Committee on CARCINOGENICITY

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

Proposed strategy for discussion of alternative paradigms for assessing carcinogenic risk

This paper presents a background of and strategy for the Committee to discuss alternative paradigms for assessing carcinogenic risk, to enable Part d of the COC Guidance Statement G07 (Alternatives to the 2-year Bioassay) to be drafted.

Introduction

1. The Committee of Carcinogenicity (COC) guidance statement series provides the Committee's views on all aspects of carcinogen risk assessment.

2. Guidance statement G03 – Hazard Identification and Characterisation: Conduct and Interpretation of Animal Carcinogenicity Studies provides advice on hazard identification and characterisation of chemical carcinogens using animal carcinogenicity studies, in which the objective is to observe the development of neoplastic lesions during or after exposure of groups of animals for a major portion of their life span.

3. Guidance statement G07 – Alternatives to the 2-year Bioassay discusses approaches that have been proposed as alternative strategies to the current paradigm using lifetime bioassays. G07 comprises four parts. Parts a (alternative *in vivo* assays) and b (cell transformation assays) have been published (COC, 2016). Parts c (developing methodologies and strategies, e.g. toxicogenomics) and d (alternative testing paradigms, such as histopathology and proliferative markers in subchronic rodent studies) remain to be addressed.

4. Alternative testing paradigms, which will form G07 Part d, were discussed as a new item in the 2013 horizon scanning, and several references were provided. The list of priorities was reviewed at the November 2015 horizon scanning, and it was agreed that this was a high priority piece of work.

5. Members are asked to consider this introductory paper and to advise on the area and the literature reviewed, with the aim of focussing the COC's deliberations on this issue. A more in-depth discussion of this topic is planned for a future meeting.

Background and evidence base

6. The conduct of 2-year bioassays in rodents for carcinogenicity risk assessment is being questioned for a number of reasons:

- Ethical considerations. Studies require the use of a large number of animals. In line with the 3Rs principle, efforts are being made to reduce animal use and to develop more-refined testing strategies. In addition, legislation prohibiting animal testing is being introduced for some sectors, for example a complete ban on animal tests for cosmetic ingredients in Europe from 2013.
- Logistics. Studies are time-consuming and expensive to perform.
- Accuracy and specificity to predict carcinogenicity in humans. The conditions under which chemicals are tested are not necessarily relevant to human exposure – there are issues relating to the use of the maximum tolerated dose (dose extrapolation) and modes of carcinogenic action, particularly in rodents, (species extrapolation) that may not be relevant to human risk assessment, leading to a high false-positive rate (see, for example, Ames and Gold, 1990; Gold et al., 1992).

Alternative approaches are being developed to enable decisions to be made about carcinogenic potential without the requirement to conduct 2-year studies in rats and mice, and to provide informative mechanistic information.

Alternative tests

7. Alternative approaches to the 2-year bioassay can be listed in three broad categories (Doktorova et al., 2012):

- *In vitro tests* (short-term assays, such as genotoxicity/mutagenicity tests) provide a useful screen to indicate positive or negative carcinogenic potential, particularly for genotoxic compounds. However, they produce high numbers of positive results, have limited capacity to identify nongenotoxic carcinogens and lack dose–response characterisation.
- *In silico* assays using structure-based computational predictions are becoming more useful as this database increases, but the predictability for carcinogenicity is currently not very strong. Their main use to date has been for genotoxicity. They are not quantitative.
- Short- or medium-term *in vivo* assays, using, for example, modified animal models that are more susceptible to carcinogenicity in the short term (e.g. genetically modified mouse strains, see G07 Part a) or traditional, repeat-dose toxicity studies with the incorporation of additional endpoints to increase sensitivity/specificity to identify potential human carcinogens.

Alternative testing paradigms

8. A number of authors have proposed modifications of, or alternatives to, the standard, dual-approach testing strategy for carcinogenic potential based on genotoxicity tests plus lifetime bioassay in rats and mice.

9. In 1998, the US FDA reviewed the use of 2-year rodent studies and alternative strategies for carcinogenesis testing and stated an aim to move away from reliance on the results of one test (the lifetime bioassay) towards a decision-making process based on a profile of data, using a weight-of-evidence approach that takes into

account the increased knowledge of carcinogenic mechanisms that has been gained since the 2-year bioassay was adopted as a routine screen in the 1970s (Schwetz and Gaylor, 1998). A conceptual strategy was proposed, including a preliminary evaluation for genotoxicity to include data on physical chemical properties, structure alert information, information from computer-based prediction systems and the results of a genetic toxicity screen, and subsequent tests to include transgenic mouse models and then possibly a 2-year study. The inclusion of data relating to nongenotoxic mechanisms of carcinogenicity would be important, including the mechanisms identified in Table 1. It was proposed to evaluate these new test systems in parallel with the conduct of traditional 2-year bioassays.

Hormone modulation
Steroid (estrogen, androgen, retinoid)
Growth factor perturbation
Cell proliferation (mitogenic, cytotoxic)
Specific tissue responses
Bladder, stones
Liver, necrosis
Kidney, $\alpha_2\mu$
Forestomach
Inhibition of apoptosis
Specific mechanisms
β-Agonist, uterine tissue
H_2-antagonist, glandular stomach
Peroxisome proliferation
Cell-to-cell communication
P450 induction
Spindle fiber effects
Altered methylation status

Table 1 Measures of altered cell function (from Schwetz and Gaylor, 1998).

10. The International Council on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) in its Guideline S1 proposed that carcinogenicity testing for regulatory purposes be based on a 2-year test in one (rather than, historically, two) rodent species, supplemented with other data (ICH, 1998). Discussions are ongoing regarding revision of ICH S1 regulatory guidance for rodent carcinogenicity testing to support the registration of small molecule pharmaceuticals, with an aim now to define situations where complete waiver of a 2-year bioassay would be justified (ICH, 2012). These discussions take into account mechanisms of on-target and off-target pharmacology, genotoxicity, microscopic (histopathologic) risk factors for carcinogenicity identified in rat studies up to 6 months in duration, exposure margins in rat toxicity studies, hormonal activity, immunosuppression, results of nonrodent toxicity studies, and results of medium-term alternative mouse studies.

11. The ICH S1 revision process was reviewed by Morton et al. (2014), who suggested a weight-of-evidence decision process guiding the conduct of rodent carcinogenicity studies, illustrated in Figure 1. This includes options for a single mouse (6-month transgenic or 2-year) carcinogenicity study when human cancer risk is low because the weight of evidence suggests there is unlikely to be neoplasia in rats that is relevant to human cancer (Categories 3a, 3b), and no rodent carcinogenicity study with an appropriate label warning when a compound is expected to cause human cancer (Category 1). When human cancer risk is uncertain and rodent carcinogenicity studies will contribute to risk assessment, two rodent carcinogenicity studies would be required (Category 2).

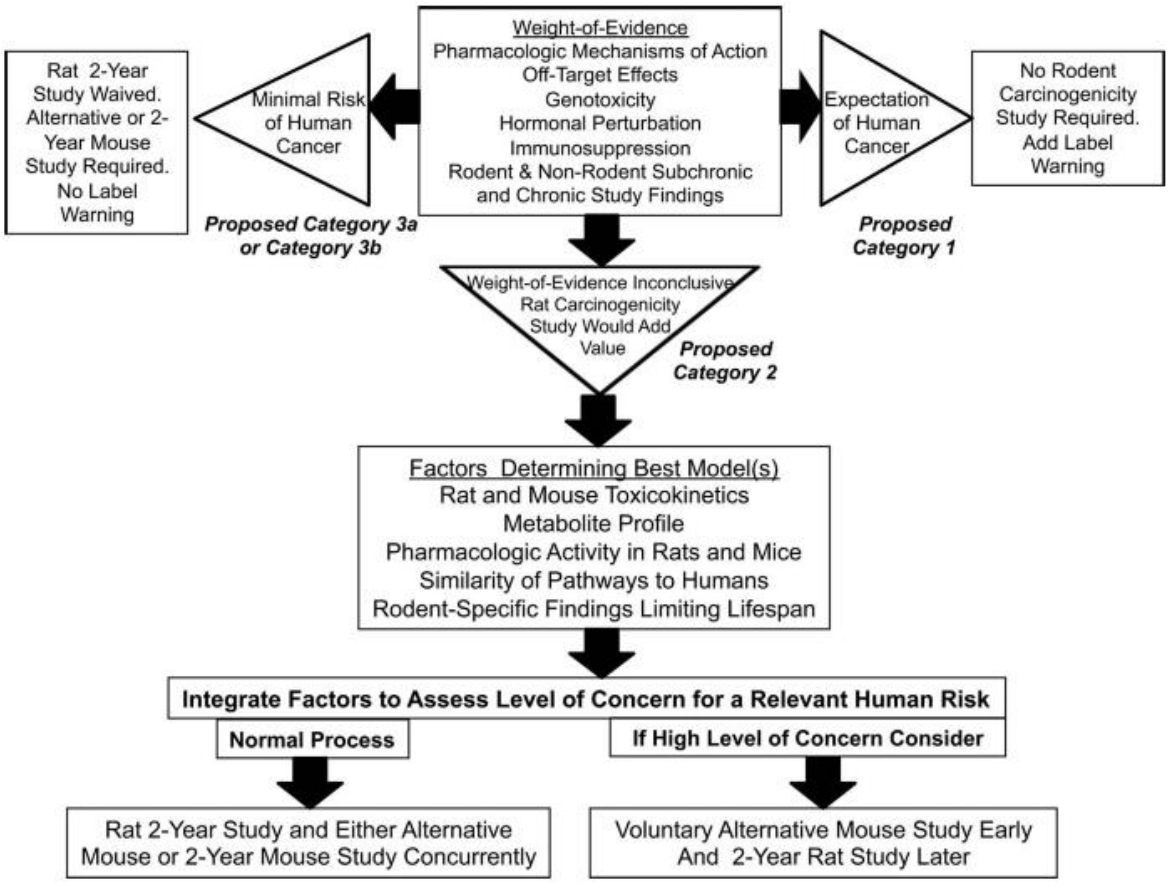

Figure 1 Suggested weight-of-evidence decision process guiding the conduct of rodent carcinogenicity studies for the assessment of small-molecule pharmaceuticals (from Morton et al., 2014).

12. ICH are currently evaluating prospectively the reliability of a less-than-lifetime strategy, through data generated by companies (ICH, 2016), and will base their guidance on the outcome of this exercise.

13. Cohen (2004, 2010a) has presented a number of reviews and opinion pieces, which argue that the 2-year rodent bioassay is no longer necessary or appropriate for the evaluation of possible carcinogenic risk to humans and that its use should be discontinued. An alternative model is presented that is based on shorter term tests, with an emphasis on mode of action and interpretation of the relevance to humans of findings in rodents. This model is represented as a tiered approach, incorporating a

short-term screen for genotoxicity, immunosuppressive and oestrogenic activity (known mechanisms of carcinogenesis in humans) using *in vitro* and *in vivo* tests, and the concurrent conduct of a 13-week assay using multiple doses to evaluate endpoints showing evidence of toxicity/cell proliferation. The premise is that increased carcinogenic risk occurs via 1. increased net rate of DNA damage per cell division, occurring in pluripotential cell populations, and 2. increased number of DNA replications (i.e. increased cell proliferation or decreased cell loss). The testing paradigm in Figure 2 is proposed.

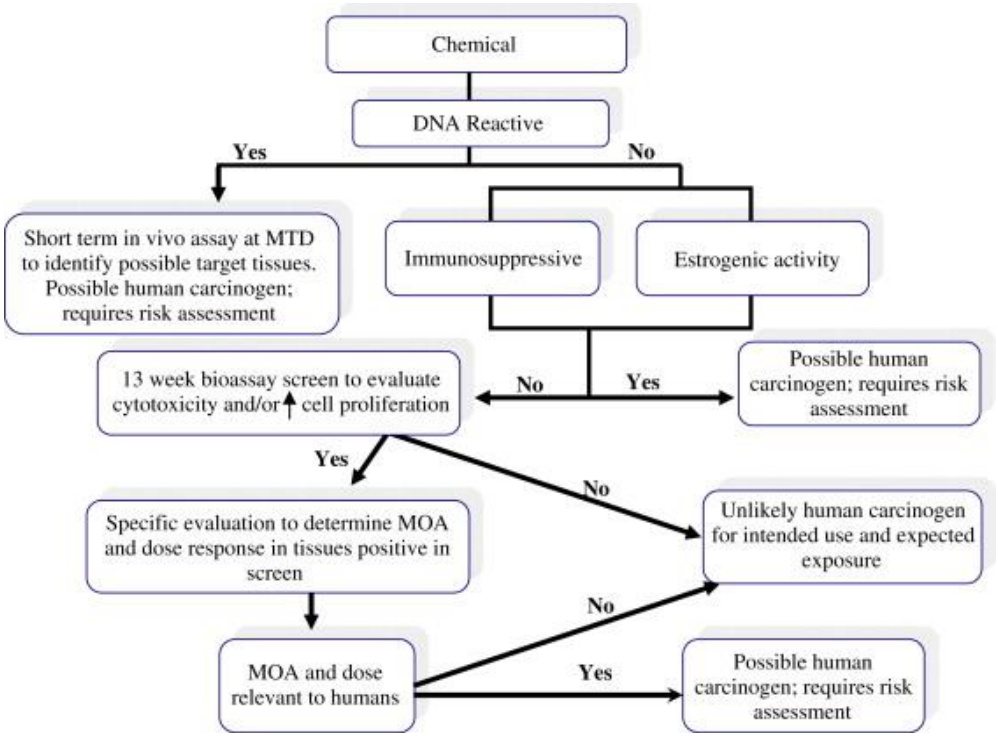

Figure 2 Sequential evaluation of a chemical for possible human carcinogenicity (from Cohen, 2010a).

14. The key events in this schedule involve precursor changes that can be identified earlier than the appearance of tumours, i.e. in 13-week studies in rats and mice rather than from 2-year studies.

Evaluations of alternative testing strategies

15. A number of groups have tested alternative schemes for carcinogenicity prediction, using existing toxicological databases.

16. In a study including 87 chemicals in the NTP database, Allen et al. (2004) showed that evaluation of three features (hepatocellular necrosis, hypertrophy, and cytomegaly) in the livers of rats treated for 90 days was effective to detect 7 of the 11 compounds that produced an increased incidence of liver cancer in a 2-year rat bioassay (with 16 false positives). The addition of liver weight as an extra criterion improved the sensitivity of the screen (11/11), but reduced specificity (32 false positives). Cohen (2010b) used these findings as a basis for a detailed discussion of liver carcinogenesis as an example of the use of short-term investigations in cancer

risk assessment. This concluded that the set of four short-term tests defined by Allen and colleagues can be used in combination with follow-up mechanistic studies to obtain an overall classification as to mode of action for hepatocarcinogenesis, without the requirement for a 2-year bioassay, and that this process could also be applied to evaluation for carcinogenic potential in other tissue types. In a commentary on the paper of Cohen, Long (2010), however, raised the points that this method would have a low probability of identifying rodent liver carcinogens that act through a previously undescribed mode of action and would have questionable applicability to tissues other than the liver, where precursor lesions have been less well characterised. It was further suggested that the evaluation of all necessary precursor lesions in all relevant tissues may in fact turn out to be more resource consuming than the conduct of standard 2-year bioassays (Long, 2010).

17. The International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute (HESI) conducted a retrospective analysis of the NTP database to test the hypothesis that the signals of importance for human cancer hazard identification can be detected in shorter term studies than the 2-year bioassay (Boobis et al. 2009). Sixteen chemicals were selected on the basis that they were positive in liver, kidney or lung in lifetime rodent carcinogenicity bioassays, and that genotoxicity and short-term rodent study data were available. A detailed assessment of tumours induced by non-genotoxic carcinogens suggested that conventional endpoints representing cellular changes indicative of a tumourigenic endpoint (cytotoxicity, hyperplasia, local irritation) could be identified in 13-week studies for most, but not all, of the chemicals examined, indicating that additional endpoints are required to identify some signals not determined by routine evaluation. Other issues that were noted included the requirement for a reliable battery of genotoxicity tests (some chemicals produced equivocal results), further definition/evaluation of short-term tests for immunosuppressive effects, and further efforts to determine false-positive rates of this approach.

18. In a comparison of data from short-term dose-ranging studies and 2-year carcinogenicity studies in rats for 60 pharmaceutical compounds in the FDA database, short-term indicators such as hyperplasia, hypertrophy, increased organ weights, tissue degeneration or atrophy, and mineralisation were not reliable predictors of tumour outcome in the corresponding tissues in carcinogenicity bioassays (Jacobs, 2005). The tissues examined in this study were liver, kidney, mammary, thyroid, adrenal, urinary bladder and lung.

19. Extending the work of Jacobs, Reddy et al. (2010) tested a ‘whole animal negative predictivity’ strategy, concluding that the complete absence of histopathological evidence of preneoplasia in all tissues in short-term toxicity studies *is* a reliable indicator for negative tumour outcome in a 2-year carcinogenicity bioassay, providing support for using these data as part of carcinogenic risk assessment. In this study, 2-year rat bioassay data for 80 pharmaceuticals (30 carcinogens and 50 noncarcinogens) were compared with findings from corresponding 6- or 12-month toxicity studies. The model specified the presence of preneoplasia (hyperplasia, cellular hypertrophy, and atypical cellular foci) in any single tissue (25 of the 30 carcinogens) as positive, and the absence of preneoplasia in all tissues (35 of the 50 noncarcinogens) as negative: sensitivity 83% (25/30), specificity 70% (35/50), negative predictive value (NPV) 88% (35/40), positive

predictive value (PPV) 63% (25/50)[1]. The authors considered that the positive 2-year bioassay results for the five false negatives were of questionable relevance to carcinogenicity in humans, also noting that these were all approved compounds currently marketed for non-life-threatening specifications.

20. Sistare et al. (2011) further tested the 'whole animal negative predictivity' strategy proposed by Reddy and colleagues, using an expanded database including 182 pharmaceutical compounds (66 positive and 116 negative in 2-year rodent carcinogenicity studies). In this study, negative outcome was specified as the absence of all of the following three criteria:

- genotoxicity,
- any knowledge or significant evidence of hormonal perturbation activity, and
- evidence of histopathologic risk factors of rat neoplasia in all tissues examined in the corresponding chronic rat toxicology study conducted at similarly matching doses to those used in 2-year carcinogenicity studies.

21. Immunosuppression was not included as a criterion, on the basis that results in rat carcinogenicity tests do not reliably reflect human risk for this effect (see below). Using this 3 test criteria–negative prediction strategy, 106 compounds were positive and 76 negative: sensitivity 79% (52/66); specificity 53% (62/116); NPV 82% (62/76); PPV 49% (52/106). Sensitivity was similar when considering endpoints at 6 or 12 months. The sensitivity of microscopic findings to predict rat neoplasia (i.e. to identify true positives) on an organ-by-organ basis was lower than on a whole-animal basis: a total of 42 true positives were identified from positive microscopic findings, and for 9 of these, the site of the tumour in the carcinogenicity study did not match any of the positive tissues in the repeat-dose toxicity study. The authors asserted that in fact a limited number of tissues (~10) serve as 'sentinels' for the majority of all tumour types seen in the 2-year studies. The human health relevance of positive 2-year rat bioassays for the 14 false-negative compounds was considered to be questionable, discussed in detail on a case-by-case basis. Ten of these compounds were marketed, 2 were not marketed for reasons unrelated to the rat carcinogenicity findings and 2 were still in development despite the positive rat carcinogenicity findings.

22. An evaluation of 78 IARC Group 1 and 2A chemicals was similarly carried out. Sixty-seven were positive for genotoxicity. Also taking into account a case-by-case consideration of findings for the the 11 non-genotoxic compounds, the authors concluded that the combined use of the three specified criteria was sufficiently predictive of rat carcinogenicity and may provide a strategy by which the 2-year rat assay, as well as the 2-year mouse assay, may not be necessary.

Alternative tests – endpoints

23. Although examination of tissues from standard, repeat-dose toxicity studies for markers indicative of preneoplasia has been shown to be useful to predict the outcome of corresponding 2-year rodent carcinogenicity assays, additional endpoints are required to improve sensitivity and specificity. Proposals include DNA labelling

[1] Sensitivity=TP/(TP+FN)X100, Specificity=TN/(TN+FP)X100, PPV=TP/(TP+FP)X100, NPV=TN/(TN+FN)X100 (TP=true positive, TN=true negative, FP=false positive, FN=false negative)

indices, blood and urine chemistry, organ weights, measures of apoptosis, micronucleus or comet assays, and the incorporation of carcinogen-specific molecular (e.g. 'omics') signatures from *in vivo* assays (Boobis et al., 2009; Cohen 2010a,b; Rothfuss et al., 2011; Doktorova et al., 2012).

24. Two particular properties of non-DNA-reactive compounds that are known to be significant indicators of carcinogenicity in humans are immunosuppression and oestrogenic effects.

Immunosuppression

25. Cohen (2010b) proposed that immunosuppression can be evaluated by histopathological evaluation of the lymphoid system, i.e. thymus, lymph nodes and spleen, in 13-week studies. In their study using the NTP database, Boobis et al. (2009) evaluated immune system markers including changes in haematology (total leukocyte, segmented neutrophil, lymphocyte, and monocyte counts), changes in spleen and/or thymus weights, and histopathological findings in the bone marrow, spleen, thymus, and lymph nodes. In fact, none of the 16 chemicals evaluated were found to have caused direct immunosuppression in 13-week rodent studies and the authors noted that further work, using a range of known positive and negative compounds, is required to develop endpoints for immunosuppression.

26. Sistare et al. (2011) did not incorporate immunosuppression as a category in their evaluation, pointing out that 2-year rat carcinogenicity studies do not reliably detect this human risk. It was noted there are likely to be significant differences between broad-based immunosuppressants and selective immune modulatory compounds that would be important to understand in helping to provide perspective for human risk assessment.

27. As the aetiology of some immunosuppressive agents in human cancers is via the facilitation of certain viral infections, Alden et al. (2011) suggested that transgenic mouse models harbouring specific viral infections may be useful to assess the potential of compounds to be carcinogenic through immunosuppressive effects.

28. In a critical review of the use of rodent models to assess the carcinogenicities of immunosuppressive agents, Bugelski et al. (2010) concluded that both the 2-year rodent bioassay and alternative models (neonatal mice; genetically engineered mice; UV/ionizing radiation/chemical carcinogens, including two-stage initiation/promotion protocols; viral tumorigenesis; tumour transplantation systems [allografts and xenografts]) are of little use in detecting risk of neoplasia caused by nongenotoxic immunomodulatory compounds.

29. A recent HESI/FDA workshop on immunomodulators and cancer risk underlined the point that this area is complex, immunosuppressive properties do not necessarily equate to increased carcinogenic potential, and that a similar cancer risk should not be assumed for all immunomodulatory molecules. A weight-of-evidence approach to carcinogenic risk assessment of immunosuppressants was proposed, including data from targeted immune function tests. However, the quantitative relationship between these endpoints and cancer risk is currently not well established (Lebrec et al., 2016).

30. It is also worth noting that the EPA recently proposed that immunotoxicity endpoints incorporated into repeat-dose studies may be accepted as an alternative to specific immunotoxicity studies in applications for pesticide registration, based on a retrospective evaluation of the utility of the specific immunotoxicity studies (US EPA, 2013).

Hormonal perturbation

31. Cohen (2010a) argued that oestrogenicity can be assessed in 13-week studies by histopathologic evaluation of oestrogen-dependent tissues, such as breast, endometrium and cervix. Changes in rodent endocrine tissues, other than oestrogenic activity, are generally not considered to be predictive of carcinogenic activity in humans (Cohen et al., 2004). In their evaluation using the 'whole animal negative predictivity' strategy for carcinogenicity of pharmaceutical compounds, Sistare et al. (2011) used a weight-of-evidence approach to assess hormonal perturbation, which included evidence of microscopic and/or macroscopic changes in multiple endocrine tissues within a sex, measurements of changes in hormone levels, and knowledge of pharmacological mechanism of action (hormone receptor binding, alteration of hormone levels, alteration of activity of endogenous hormones). It was noted that many of the true-positive compounds were hormonal agents or known to cause hormonal changes in rats. These were associated with ovarian granulosa cell, bone, mammary, testicular, pancreatic and/or thyroid tumours, and all had earlier documented effects on hormones or hormonally regulated tissues in the rat in tissues related to the tumours seen in the lifetime bioassay. These effects were considered in many cases to be due to indirect, rodent-specific hormonal mechanisms and the authors noted that it is well known that chronic hormonal perturbation is a risk for tumourigenesis in rats that may or may not always translate to humans.

Summary

32. Short-term, *in vivo a*lternatives to the 2-year rodent carcinogenicity bioassay are being developed, based on 1. evaluation of routine endpoints in subchronic rodent studies and 2. the development of additional endpoints to improve sensitivity and specificity.

33. A number of theoretical paradigms have been proposed, based on tiered strategies and weight-of-evidence approaches, incorporating screens for genotoxicity, immunosuppressive potential, oestrogenicity, and cell proliferation, to be performed either before, alongside, or in replacement of the 2-year rodent bioassay to predict whether a substance is likely to be a human carcinogen.

34. Models have been tested using existing toxicological databases. The ICH have an ongoing initiative to test strategies prospectively, in relation to carcinogenicity testing of pharmaceuticals. Short-term tests have shown utility in predicting results in 2-year bioassays. Good negative predictive value has been reported using 'whole animal negative predictivity' models although positive predictive values are lower. It has been proposed that these models can be useful in earmarking compounds that are of potential concern and require further investigation, i.e. for hazard identification.

Strategy for review

35. The Toxicology Unit at Imperial College will prepare a discussion paper using the literature base above as a starting point for more in-depth review of this topic. Specific aspects suggested to be emphasised are:

- Available alternative testing paradigms and approaches
- Ongoing work developing other paradigms and approaches of which the Committee is aware
- Evaluation of the available paradigms, and those in development – including how well they predict rodent or human carcinogenicity
- Endpoints of relevance, methods to evaluate these endpoints, and how they are combined in the models
- Any other aspects raised by Members

36. This discussion paper and the Committee's evaluation thereof will form the basis of a draft for Part d of the COC Guidance Statement G07.

Questions for the Committee

37. Members are asked to advise on the area, to inform the preparation of this discussion paper, with the aim of focussing the COC's deliberations on this issue, and to consider the following questions:

i. Does the Committee have any comments on the evidence base described?
ii. Does the Committee have any suggestions regarding additional aspects to be included in a review of this area, for example, in relation to:
    - tests for immunosuppression and oestrogenicity/hormonal perturbation
    - evaluation of cell proliferation *in vivo*
    - studies that have tested alternative strategies using existing toxicological databases?
    - other relevant literature?
    - ongoing studies of relevance?

Imperial College Toxicology Unit & COC Secretariat
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