potential carcinogens are often based on single endpoints and animal studies using high-dose exposures. Recent increases in our knowledge of the aetiology of cancer, interest in low-dose relevant exposure and combined exposure to multiple chemicals means that new assessment paradigms need to be developed. As a response to ethical and cost concerns, 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.

49. For potential carcinogens, ten key Hallmarks of Cancer have been proposed for outlining essential temporal and spatial alterations in cell physiology that define malignant growth. These Hallmarks could form the basis for a new paradigm for assessing the risk of chemical carcinogens in mixtures. This could include chemicals affecting different Hallmarks (stages) in the development of malignancy, although

individual chemicals may not be classified carcinogens. However, this is also a potential problem as individual chemicals affecting specific Hallmarks should not necessarily be classified as carcinogens.

50. At present, this approach 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 non-animal techniques and assays and the testing of the new paradigm using combined exposure to known multiple chemicals.

Questions for the Committee

51. Members are asked to consider this paper and in particular:

i. How does the Committee wish to take forward the guidance statement on effects of combined exposure to chemicals on carcinogenicity?

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Abbreviations

AOP – Adverse Outcome Pathway

B(a)P – Benzo(a)pyrene

BMD – Benchmark Dose

NMBA – N-nitrosomethylbenzylamine

NOAEL – No Observed Adverse Effect Level

QSAR – Quantitative Structure-Activity Relationship

ROS – Reactive Oxygen Species

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