CC/2018/03

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

Risk Assessment of the Effects of Combined Exposures to Chemical Carcinogens - an update

Introduction

1. COC Guidance Statement G08 is the Committee's guidance on risk assessment of mixtures of chemical carcinogens. This was originally written as the COC Statement on the Risk Assessment of the Effects of Combined Exposures to Chemical Carcinogens in 2010 (COC, 2010) (presented in Annex 1) and has served as G08 since the website was migrated. The document was based on the Committee's review of the scientific literature of developments in the assessment of chemical mixtures in 2008 and set out overarching principles of chemical mixtures assessment, discussed the applicability of these approaches to combinations of carcinogens and examined some specific examples of mixtures of carcinogens and the potential for synergistic interactions.

2. Since the original statement was published, many authoritative and regulatory bodies have developed risk assessment frameworks and/or guidance on chemical mixture assessments. This discussion paper outlines some of these new developments. The Committee are asked to consider this paper, together with the original statement, and propose content for a reformatted and revised version of G08.

Background

3. There are a number of widely used risk assessment frameworks for chemical mixtures providing detailed guidance for the evaluation of their toxicity. For example, the COT report 'Risk Assessment of Mixtures of Pesticides and Similar Substances' (COT, 2002) formulated advice on the risk assessment of the potential toxicities of multiple residues of pesticides and veterinary medicines in food. Other, more recent, initiatives include those by the UK Interdepartmental Group on Health Risks from Chemicals (IGHRC) (IGHRC, 2009), the WHO International Programme of Chemical Safety (IPCS) (IPCS, 2009), the Scientific Committee on Health and Environmental Risks (SCHER) (SCHER, 2012) and the European Food Safety Authority (EFSA) (EFSA, 2013), which have generated robust frameworks that have a broad application across different chemical risk assessment scenarios. These frameworks are continually being refined (Boobis et al., 2006; Meek et al., 2011; Price et al., 2012) but remain largely used to consider nonspecific toxicological endpoints that generally have thresholds for effect. Whilst these strategies can be applied to

mixtures of carcinogens, there are few that specifically address the complexities of carcinogenesis. More recently the 'low dose mixture hypothesis', as examined in the Halifax project, focuses specifically on carcinogenesis (Goodson et al., 2015; Miller et al., 2017).

Types of action

4. Mixtures are generally classified based on the characteristics of the components which define the possible outcomes. Three basic types of action are widely recognised (COT, 2002; IGHRC, 2009; Meek et al., 2011; SCHER, 2012):

- Similar action (also known as non-interaction, dose/concentration addition);
- Dissimilar action (also known as non-interaction, independent joint action, response/effect addition); and
- Interaction (also known assynergism/potentiation or antagonism/inhibition).

5. These were defined in the COC 2010 statement (Annex 1) and remain the mainstay of mixtures risk assessment. It is noted that as there are no agreed definitions for describing the risk assessment of chemical mixtures and terminology can differ between different authoritative and regulatory bodies.

6. ***Similar action*** is the concept whereby combinations of chemicals have the same target organ and act via the same mode of action (MOA) (see para 14). These are commonly referred to as belonging to a common mechanism group (CMG) or a common assessment group (CAG) (see para 15). It is assumed that there is no interaction between individual chemicals. Similar action is also referred to as 'dose, or concentration addition'. The overall effect of the mixture is determined by the sum of the effects of the respective doses and the relative potencies of the components.

7. ***Dissimilar action*** assumes individual chemicals have different MOAs and the nature and specific site of action may also differ. The effect of each chemical does not modulate or contribute towards the effects of the other constituents of the mixture and, hence, the health effects of exposure to the mixture are expected to be qualitatively and quantitatively similar to those produced by individual components when administered alone. Effect addition is the summation of the individual responses of the different mixture components and toxicity is predicted from the dose response curves of the individual chemicals.

8. ***Interaction*** is present when the observed effect of two or more chemical exposures differs from the effect that would be expected if the exposures had additive effects. Synergism and potentiation are terms used to describe responses that are greater than additive, and antagonism and inhibition are used for responses that are less than additive.

9. There are a number of possible mechanisms by which interaction can occur. These include toxicokinetic interactions that cause deviations from additivity (e.g.

modification of absorption, uptake or clearance mechanisms); metabolic interactions where one chemical alters the metabolism of other mixture components; and toxicodynamic interactions where a biological response resulting from exposure to one individual chemical is impacted by another (e.g. the result of ligand-receptor interactions).

Approaches to the risk assessment of chemical mixtures, terminology and tools

10. The risk assessment of whole mixtures can be carried out on data on the mixture itself if available (e.g. tobacco smoke; herbal medicines). However, it is more commonplace for risk assessment to be undertaken using component-based approaches. Using this approach, data on the individual chemicals and knowledge of their toxicities can be assessed together depending on whether the mixture is considered to exhibit 'similar action', dissimilar action' or 'interaction. It is acknowledged that establishing whether interactions occur is difficult. A number of tools have been developed to assist with component-based risk assessment, as outlined in the following paragraphs. The terminology given is an amalgamation from a number of sources, broadly describing the same tools.

11. ***Aggregate exposure*** refers to exposure to a single chemical from multiple routes/sources (IGHRC, 2009; IPCS, 2009; SCHER, 2012; WHO, 2017).

12. ***Combined exposure and/or cumulative exposure*** generally refer to exposure to multiple chemicals from multiple routes/exposures but is also used to describe multiple chemicals by a single route (SCHER, 2012; WHO, 2017). More broadly it is defined as the demographic, spatial and temporal characteristics of exposure to multiple single chemicals and physical stressors through all relevant pathways (e.g. food, water, residential uses, occupational exposure) and routes (e.g. oral, dermal, inhalation) (IPCS, 2009; Moretto et al., 2017). This can include non-simultaneous exposures, which may be important when considering carcinogenesis.

13. ***Cumulative Risk Assessment*** (CRA) is defined as the risk assessment of combined exposures to multiple chemicals (Meek, 2013; Moretto et al., 2017).

14. ***Mode of action*** is a widely applied principle used when evaluating the effects of combined exposures to multiple chemicals. MOA is a biologically plausible sequence of key events leading to an observed effect supported by robust experimental observations and mechanistic data (Boobis et al., 2006). Chemicals that act by the same MOA can be evaluated using dose addition.

15. ***Common mechanism group*** (CMG) or ***Cumulative assessment group*** (CAG) are techniques used in mixture risk assessment to group chemicals with the same MOA, which can then be assessed as a single chemical (IPCS, 2009). Most simply, this applies to chemicals that act through the same molecular target to elicit the same effect(s), for example, a receptor such as the aryl hydrocarbon receptor (AhR) receptor or the oestrogen receptor (ER). More broadly, chemicals acting

independently on the same rate-limiting key event would be anticipated to exhibit dose additivity in their carcinogenic response. Chemicals within a CMG are assessed using 'similar action' principles. Note that CAG appears to be principally used by EFSA (EFSA, 2013).

16. ***Relative Potency Factors*** (RPF), ***Potency Equivalency Factors*** (PEFs) or ***Toxic Equivalency Factors*** (TEF) are used for mixtures consisting of a single class of structurally similar chemicals. The RPF, PEF or TEF express the relative potency or toxicity of a given chemical within a CMG to an 'index compound'. The index compound is generally the one for which toxicity and absorption, distribution, metabolism and excretion profiles are best characterised, This normalises the toxicities of chemicals within a CMG to a single compound (SCHER, 2012; WHO, 2017). The concentration of each component of the mixture is multiplied by its TEF, and all the components summed to give the ***Toxic Equivalency Quotient*** (TEQ) expresses the toxicity of a mixture of chemicals within a CMG in terms of an equivalent dose of the 'index compound' (IGHRC, 2009*).*

17. ***Hazard Index*** (HI) is an approach that is widely used for component-based assessments for chemicals within a CMG for which data are available on the components (IGHRC, 2009). The Hazard Quotient (HQ) is calculated as the ratio of the exposure to an individual health-based reference level or value (RL or RV), for example, TDI. The HI is then calculated as the sum of the HQ's. If HI > 1, the total concentration of mixture components exceeds the level considered to be without harm.

$$HI = \frac{E_1}{RL_1} + \frac{E_2}{RL_2} + \ldots + \frac{E_n}{RL_n} \quad \text{or} \quad HL : \sum_{i=1}^{n} \frac{E_i}{RL_i}$$

Where: HI is the hazard index; E represents the exposure level of each individual component; and RL represents a reference level for each individual component.

When the RV of a certain compound is based on an effect that is not the group effect (common toxic effect), or the applied assessment factor includes adjustments not related to the endpoint of concern, then the HQ can be refined by identifying the RV for the group effect and adjusting the hazard quotient, accordingly. This approach is commonly used in scenarios where components are identified to be in a CMG, such as the trihalomethanes (THM) in drinking water assessment (see para 41).

Scientific frameworks and guidance

18. Many organisations have developed and published guidance and scientific frameworks for the risk assessment of chemical mixtures (EFSA, 2013; IGHRC, 2009; Meek et al., 2011; Price et al., 2012; SCHER, 2012; Sexton, 2012; Solomon et al., 2016). These are underpinned by some basic mathematical assumptions and approaches. Frameworks are generally based on a problem formulation step, followed by the application of a tiered approach, which considers co-exposures in the context of the margin of exposure (MOE). If the MOE for preliminary tiers indicate

that there is no concern, then further assessment is not required. Many of the established frameworks are generic in nature and there are few specific references to the applicability of the approach to mixtures of carcinogens or the process of carcinogenesis.

19. When compiling the 2010 statement, the COC considered the COT report 'Risk Assessment of Mixtures of Pesticides and Similar Substances' (COT 2002) and the initiative coordinated by the UK IGHRC (IGHRC 2009). Although the WHO IPCS framework was also considered, this was not published at that time. Whilst the COC 2010 statement concluded that procedures for the risk assessment of combined exposures to multiple chemicals provided solid guidance for anyone required to evaluate the toxicity of chemicals, it was noted that there is no specific guidance on the assessment of the impact of combined exposure to carcinogens or to carcinogens and other chemicals with regards to cancer.

20. A number of frameworks have also been developed since the publication of the 2010 statement. These, together with the IGHRC framework included in the 2010 statement, are summarised in the paragraphs below.

IGHRC framework

21. The IGHRC developed a stepwise, tiered approach for the risk assessment of chemical mixtures (detailed in Annex 2). The recommended risk assessment process involves defining the mixture, evaluating the likelihood of exposure, applying tools such as ‘dose addition’ and finally, if these steps indicate concern, the inclusion of risk management steps. The HI and TEF approaches for the evaluation of mixtures are advocated (IGHRC, 2009).

WHO/IPCS framework

22. The WHO/IPCS framework for the risk assessment of the combined exposure to multiple chemicals is a tiered approach based upon increasing refinement of hazard and exposure assessments (Annex 3) (IPCS, 2009; Meek, 2013; Meek et al., 2011). The framework was developed as a robust approach to estimate risk and to identify risk management scenarios where co-exposures to multiple chemicals are anticipated. It details specific terminology to facilitate interpretation of different exposure scenarios – in particular, the following are highlighted:

- *“single chemical, all routes” (referenced in some jurisdictions as “aggregate” exposure) - same substance from multiple sources and by multiple pathways and routes of exposure; and*

- *“multiple chemicals by a single route” is distinguished from exposure to “multiple chemicals by multiple routes”*

23. An initial problem formulation step includes the evaluation of the nature of chemical exposure, the availability of data on mixture components, the likelihood of

[co-]exposure of chemicals and the rationale for considering compounds within an assessment group.

24. The framework, based on established PODs for the mixture constituents, takes into account ADME characteristics (absorption, distribution, metabolism, excretion) and chemical MOA and works towards establishing whether the cumulative MOE is adequate. The IPCS framework also recommends that combined exposure to multiple chemicals should be defined in the context of whether or not the components act by similar or different modes of action (i.e. “Single Mode of Action” or “Multiple Modes of Action”) (IPCS, 2009).

25. The applicability of this framework is well established and a number of detailed case studies are available; for example a general evaluation of substances potentially detectable in surface water (WHO, 2017), or the risk assessment of polybrominated diphenyl ethers, as a group (Meek et al., 2011).

SCHER opinion

26. The SCHER, the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and the Scientific Committee on Consumer Safety (SCCS) report, ‘Opinion on the Toxicity and Assessment of Chemical Mixtures’ constitutes advice to the European Commission on issues relating to chemical mixtures (SCHER, 2012). It examines scientific evidence for the effects of combinations of chemicals and different approaches for the assessment of mixture effects. The WHO/IPCS framework is referred to and drawn on. The decision tree provided is that of the WHO/IPCS (Annex 4).

CEFIC / MIAT framework

27. The Mixtures Industry Ad-hoc Team (MIAT), created by the European Chemical Industry Council (Conseil Européen des Fédérations de l'Industrie Chimique, CEFIC), developed a generic decision tree based on the approaches recommended by SCHER and WHO/IPCS to address issues associated with combined exposures to multiple chemicals (Annex 5) (Price et al., 2012). Other approaches, such as those described by IGHRC and EFSA were also considered and it was concluded that they were broadly consistent with the one developed. The framework was applied to ‘real world’ examples of combined exposures in effluent and surface waters and it was concluded that, in general, the frameworks are useful for the prioritisation of managing potential risks from exposure to chemical mixtures.

Sexton review

28. Sexton highlighted ‘stressor-based approaches’, based on the identification of multiple stressors, including chemical exposures, and compared these to ‘effects based approaches’, driven by observed or hypothesised health outcomes, (Sexton, 2012). The key difference is that the first approach aims to prospectively identify the health effects of the defined set of stressors (bottom-up approach) whilst the second

approach aims to retrospectively identify the cause of a health outcome (top-down approach). The review notes that few detailed CRAs have been undertaken and that published work is often based around conceptual models and theoretical frameworks.

EFSA opinion

29. EFSA examined guidance published by a number of authoritative organisations (e.g. US Environmental Protection Agency (US EPA); Agency for Toxic Substances and Disease Registry (ATSDR); IGHRC; IPCS; SCHER). EFSA recommends a structured approach to the risk assessment of chemical mixtures based on those of other organisations, to include a problem formulation step that defines the hazard within a particular legal framework, the grouping of chemicals based on the mechanism of toxicity and target organ (CAG's), the use of toxicokinetic data and a tiered approach to risk characterisation. Recommendations are also given to identify priority exposure scenarios (EFSA, 2013).

ILSI/HESI framework

30. Problem formulation is also central to the International Life Sciences Institute (ILSI)/Health and Environmental Sciences Institute (HESI) Risk Assessment in the 21st century (RISK21) CRA project (Moretto et al., 2017; Solomon et al., 2016). An initial 'gatekeeper step' is applied which establishes whether the CRA is necessary and then the process is based on the principles of the RISK21 matrix (utilise existing information; consider exposure early in the risk assessment process; use a tiered approach for data development and decision making). A diagrammatic representation of the risk assessment process described is provided in Annex 4. Estimates of exposure and toxicity, are plotted on the X- and Y-axes of the RISK21 matrix, respectively, resulting in graphical, and therefore visual, means to estimate risk. A systematic approach identifies all critical factors specific to the risk assessment and a hypothesis is developed about the possibility of adverse outcomes as a consequence of exposure to the identified chemicals. Factors that may modulate the response to the chemicals are considered, including lifestyle and environmental factors or the presence of other chemicals or stressors which may alter toxicity (Solomon et al., 2016).

Regulatory approaches and guidance on the assessment of (carcinogens within) chemical mixtures

31. Whilst there are a growing number of frameworks and general guidance on the conduct of the CRA of chemical mixtures, there are fewer regulatory requirements specifying the need for a CRA to be carried out. A commentary from the European Commission provides a comprehensive review of the CRA approaches from a wide variety of regulatory environments and a number of case studies are presented (Kienzler et al., 2016). Those that recognise the need for the assessment of aggregate or combined exposures are generally concerned with the registration of intentional mixtures such as formulated pesticide or medicinal products. These

include plant protection products (Regulation No 1107/2009, 283/2013 and 284/2013); biocides (Regulation No 528/2012); human pharmaceutical (Directive 2001/83/EC); veterinary medicines (Directive 2001/82/EC); and cosmetics (Regulation No 1223/2009). However, these products are unlikely to be licensed if they contain potential carcinogens and therefore are not considered to be relevant to the current COC evaluation. The Water Framework Directive (WFD) 2000/60/EC provides methods to calculate the quality standards of a mixture but no reference to the presence of potential carcinogens is made.

32. REACH (Regulation 1907/2006) acknowledges that deliberate combinations of chemicals occur, such as formulated products, or that a substance requiring evaluation may, itself, be a mixture. Biocidal Products Regulations (BPR) state that combined exposure and mixture assessments should be considered where relevant and refer to the WHO/IPCS framework for guidance (ECHA, 2017b).

33. Guidance on the Application of the Classification, Labelling and Packaging Criteria (ECHA, 2017a) provide detailed information on classification of a mixture, utilising data on the whole mixture or individual components. It is stated that additivity principles are not normally applied for a number of hazard categories including carcinogenicity. However, additivity may be applicable if scientifically justified and supported by expert judgement. It is noted that CLP communicates hazard assessments and is not a risk assessment tool.

34. In the EU, pesticide residues in foods are regulated under Regulation EC 396/2005, which states that maximum residue levels (MRLs) should be set through consideration *"of human exposure to combinations of AS [active substance] and their cumulative and possible aggregate and synergistic effects".* The Regulation explicitly addresses the need for carrying out further work to develop methodology and technical guidelines on pesticide residues that allows aggregate, cumulative and synergistic effects to be taken into account. One potential issue that can be foreseen is that proven methodology such as the use of ADIs focus on single substances.

35. There are some examples where whole mixture approaches are utilised in regulatory environments. Medical device risk assessment, covered in ISO 10993-3 standard, provides a means to evaluate genotoxicity, carcinogenicity and reproductive toxicity of medical devices, including a step for risk assessment, and gives modified approaches for extraction procedures and sample preparation. A decision tree is provided in which specific concerns over testing extracted mixtures are addressed.

36. Herbal medicines present another example of a regulated product that may be a complex mixture; a Guideline exists for the genotoxicity assessment of herbal preparations (EMEA/HPMC/107079/2007).

37. Some bodies provide guidance specifically pertaining to the assessment of carcinogens within a mixture. In the SCHER joint report on the Toxicity and Assessment of Chemical Mixtures, the potential for a non-carcinogenic chemical to

impact synergistically in the carcinogenic process is considered. The report states that '*An example of a synergistic action is the combination of a chemical which causes a mutation with one that induces proliferation in the carcinogenic process. This represents the classical initiation–promotion model. Chemicals that interfere with cell cycle regulation, increase the permeability of skin/mucosa or alter the bioactivation/detoxication equilibrium might synergise with classical carcinogens'* (SCHER, 2012).

38. The US EPA has published guidelines for the human health risk assessment of chemical mixtures and addresses some issues associated with the presence of carcinogens in a mixture. This includes consideration of the potential modes of interaction for carcinogens, and examples of the carcinogenic risk assessment of whole mixtures (e.g. coke oven emissions) (EPA, 2000). Response addition is considered an appropriate method to assess carcinogenic risk in a further EPA report 'Considerations for Developing Alternative Health Risk Assessment Approaches for Addressing Multiple Chemicals, Exposures and Effects'. It states '*the probabilistic risk of cancer in a given dose group is typically estimated by the proportion of responders in that group. One can then estimate total cancer risk from a mixture by summing the individual cancer risks for the carcinogens in the mixture'* (EPA, 2006). With regards to carcinogenesis, guidance on CRA of pesticides acknowledges that a group of pesticides (amitrole, mancozeb and ethylene thiourea) cause thyroid follicular cell carcinogenesis by disruption of thyroid-pituitary homeostasis. Accordingly, co-exposure to these chemicals should be considered as additive when assessing risk (EPA, 2002).

39. Overall, consideration of human exposure to mixtures under EU regulation is minimal. A few exceptions include the regulation of formulated products such as human pharmaceuticals or cosmetics, but carcinogenesis is not likely to be of concern for these scenarios. There are a few examples of guidance specifically on assessment of carcinogens in mixtures. Frameworks such as the one developed by WHO/IPCS (Meek et al., 2011) are also cited as useful tools for assessing cumulative risks of environmental exposures.

Examples of the risk assessment of chemical mixtures:

40. In the 2010 statement, the Committee considered specific examples of chemical mixtures, including PAHs which, as a group, have been examined in detail with regard to potential human exposures. PAHs provide a good example of the applicability of mixture risk assessment scenarios, especially with carcinogenesis as a potential endpoint. At the same time, the COC also examined epidemiological literature for examples of evaluations of the effect of combinations of exposures on cancer incidence and the potential impact on public health. The two examples considered were combined exposure to alcohol and tobacco smoking on the incidence of a number of cancer endpoints, and combined exposure to asbestos and tobacco smoking on the incidence of lung cancer. These are detailed further in Annex 1.

41. A further mixture assessment is the use of a CMG for assessment of THMs, a group of genotoxicants formed as drinking water disinfection by-products. In drinking water risk assessment, the group is comprised of four; chloroform (CHCl3), bromodichloromethane or dichlorobromomethane (CHBrCl2) (BDCM), dibromochloromethane or chlorodibromomethane (CHClBr2) (DBCM), and bromoform (CHBr3) (WHO, 2005). An additive approach has been used for the risk assessment of total THMs, using the individual guideline values (GVs) and known concentrations of each as follows:

$$\frac{C_{bromoform}}{GV_{bromoform}} + \frac{C_{DBCM}}{GV_{DBCM}} + \frac{C_{BDCM}}{GV_{BDCM}} + \frac{C_{chloroform}}{GV_{chloroform}} \leq 1$$

where C = concentration and GV = guideline value.

New and ongoing initiatives

42. There are some ongoing initiatives including the EUROMIX project, a European consortium aiming to establish and disseminate new, validated mixtures test strategies, and an EU biomonitoring project which aim to assess risks of human cumulative exposures. These initiatives may in the future deliver information which can facilitate the development of broader risk assessment and management strategies, and as relevant the Committee will be kept informed of progress of or publications from these groups. (EUROMIX, 2017; HBM4EU, 2017).

43. Currently, EFSA have two documents relating to mixture assessments out for consultation. Procedures for the assessment of the genotoxicity of a mixture are presented in the 'statement on genotoxicity of chemical mixtures' (Annex 6), which recommends that the mixture is characterised as fully as possible. For a fully characterised mixture, which contains a substance that is a known *in vivo* genotoxicant, then the mixture should be determined to be genotoxic. The application of MOE and threshold of toxicological concern (TTC) approaches to assess risk are discussed for this scenario. For a mixture containing a high proportion of unidentified components, experimental testing of the unidentified fraction should be considered first, followed by testing of the whole mixture. Fractionation of the whole mixture is suggested, for example, if it is suspected that genotoxicants are present. Standard genotoxic testing strategies are recommended to identify the presence of genotoxicants. Negative results in adequately performed *in vitro* assays are sufficient to consider the mixture to be of no toxicological concern. Where positive findings occur in one or more *in vitro* assays, it is recommended that follow up with *in vivo* assays is considered.

44. In the second EFSA consultation on 'draft guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals' (Annex 7), a consolidated procedure for assessing the risk of a chemical mixture is provided which is considered appropriate for use in all areas of work relevant to EFSA. A tiered approach to risk assessment is

described. In the first-tier chemicals are grouped together based on exposure only, and MOA is not taken into consideration. If exposure levels are shown to be sufficiently low to ensure 'protection' from risk using this simple, conservative model, then progression to the next tier is not necessary. Successive tiers, if required, utilise increasing amounts of information (exposure, mixture composition, toxicological information) to refine the risk assessment. The framework is based on common risk assessment approaches (problem formulation, exposure assessment, hazard identification and characterisation and risk characterisation) of either whole mixtures or using component-based approaches. Specific consideration is given to the use of CAGs, dose addition assumptions and integration of evidence to enable refinements to the assessment.

45. With regards to carcinogenesis, a TTC approach is suggested for a poorly characterised mixture for which there is reassurance that a potent carcinogen is not present. The risk assessment of a whole mixture which is genotoxic, is considered as an individual chemical and a MOE approach is recommended (MOE>10,000). The EFSA statement on the genotoxicity of chemical mixtures (para 43) is referenced.

Low dose mixture hypothesis to carcinogenesis

46. An academic, literature based, investigation named the 'Halifax Project' has been undertaken to evaluate the potential for low doses of chemicals to act synergistically to induce cancer (Goodson et al., 2015). This National Institute of Environmental Health Sciences (NIEHS) funded programme of work, based on the 'hallmarks of cancer' hypothesis[1] (Hanahan and Weinberg, 2000, 2011) examined how exposure to combinations of chemicals at low doses may contribute to overall cancer burden. The underlying premise is that individual chemicals, which may not all be carcinogens, have the potential to modulate some of the characteristic cellular 'hallmarks of cancer' and, working in concert with other chemicals, may induce tumourigenesis. The low dose mixture hypothesis therefore anticipates that low doses of chemicals, acting via dissimilar modes of action and affecting one of the hallmark effects, may act together to induce cancer, when individually they would not.

47. Teams working on each hallmark identified prototypical chemicals present in the environment at low levels which had been demonstrated as having the ability to disrupt specific, key processes within carcinogenesis. The term 'low dose' was defined, according to EFSA as (an effect occurring at doses below 1 mg/kg in a routine toxicity test) and this was used as the arbitrary cut off point for assessment.

[1] This postulates that the development of a malignant, cancer genotype arises from the perturbation of a number of vital physiological processes: proliferative signalling; evasion of growth suppression; resistance to apoptosis and cell death; limitless replicative potential; sustained angiogenesis; tissue invasion and metastasis; genome instability; tumour promoting inflammation; avoiding immune destruction and dysregulated metabolism.

Further to the assessment of chemicals deemed to be present at low doses, a low dose effect, as defined by the US EPA, is a biological response occurring at a typical human exposure level, where a human blood concentration has been measured following an environmental exposure or at a dose below the lowest dose used in a toxicity test in cases where a LOAEL had been determined.

48. For each 'hallmark of cancer' prototypical disruptors of each specific hallmark process were identified. Chemicals were selected based on a number of characteristics including; a ubiquitous presence in the environment and evidence that it selectively disrupts identified target pathways or mechanisms. Eighty-five prototypical chemicals were identified, 50 of which cause effects at low doses. Heavy metals, acrylamide and nano-particles were identified to enhance genome instability (Langie et al., 2015) and nickel chloride, methylmercury and radiation impact on tumour microenvironments, with the role of oxidative stress highlighted as an important mechanism for this effect (Casey et al., 2015). Several chemicals identified as non-carcinogens were known to modulate molecular and cellular targets involved in tumour-associated inflammation (e.g. bisphenol A, atrazine, and phthalates) (Thompson et al., 2015). Chemicals considered with regard to proliferative stimuli or growth suppression included those known to affect p53 signalling, tyrosine kinase mediated growth factor signalling, or cytokine mediated proliferative responses (Engstrom et al., 2015; Nahta et al., 2015).

49. Cross hallmark relationships, where chemicals act on critical signalling pathways functioning in different target areas, were also identified and include chemicals which exert different effects at different dose levels. Many of the chemicals examined did not exhibit thresholds for effect and it was concluded that low levels of environmental chemicals acting together to induce cancer was a plausible hypothesis (Goodson et al., 2015). It is noteworthy that the authors provide caveats to their research; for example, that the disruptive actions of these chemicals have been produced under a wide variety of experimental circumstances and that they do not implicate the individual chemicals to cause cancer *per se*.

50. Exploration of the low dose mixture hypothesis of carcinogenesis is a relatively new area of investigation, particularly when attempting to address exposure to chemicals from a wide range of classes and sources with diverse biological activities. As yet, there are no studies which can directly examine these hypothetical assertions and they may not be borne out by empirical research. It has been proposed that future research should focus on the development of clinical epidemiology methods to examine environmental exposures, the use of translational toxicology (toxicogenomics) to identify biomarkers of carcinogenic transformation and understanding of the biological significance of low dose exposures (Miller et al., 2017).

Summary

51. This paper has summarised some key concepts, definitions and tools for the risk assessment of chemical mixtures. In addition, currently available frameworks

and guidance have been discussed. Although there are several published frameworks for assessing the risk of exposure to combinations of chemicals, these are broadly similar to one another, in that they all involve a problem formulation step followed by a tiered approach to risk assessment, dependent on the nature and extent of exposures. On the whole, regulatory approaches do not specifically discuss the risk assessment of mixtures containing carcinogens. Additional considerations may therefore be needed as to the suitability of using available approaches to assess the risk of mixtures containing carcinogens.

Questions to the Committee

52. Members are asked to consider the frameworks for risk assessment of mixtures of chemical carcinogens presented in this paper and in particular:

i. What are Members opinions on the applicability of the available frameworks for the assessment of chemical mixtures containing carcinogens?

ii. How would the Committee wish to structure a formal Guidance Statement on risk assessment of chemical mixtures?

iii. Does the Committee wish to make comments on the two EFSA consultation documents?

NCET at WRc/IEH-C under contract supporting the PHE COC Secretariat
July 2018

General Abbreviations/Glossary

ADME:	Absorption, distribution, metabolism, excretion
AhR:	Aryl hydrocarbon receptor
ATSDR:	Agency for Toxic Substances and Disease Registry
B[a]P:	Benzo[a]pyrene
BMD:	Benchmark dose
CAG:	Common assessment group
CEFIC:	European Chemical Industry Council (Conseil Européen des Fédérations de l'Industrie Chimique)
CMG:	Common mechanism group
COM:	Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment
COT:	Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment
CRA:	Cumulative risk assessment
CYP:	Cytochrome P450
EFSA:	European Food Safety Authority
ER:	Oestrogen receptor
ILSI/HESI:	International Life Sciences Institute / Health and Environmental Sciences Institute
HI:	Hazard index
HQ:	Hazard quotient
IGHRC:	UK Interdepartmental Group on Health Risks from Chemicals
IPCS:	International Programme of Chemical Safety
IPCS:	WHO International Programme of Chemical Safety
MIAT:	Mixtures Industry Ad-hoc Team
MOA:	Mode of action
MRL:	Maximum residue level
PAH:	Polycyclic aromatic hydrocarbon
POD:	Point of departure
RPF:	Relative potency factors
RV:	Reference value
SCCS:	Scientific Committee on Consumer Safety
SCENIHR:	Scientific Committee on Emerging and Newly Identified Health Risks

SCHER:	Scientific Committee on Health and Environmental Risks
TEF:	Toxic equivalency factor
TEQ:	Toxic equivalency quotient
THM:	Trihalomethanes
TTC:	Threshold of toxicological concern
US EPA:	US Environmental Protection Agency
WFD:	Water framework directive

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

Risk Assessment of the Effects of Combined Exposures to Chemical Carcinogens - an update

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NCET at WRc/IEH-C under contract supporting the PHE COC Secretariat
July 2018

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

STATEMENT ON THE RISK ASSESSMENT OF THE EFFECTS OF COMBINED EXPOSURES TO CHEMICAL CARCINOGENS

Introduction

1. Testing and risk assessment are usually carried out on individual chemicals whereas humans are exposed to multiple chemicals both simultaneously and sequentially. At the horizon scanning exercise in 2007 we decided to review current developments in the testing and assessment of chemical mixtures with regard to carcinogenicity. For this review, "mixtures" was defined as combined exposure to more than one carcinogen, or to a carcinogen and other chemical(s) with potentially modifying effects, either simultaneously or at different times. The purpose of the review was to examine the data in the scientific literature on this topic, with a view to providing advice on the potential carcinogenic action of these combined exposures and on methods for testing and assessment of such effects.

2 Carcinogenicity is a multistage process. In simple terms, the main components of this process are initiation and promotion. *Initiation* is caused by changes in the cellular genetic material due to an induced or spontaneous mutation or gene rearrangement. The initiated cell has an altered response to external stimuli resulting in cell growth or programmed cell death (apoptosis) and is vulnerable to abnormal division or to escape from signals for apoptosis. *Promotion* is any process which gives the initiated cell a growth advantage over normal cells. Clonal proliferation of the initiated cell produces cancer. Chemicals can cause initiation and/or act to enhance promotion (promoters). The action of any particular chemical could potentially be influenced by other chemicals to which an individual is exposed, either simultaneously or at a different time.

3 When a chemical (or its metabolite) causes initiation by interacting directly with the genetic material, it is referred to as a "genotoxic carcinogen" and the process as "genotoxic carcinogenicity". Chemicals which cannot be shown to interact directly with, or cause damage to, DNA in a number of short-term screening tests, but which are capable of inducing cancer, are referred to as non-genotoxic carcinogens.

4. Our sister committee, the Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM[2]), has reviewed the literature pertaining to the evaluation of mixtures of potential mutagens (COM 2009). The COM focused on the possible occurrence of synergistic interactions, the possible mechanisms that may underpin these interactions, and whether these findings were likely to have any implications for human health risk assessments. It concluded that there were some examples where interaction with regard to mutagenicity occurred but that these required further evaluation before the significance to public health could be determined. Our attention was drawn to the COT report 'Risk Assessment of Mixtures of Pesticides and Similar Substances' (COT 2002) and also to initiatives

[2] A list of all abbreviations in this statement is given at the end of the document.

such as those organized by the UK Interdepartmental Group on Health Risks from Chemicals (IGHRC 2009) and World Health Organisation (WHO)/International Programme on Chemical Safety (IPCS, draft document). Both of the latter organisations have developed framework procedures for the risk assessment of combined exposures to multiple chemicals which will provide solid guidance for anyone required to evaluate the toxicity of chemicals. However, we note that, within these documents, there is no specific guidance on the assessment of the impact of combined exposure to carcinogens or to carcinogens and other chemicals with regards to cancer.

5. The papers presented to us on this topic discussed general principles and gave some examples of where attempts had been made to evaluate combined actions of different carcinogens. The different types of combined actions used to characterize the possible outcomes between compounds in a mixture, as detailed in the COT report on pesticides and similar substances, have been classified as follows:

1. Simple similar action (non-interaction, dose addition)
2. Simple dissimilar action (non-interaction, response addition)
3. Interaction (synergism/potentiation or antagonism/inhibition)

Simple similar action (also referred to as simple joint action) is the concept whereby combinations of chemicals have the same target organ acting via the same mechanism (or mode) of action. It is also occasionally referred to as 'dose or concentration addition' although, strictly speaking, this is the effect, not the concept. In simple similar action, the effect of the components of a mixture is determined by their respective doses and potencies. The combined effect is estimated from the summation of the potency-normalised doses and toxicity can be predicted from the dose response curve of a 'reference' compound, to which the others are normalised.

Simple dissimilar action (also referred to as independent joint action, simple independent action, effect/response addition) is assumed when individual chemicals have different modes of action and, possibly, the nature and site of action also differ. The effect of each chemical does not modulate or contribute towards the effects of the other constituents of the mixture and, hence, the health effects of exposure to the mixture are expected to be qualitatively and quantitatively similar to those produced by individual components when administered alone. Effect addition is the summation of the individual responses of the different mixture components and toxicity is predicted from the dose response curves of the individual chemicals.

Interaction is present when the observed effect of two or more exposures differs from the effect that would be expected if the exposures had additive effects. Synergism and potentiation are terms used to describe responses that are greater than additive, and antagonism and inhibition are used for responses which are less than additive.

6. The possible mechanisms underlying an interaction are often divided into three categories: direct chemical-chemical, toxico/pharmacokinetic, and toxico/pharmacodynamic mechanisms. It is emphasized that the nature of the interaction can change with altered exposure conditions (for example, dose,

duration, sequence of exposure and the relative proportions of the components of the mixture). How these concepts and definitions can be applied to experimental and human epidemiological exposure scenarios are described in paragraphs 22 to 25. In both cases, the definition of non-additivity will depend on the nature of the outcome measured and the shape of the dose- (or exposure-) response model fitted.

7. The review was undertaken taking into account these theoretical classifications and principles. However, it is recognized that the nature of potential combination effects do not fall neatly into categories and some mixtures may have more than one type of effect. Initiation and promotion are discrete stages of carcinogenesis and therefore likely to be subject to the influence of different chemicals, as indicated by the development of initiation/promotion experimental carcinogenesis models. We also considered that it would facilitate the review if we examined examples of synergistic reactions which occur within the different stages of the carcinogenic process, as this may shed light on the mechanisms whereby carcinogens can interact. Finally, we sought to understand how the theoretical application of the general principles involved in evaluating the combined exposures to mixtures of chemicals can be applied to relevant environmental or occupational exposure scenarios.

8. With regard to evaluating synergistic responses, it was noted that the COM, in its review of mixtures, assessed papers according to the criteria laid out in Borgert (2001). The essential criteria were:

1. Dose-response relationships for the individual mixture components are adequately characterised.
2. An appropriate non-interaction or additivity hypothesis should be, *a priori*, explicitly stated and used as the basis for assessing combination effects.
3. Combination of mixture components should be assessed across a sufficient range of concentrations and mixture ratios to support the goals of the study

However, we were unable to use these criteria for the papers we reviewed, as the requirement for detailed dose response data was rarely met. Mutagenicity/genotoxicity, which was the subject of the COM review, is at most, only a contributory factor of the carcinogenic process. To evaluate accurately the effects of mixtures of chemicals on the entire carcinogenic process would necessitate life-time carcinogenicity studies of mixtures of carcinogens. These studies would need to include groups of animals receiving different doses of both the mixtures and the individual chemicals to determine the dose responses for both. This would entail large and complex studies which would be expensive and require ethical consideration in view of the high number of animals needed.

Mode of Action concept and Simple similar action

9. A widely applied principle when evaluating the effects of combined exposures to multiple chemicals is the Mode of Action (MOA) concept. MOA is a biologically plausible sequence of key events leading to an observed effect supported by robust experimental observations and mechanistic data. Chemicals acting by dose addition can be said to act by the same MOA and the term common mechanism group (CMG) is frequently used in mixture risk assessment for a group of chemicals with

the same MOA. Most simply, this applies to chemicals which act through the same molecular target to elicit the same effect(s), e.g. a receptor, such as the AhR receptor or the oestrogen receptor. More broadly, chemicals acting independently on the same rate-limiting key event would be anticipated to exhibit dose additivity in their carcinogenic response.

10. In the UK, the method used to assess the risk of carcinogens depends on their MOA. As noted above, genotoxic chemicals react with and mutate DNA, and non-genotoxic carcinogens act by other mechanisms. From what is known about the MOA of genotoxic carcinogens, it is currently assumed that, in the absence of mechanistic data to suggest a threshold for genotoxicity, no thresholds for carcinogenicity exists. The predominant risk assessment advice is to keep exposures as low as reasonably practicable (ALARP) so as to minimise risk. In addition, risk estimates can be calculated using the dose response in epidemiology or animal studies to give estimates of risk for human exposure. Many non-genotoxic carcinogens induce tumours as a secondary adverse effect arising from an initial toxicological effect, which has a threshold. It follows that there is no carcinogenic risk at dose levels that do not produce the primary toxicological event i.e. at doses below the threshold. In these cases, a risk assessment approach is employed in No Observed Adverse Effect Level, is divided by uncertainty factors to take account of the possible interspecies and intraspecies differences to produce a tolerable daily intake (ref COC guidelines). From what is known about mechanisms of effect, it is currently assumed that, in the absence of mechanistic data to suggest a threshold for genotoxicity, no threshold for carcinogenicity exists. The predominant risk assessment advice is to reduce exposures to as low as reasonably practicable (ALARP) so as to minimise risk. In addition, the dose response in epidemiology or animal studies can be used to generate advice about the level of concern for humans at various levels of exposure.

11. When there is evidence that the members of a group of chemicals elicit their effects by the same MOA, and do not themselves interact chemically, their combined effects can be determined by using Relative Potency Factors (RPF) or Toxic Equivalency Factors (TEF). These RPFs/TEFs are expressed relative to an ‘index compound’ and are used to normalize the toxicities of chemicals within such a common mechanism group to a single compound, which is generally the one for which toxicity and absorption/distribution/metabolism/excretion (ADME) profiles are best characterised. The RPF/TEF for each chemical is derived from information such as its point of departure for one or more end-points relative to that of the index chemical in *in vivo* and *in vitro* systems, QSAR and expert judgement. RPF/TEFs can be used either to enable a risk assessment of a mixture of chemicals by using the tolerable daily intake of the best characterised member of the group (the 'index compound'), or to calculate a risk estimate for a mixture of genotoxic carcinogens. However, in the case of mixtures of genotoxic carcinogens, the predominant advice remains to keep exposures as low as reasonably practicable (ALARP), as stated above.

12. The TEF system was first developed to facilitate risk assessment for polychlorinated dibenzo-*p*-dioxins and related chemicals. Detailed evaluations of the TEFs for dioxins and dioxin-like compounds have been undertaken and published by WHO/IPCS (van de Berg et al 2006). Carcinogenic potential is not an endpoint

which has been used in the past when setting TEFs because of the lack of carcinogenicity data on individual congeners. A validation study has been carried out with 3 individual dioxins or dioxin-like compounds and this broadly supported the concept of dose addition and TEFs for carcinogenicity of mixtures of these chemicals (Walker et al 2005). However the database is very limited.

13. Oestrogens are also considered to form a CMG and there are some approaches using *in-vitro* screening which provide robust information on dose additivity (Charles et al 2002, Payne et al 2001). However, there is a paucity of studies investigating *in vivo* responses to mixtures of oestrogens. Moreover, there can be exceptions to the concept of dose additivity for groups of similar chemicals. For example, oestrogens may act through either ERα or ERβ to produce stimulation or inhibition of cell proliferation. In such cases, where the biological actions at each receptor are opposed, the effect will not necessarily be additive, and may be different in different organs depending on whether the oestrogen acts as an agonist, antagonist, or partial agonist in that organ or tissue. A further difficulty in assessing the carcinogenic potential of oestrogens is that, even if the biological effects can be benchmarked against a well characterised member of the oestrogen group such as 17β-oestradiol, the Toxic Equivalency approach cannot be used to calculate the potential increase in the risk of cancer because of the difficulty in identifying an appropriate point of departure for the tumour inducing effect in animals, or humans.

14.. Other groups of similar chemicals may all demonstrate carcinogenic potential but may not necessarily act by the same MOA. In this case it would not be appropriate to use TEFs for evaluation of the potency of a mixture. For example, the available evidence indicates that it is inappropriate to use TEFs to assess the potential oral carcinogenicity of combined exposures to polycyclic aromatic hydrocarbons (PAHs), most of which have no oral carcinogenicity data. There are inconsistencies in the response to the different PAHs, dependent on the test system used to evaluate toxicities, evidence of interactions between different PAHs (see below) and no clearly appropriate index compound. An alternative approach has been derived for the carcinogenic risk assessment of mixtures of PAHs in food by the European Food Safety Authority (EFSA) (European Food Safety Agency, 2008). This entailed using a 'surrogate marker' approach, based on benchmark dose values derived from the 2-year carcinogenicity study in which mice were fed two mixtures of coal tar containing several PAHs. A group of four PAHs (PAH4) was recommended as the appropriate surrogate marker for the presence of PAHs in food, based on their concentrations in food and in the tested mixtures. In this model, the possibility of interactions was taken into account. Whereas both methods involve uncertainties, we agree that, in this case, the EFSA surrogate marker approach is to be preferred to the Toxic Equivalency approach.

15. When assessing the risks from exposure to combinations of chemicals, it is considered important to understand dose-response relationships. Extrapolation of the effects seen at high doses to possibly more relevant low doses is likely to be especially complex if there are a number of chemicals to be taken into account, particularly if the MOAs are not well characterised. *In-vitro* studies are frequently used to investigate hypotheses that relate to combined exposures to chemicals and some examples of these studies were evaluated and are described below (para 18). Some of these studies are valuable in that they provide information about MOAs or

specific molecular targets, confirm whether a chemical within a group acts as an agonist of antagonist (e.g oestrogens), and/or provide insight into the mechanism of an interaction. However, as it is not possible to derive points of departure (POD) or benchmark indices for the critical effect, we consider that information from *in vitro* studies should be used as a qualitative measure only, and over-interpretation of dose-response relationships is to be avoided.

Simple dissimilar action

16. Application of this principle to the evaluation of cancer as an endpoint is complicated and there are insufficient experimental data on how chemicals with diverse MOAs would act in combination with regard to the induction of tumours. Consequently, an examination of the potential complexities of combined exposures to such chemicals was considered to be outside of the scope of the current review. However, in general terms, it would be appropriate to use response addition to assess the combined effects of two carcinogens which act by different modes of action and which do not interact.

Interactions

Toxicological data

17. An interaction at a key event in the carcinogenic process may be reflected in non-additive effects on carcinogenic response and we aimed to examine the potential for chemicals to interact at different stages. The following stages in the carcinogenic process were identified as examples of potential points for interaction: ADME processes, DNA adduction, mutagenicity, early preneoplastic changes, proliferation, apoptosis and neoplastic transformation. Initially, the toxicological literature was reviewed for examples of interactions and we examined in the first instance polycyclic aromatic hydrocarbons (PAHs) and heterocyclic amines (HCAs). It is noteworthy that most studies of interactions, including studies conducted *in vitro,* did not conform to the criteria laid out by Borgert, as described previously.

18. PAHs are a group of chemicals which have been evaluated with the consideration that human populations are exposed to mixtures, including complex mixtures such as those found in coal tar and urban dust particulate matter. *In vitro* and *in vivo* approaches were used in the papers retrieved to assess potential synergistic responses including: the production of PAH-DNA adducts, tumour formation using initiation promotion models, and effects on the cytochrome P450 (CYP) family of enzymes, particularly CYP1A1 and CYP1B1. There was some evidence that some PAHs, including those within a complex mixture, may have the potential to decrease the potency of others by altering metabolism. For example, a significant reduction in PAH-DNA adducts was observed when coal tar extract (Standard Reference Material, SRM_{1597}) was co-administered with benzo[a]pyrene (B[a]P) and dibenzo[a,l]pyrene (DB[a,l]P). In human breast epithelial cells (MCF-10A), reduced DNA binding was associated with induction of CYP1A1 and 1B1 (Mahadevan et al 2005). In V79 cells expressing CYP1A1 or 1B1, reduction in DNA adducts was more apparent in the CYP1B1 expressing cells (Mahadevan et al 2007). EROD activity indicated that SRM competitively inhibited the activity of both isoforms, more strongly on CYP1B1. *In vivo,* SRM_{1597} reduced the number of

tumours induced by DB[a,l]P in a SENCAR mouse skin model, but did not have the same effect on B[a]P induced lesions (Marston et al 2001).

19. The studies provided some examples of how chemicals, including complex environmental mixtures, can impact on the carcinogenic potential of other PAHs. In testing the hypothesis of competitive inhibition of enzymes responsible for the metabolic activation of PAHs, it was broadly demonstrated that tumour promotion and DNA adduction were affected by the mixtures and that this could be explained, in part, by altered CYP activity. For example, it is proposed that B[a]P is more readily activated by CYP1A1 than by CYP1B1, such that the competitive inhibition of the former isoform would result in reduced activity. Furthermore, it was generally shown that the effects of environmental mixtures on the metabolism of DB[a,l]P were different from the effects on the metabolism of B[a]P. This probably indicates the complexity of the interactions, both metabolic and genotoxic, involved in the processes and the dose dependency of these interactions. Moreover, the majority of interactions described involved toxicokinetic alterations and it is difficult to put these into context with interactions downstream in the carcinogenic process.

20. There are many reservations when interpreting these data. Although it is known that PAHs are inducers of xenobiotic metabolism, induction would be thresholded and the extent of induction would be dependent on dose, dose route and tissue examined. Differences were observed between results obtained *in vitro* and *in vivo* and between different models. The relevance of the SENCAR mouse skin model for the evaluation of carcinogenicity is also questionable. As such, it is difficult to extrapolate the altered risk of chemicals observed in the models used and the implications for human risk assessment are uncertain. It was concluded that analysis of *in vivo* studies with regard to potential interactions is complicated since pathways of activation and detoxification are inextricably linked and it is difficult to determine how these toxicokinetic interactions may contribute to the overall carcinogenic process, particularly at low levels of PAHs likely to occur following dietary or environmental exposure.

21. Heterocyclic amines (HCAs) are another class of chemicals which have the potential to interact with one another. A number of studies were retrieved which had assessed potential interactions of food heterocyclic amines using liver foci initiation promotion models in rats. The HCAs examined were Trp-P-1, Glu-P-2, IQ, MeIQ and MeIQx, Trp-P-2, Glu-P-1, MeAαC, AαC and PhIP (see abbreviations). As an example, these were administered as 1/1, 1/5, 1/10, 1/25 or 1/100 of the known carcinogenic dose[3] and as combinations of the first four HCAs at 1/5 and 1/25 of the dose or all 10 at 1/10 and 1/100 of the dose. GST-P-positive foci >0.1mm were the selected endpoint (Ito et al 1991, Hasegawa et al 1994 a,b). It was claimed that some HCAs may act synergistically in promoting tumours through a hypothesised CYP induction mechanism and this was apparent at low doses claimed by the authors to be relevant as a human consumption scenario. However, we find it difficult to draw useful conclusions from these studies for a number of reasons. Firstly, the initiation-promotion study protocols which have been used to examine interactions between the HCAs were overly complex. The partial hepatectomy protocol fixes mutations occurring during the period of regrowth and, since there was

[3] Described in Ito et al (1991) as 'the dose used in the carcinogenicity studies'.

no consistent synergistic response in this very sensitive model, the relevance to human health is questionable. The way in which the authors have analysed the results (subtracting a high background incidence from the induced incidence) is likely to be subject to significant error. In addition to the high variability and high background tumour incidence, only limited dose response data were provided. No null hypothesis was given and, therefore, no statistical comparison of the tested hypotheses was possible. We do not agree with the conclusion from these studies that there was clear evidence of synergy close to the observed NOEL for CYP induction. However, this may be artefactual. It is unlikely that subtle effects seen at high doses will occur at low, environmentally relevant exposures. Furthermore, the studies which evaluated HCAs were unconvincing and we suggest that less complex protocols might lead to more informative studies.

Epidemiological data

22. In the absence of clear evidence of interactions in carcinogenicity from the toxicological literature studied, we also examined the epidemiological literature for examples of evaluations of the effect of combinations of exposures on cancer incidence and the potential impact on public health. The two examples which we considered were combined exposure to alcohol and tobacco smoking on the incidence of a number of cancer endpoints, and combined exposure to asbestos and tobacco smoking on the incidence of lung cancer. From these data it was hoped to determine whether an understanding of the mechanisms which lead to interactions with regard to carcinogenicity could be useful in improving the assessment of the risk of combination of chemicals following exposure to man. Our comments on the data reviewed are given in the Annex to this statement.

23. In epidemiology, as in toxicology, interaction is present when the observed effect of two or more exposures differs from the effect expected if the exposure had additive, joint effects (Siemiatycki et al 1981). The term “additive effects” has to be interpreted in terms of the model fitted to the data. It is possible to work on the scale of absolute measures, such as cumulative risks, or of relative scales, such as relative risks. The epidemiologic literature refers to both types of scale, with the null hypothesis of no interaction modelled as multiplicative on the relative scale (as in logistic regression), and as additive on the absolute scale (de Klerk et al 1989).

24. There are several limitations in epidemiological studies that attempt to investigate interactions: (a) in the first place, investigation of interactions requires the data set to span a range of combinations of the variables concerned, and an observational study may not necessarily exhibit this range (b) statistical power is usually limited, because one needs a sample size approximately four times larger than for a single exposure to investigate the joint effect of two exposures; (c) in epidemiological studies where the exposure assessment is weak and/or prone to misclassification, estimates of risks and of interactions may be distorted. Low statistical power may lead to both false positive and false negative results, while exposure misclassification mainly leads to false negatives. Also, technical issues arise when managing large sets of data with high-degree order interactions (typically in the context of gene-environment interaction or genome-wide association studies). Although mathematical and computational tools have become available to tackle

such complex analyses, it remains very difficult to go beyond a two-way interaction with confidence.

25 A potential important improvement of the study of interactions in humans might come from the development of intermediate biomarkers, but this field is currently underdeveloped. Using biomarkers it is possible to follow the fates of the individual active components of a mixture in the body, to investigate their links/reactions with relevant target molecules, and eventually to devise risk assessment models.

26. In general, it was considered that assessing the potential interactions that may occur during the biological responses to carcinogenic chemicals (both increased and decreased effects) was fraught with difficulties. Firstly, it is recognised that extrapolating data from the majority of methodologies used to substitute for carcinogenicity bioassays to possible carcinogenic responses in humans is extremely difficult. *In vitro* studies can give qualitative information on the relative carcinogenic hazard at best. The complexities involved in the carcinogenic process, including the possibility that two chemicals could be present in the body at very different times, yet provoke a synergistic response, make the evaluation of risks posed by potentially carcinogenic chemicals entirely different from the evaluation of the vast majority of chemical toxicities. It could be postulated that the combination of any chemical which causes a mutation with one that induces proliferation will act synergistically with regards to the induction of tumours. This is analogous to the well-established phenomenon of initiation-promotion.

27. It is also of note that dose responses to chemicals can be more complex than simple high or low dose effects; it is possible that MOA's will also change with increasing dose, thus further complicating the interpretation of data when extrapolating. Metabolic interactions may occur although it is considered more likely that they will impact on a genotoxic event in the carcinogenesis process as this will only require a short period of alteration; a non-genotoxic mode of action will be affected only by a metabolic change over a prolonged period. In addition, the extended time taken before tumours occur following chemical exposure make it difficult at present to evaluate responses in test systems other than life-time bioassays in rodents. Epidemiological studies are expensive and investigation of interactions necessitates the existence of populations that have been exposed to the individual components of the mixture and other populations that have been exposed to the mixture. This is not a common situation for chemicals, for example, occupational and environmental exposure to the carcinogenic PAHs is always to a mixture of PAHs. Thus, epidemiological studies are not a practical alternative to animal studies in this case.

Conclusions

28. Humans are exposed to mixtures of chemicals, including carcinogens and it is not possible for the risk assessment process to account for the combined action of every possible mixture of carcinogens at all possible levels of exposures over all possible time frames. Nevertheless, some general principles were stated :

- Mixtures of chemicals which act via the same MOA and which do not react chemically with one another, such as polychlorinated dibenzo-*p*-dioxins, can be assessed using the concept of dose additivity and relative potency factors/toxic equivalency factors.
- Although there may be a substantial margin between exposure to a carcinogen and either its no observed adverse effect level (in the case of a non-genotoxic carcinogen) or another POD (in the case of a genotoxic carcinogen), it is possible that simultaneous exposure to two carcinogens which have the same MOA may result in a lower margin of exposure. Risk assessors should be alert to this possibility when assessing a chemical which commonly occurs together with one or more other chemicals which have the potential to cause cancer.
- There are several stages in the carcinogenic process at which carcinogens might interact, for example, ADME processes, DNA adduction, mutagenicity, early preneoplastic changes, proliferation, apoptosis and neoplastic transformation. MOA analysis may be of value here, in determining critical steps at which interaction might be anticipated. Potential interactions in genotoxic MOAs have been addressed in the statement generated by the COM.
- It is postulated that otherwise non-carcinogenic chemicals, such as anti-apoptotic chemicals or chemicals which interfere with cell cycle regulation, which alter ADME processes or which increase permeability of the skin or oral mucosa, might have the potential to interact synergistically with known carcinogens
- The assessment of potential interactions in the context of carcinogenicity is complex due to the multistage nature of the process. However, we do not advocate standard carcinogenicity studies on mixtures of chemicals except in exceptional circumstances. Such studies would be costly and would require ethical consideration given the high number of animals required.
- *In vitro* studies of interactions should be hypothesis driven, attempt to characterize the dose-response and use models relevant to *in vivo* carcinogenicity. These studies should adhere to the criteria laid out in Borgert et al (2001). Models used to evaluate the synergistic interactions between PAHs and between HCAs were, in general, complex and may not truly reflect the situation for carcinogenesis. Thus extrapolation of results for risk assessment in humans is difficult.
- Overall, *in vitro* studies can be used to confirm molecular targets or provide insight into MOA identification but are not of value for the evaluation of relative potencies of chemicals or interactions at environmentally relevant exposure levels.
- In terms of the risk assessment for potential interactive effects of carcinogens, exposure to a non-genotoxic carcinogen at or below the no-effect level for the critical effect contributing to the interaction is unlikely to result in an interaction with a chemical which has a different MOA. In the case of genotoxic carcinogens, in principle, effects could occur at any level of exposure which could lead to interaction. This supports the view that exposure to genotoxic carcinogens should be as low as reasonably practicable.

Annex

Examples of multiple exposures and potential interactions in humans

Alcohol and tobacco smoking:

1. Alcohol and tobacco smoking are each known to be predominant risk factors for a number of cancers i.e. cancers of the mouth, neck and squamous cell carcinoma of the oesophagus. The studies reviewed show that these two factors act in a greater than additive manner to produce these cancers with effects apparent at moderate as well as high intakes (Lagergren et al 2000, Lee et al 2008). In some instances, the multiplicative increases are very large (odds ratios of up to 177). However, this synergism is not apparent for oesophageal adenocarcinoma and cancers of the gastric cardia (Sjodahl et al 2006).

2. The mechanism for the synergistic effect is not well understood and we considered a number of plausible hypotheses. Firstly, the induction of cytochrome P450 (CYP) enzymes by ethanol is suggested as a potential mechanism. There is evidence that ethanol induces CYP isoforms which are capable of metabolically activating some carcinogenic nitrosamines found in tobacco smoke. Induction of the CYP 2E1 isoform at extra-hepatic sites such as the oesophagus, combined with decreased first-pass metabolism of tobacco associated nitrosamines in the liver due to competitive inhibition by ethanol, is predicted to lead to increased concentrations of DNA-reactive nitrosamine metabolites leading to elevated cancer risk (Lecheveral et al 1999, Godoy et al 2002, Anderson et al 1995). A second plausible hypothesis, based on *in vitro* data which are convincing but not extensive, suggests that alcohol increases the permeability of the oral mucosa to carcinogenic nitrosamines. This may also contribute to the synergistic effect observed (Du et al 2000, Azzi et al 2005).

3. We agree that the metabolic interaction hypothesis is plausible. However, we concluded that, although the permeability mechanism looks reasonable, it was not clear whether the *in vitro* results could be extrapolated to the *in vivo* situation. We suggest that consideration should also be given to the interaction of alcohol and growth factors and the effect of local irritation of tissues. In addition, although the metabolic argument is convincing, this scenario could also be true of exposures to other chemicals which induce CYP2E1 and it was noted that there are no clear indications that there are similarly other synergistic carcinogenic interactions with alcohol.

Cigarette smoking and asbestos

4. Exposure independently to cigarette smoke or to asbestos causes lung cancer and it has been claimed that combined exposure results in a synergistic effect on lung cancer induction (Selikoff et al 1968, Lee 2001). The exact nature of the interaction between asbestos and tobacco smoking in the induction of lung cancer has been debated among researchers. From the published literature, most systematic reviews have found a marked heterogeneity in the magnitude of the joint effect, with the interaction ranging from less than additive in some studies to multiplicative in other studies. Despite extensive investigations exploring the

interaction between cigarette smoke and asbestos, the precise mechanisms involved at the cellular and molecular level are unclear. Asbestos and tobacco are both complex carcinogens and it is believed that they can both act at more than one stage of carcinogenesis and, hence, have interdependent effects on the multistage process of lung cancer (Vainio and Boffetta, 1994).

5. A number of authors have proposed a synergistic interaction between cigarette smoke and asbestos and various mechanisms have been proposed as the potential explanation. These include:

- cytotoxic, genotoxic and clastogenic nature of asbestos and tobacco smoke – supra-additive effects have been noted for mutation frequency, sister chromatid exchange, and DNA strand breaks in a variety of test systems (Lohani et al 2002, Kelsey et al 1986, Jung et al 2000)
- the generation of oxidative damage - both cigarette smoke and asbestos fibres generate reactive oxygen species and synergistic responses in models evaluating this have been observed. However mechanistic insights into or hypotheses about this interaction are not well developed.
- enhancement of the penetration and accumulation of asbestos in the lung by tobacco smoke – demonstrated in a number of models including following the assessment of asbestos fibres in the airways of smokers and non-smokers (McFadden et al 1986 a,b).
- the potential for asbestos to act as a delivery system for tobacco carcinogens into the lung, for example by enhancing the diffusion of lipophilic carcinogens, was shown to be unlikely (Gerde et al 1994).
- the enhancement of somatic mutations in KRAS, FHIT and p53 genes. – some associations of smoking and/or asbestos exposure and lung cancer with these genes have been postulated although specific mechanisms have not been not described.

6. Overall, it was difficult to draw conclusions from the studies evaluating the proposed synergy between asbestos and tobacco as the interaction models need to be studied in depth to understand whether the interaction is additive or multiplicative and to evaluate in detail the hypothesised mechanisms for the interactions and whether they are relevant to understanding risk in man. The definition of additivity in an experiment appears to depend upon which model fits the individual chemicals evaluated. Furthermore, the importance of different types of asbestos needs to be addressed; different types of asbestos may fit different dose response models. Exposure misclassification might also lead to substantial uncertainty in epidemiological studies; this distortion in risk estimates means it is impossible to differentiate between interaction models. We consider that there is some evidence that there might be a synergistic interaction, but it is not strong. It should be noted that, whilst mesothelioma risk stays constant over time following cessation of inhalation of asbestos, lung cancer risk reduces in reformed smokers. This probably reflects the fact that asbestos fibre remained in the lung whereas the amount of smoke residue is considered to be significantly reduced once smoking stopped.

7. Overall, without an understanding of the specific mechanisms, it is concluded that it is hard to interpret the short term studies retrieved, although it is possible to suggest plausible hypotheses. Epigenetic mechanisms may also play a part, or

asbestos exposure might increase uptake of carcinogens from tobacco smoke. We consider that examination of the p53 mutational spectra might offer some insights, as this is well defined for mutations arising as a result of exposure to tobacco smoke. It might also be interesting to examine the anatomical location of lung tumours, for example at bifurcations of the airway, which might help elucidate a mechanical mechanism.

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General Abbreviations/Glossary

ADME:	Absorption, distribution, metabolism, excretion
B[a]P:	Benzo[a]pyrene
CMG:	Common mechanism group
COM:	Committee on mutagenicity
COT:	Committee on toxicity
CYP:	Cytochrome P450
DB[a,l]P:	Dibenzo[a,l] pyrene
DNA:	Deoxyribonucleic acid
ER:	Oestrogen receptor
EROD:	Ethoxyresorufin-O-deethylase
GST-P:	Glutathione-S-transferase-placental
HCA:	Heterocyclic amine
MOA:	Mode of action
MCF-10A:	A human breast epithelial cell line
PAH:	Polycyclic aromatic hydrocarbon
POD:	Point of departure
SRM1597:	Coal tar extract standard reference material
TEF:	Toxic equivalency factor

V79: A Chinese hamster cell line

HCA Abbreviations:

Trp-P-1: 3-amino-1,4-dimethyl-5H-pyirodo[4,3-b]indole

Trp-P-2 : 3-amino-1-methyl-5H-pyridoI[4,3-b]indole

Glu-P-1: 2-amino-6 methyldipyrido[1,2-α:3',2'-d]imidazole

Glu-P-2: 2-amino-dipyrido[1,2-α:3',2'-d]imidazole

IQ: 2-amino-3-methylimidazo[4,5-f] quinoline

MeIQ; 2-amino-3,8-dimethylimidazo [4,5-f] quinoline

MeIQx: 2-amino-3,8-dimethylimidazo[4,5-f] quinoxaline

MeAαC: 2-amino-3-methyl-9H-pyrido[2,3-b]indole

AαC: 2-amino-9H-pyrido[2,3-b]indole

PhIP: 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine

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Risk Assessment of the Effects of Combined Exposures to Chemical Carcinogens - an update

IGHRC decision tree for the risk assessment of chemical mixtures

Figure 1 in IGHRC (2009). Chemical Mixtures: A Framework for Assessing Risk to Human Health (CR14). IEH Consulting Ltd.
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Figure 1 in WHO/IPCS framework for the assessment of combined exposures to multiple chemicals

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Risk Assessment of the Effects of Combined Exposures to Chemical Carcinogens - an update

Diagrammatic representation of the risk assessment process described by ILSI/HESI

Figure 1 from Solomon, K.R., Wilks, M.F., Bachman, A., Boobis, A., Moretto, A., Pastoor, T.P., Phillips, R., and Embry, M.R. (2016). Problem formulation for risk assessment of combined exposures to chemicals and other stressors in humans. Crit Rev Toxicol *46*, 835-844

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Risk Assessment of the Effects of Combined Exposures to Chemical Carcinogens - an update

Decision tree developed by CEFIC/MIAT

Figure 1 from Price, P., Han, X., Junghans, M., Kunz, P., Wattts, C., and Lewverett, D. (2012). An application of a decision tree for assessing effects from exposures to multiple substances to the assessment of human and ecological effects from combined exposures to chemicals observed in surface waters and waste water effluents. Environ.Sci. Eu *24*, 34 -47.

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Risk Assessment of the Effects of Combined Exposures to Chemical Carcinogens - an update

Public consultation on Statement on Genotoxicity Assessment Chemical Mixtures –EFSA

http://www.efsa.europa.eu/en/consultations/call/180626

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Public consultation on MIXTOX Guidance

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