This is a draft paper for discussion. It should not be quoted, cited or reproduced.

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

DISCUSSION PAPER ON BENZIMIDAZOLES AND OTHER TUBULIN BINDING COMPOUNDS: THE POSSIBILITY OF GROUPING BASED ON A COMMON MECHANISM OF TOXICOLOGICAL ACTION

Introduction

1. The COT published its report on Risk Assessment of Mixtures of Pesticides and Similar Substances in September 2002. One of the recommendations of the report was that a scientific and systematic framework should be established to decide when it is appropriate to carry out combined risk assessments of exposures to more than one pesticide and/or veterinary medicine. The COT recommended that the default assumptions in risk assessments of combined exposure should be that chemicals with different toxic actions will act independently, and that those with the same toxic action will act additively. In the latter circumstances the COT recommended that a toxic equivalency approach might be considered.

2. One of the actions which has been taken forward by government departments and agencies as a result of the COT recommendations has been the formation of a 'Science Group' to consider which pesticides and similar substances can be grouped together based on common mechanisms of mammalian toxicity, and to take forward the risk assessment of such common mechanism groups (CMGs).

3. One class of substance highlighted in the COT report as requiring further investigation as a possible CMG was the benzimidazoles. This was selected as one of the first groups to be investigated by the Science Group as it was considered that a group of substances whose mode of action had not been fully characterised even though they had broad evidence for similar mechanisms of action for cellular effects would be a suitable test case of the procedure for grouping CMGs.

4. The process agreed for identifying and grouping substances in CMGs is based on the approach currently being used by the US Environmental Protection Agency (US EPA). This is summarised in Table 1, below:

Table 1: Process for evaluating substances for inclusion in a common mechanism group

This is a draft paper for discussion. It should not be quoted, cited or reproduced.

<table>
<tr><td>Stage 1</td><td>Initial grouping of chemicals based on any or all of the following:
Structural similarity
Mechanism of pesticidal action
General mechanism of mammalian toxicity
A specific toxic effect
Add other substances that are metabolic precursors to any of the substances identified above</td></tr>
<tr><td>Stage 2</td><td>Definitively identify those substances from stage 1 that cause a common toxic effect
Evaluation and comparison, and exclusion of any substance that does not induce a common toxic effect with at least one other substance</td></tr>
<tr><td>Stage 3</td><td>Determination of the toxic mechanism of action leading to the common toxic effect
As a minimum identify the major biochemical events that are most responsible for causing the common toxic effect</td></tr>
<tr><td>Stage 4</td><td>Comparison of mechanisms of toxicity of each substance</td></tr>
<tr><td>Stage 5</td><td>Final refined grouping into common mechanism group
Identify and retain pesticides and other substances that cause a common toxic effect by a common mechanism
Eliminate those substances that cause the common toxic effect by different mechanisms</td></tr>
</table>

5. It is suggested that for benzimidazoles, these stages could equate to the following:

Stage 1: *In vitro* evidence for mutagenicity and/or aneugenicity and/or pesticidal/veterinary action on tubulin.
Stage 2: Clear evidence of aneugenicity *in vivo*
Stage 3: Evidence for binding to mammalian tubulin
Stage 4: Rationale for mechanism of aneugenicity
Stage 5: Stages 1-4 considered together

6. This is further discussed in paragraph 23.

7. The compounds that have been initially grouped (stage 1) are listed below. These include benzimidazoles used as pesticides (fungicides) and/or as veterinary medicines (anthelmintics, except for one compound). Some of these compounds are metabolites of other compounds listed, but they have been listed separately since they are also individually marketed. In addition four other pesticide and veterinary medicine active substances for which there is evidence for a potential for microtubule disruption have been identified and included.

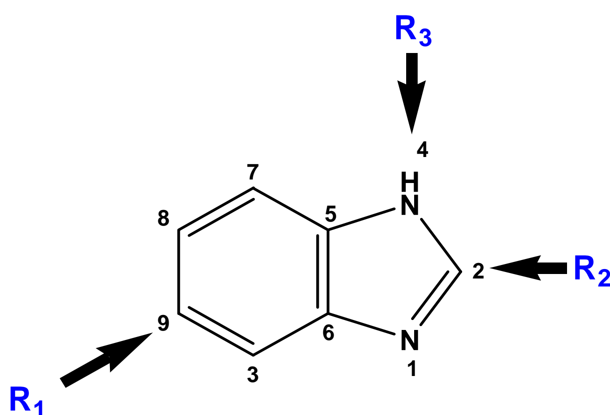
Albendazole
Albendazole oxide
Benomyl
Carbendazim
Chlorpropham
Febantel
Flubendazole
Fuberidazole
Mebendazole
Netobimin
Omeprazole
Oxfendazole
Pendimethalin
Propyzamide
Thiabendazole
Thiophanate methyl
Triclabendazole
Trifluralin

This is a draft paper for discussion. It should not be quoted, cited or reproduced.

Febendazole Oxibendazole

8. Sixteen of the 20 substances listed above contain the benzimidazole ring (see Figure 1), which is believed to be responsible for the pesticidal/veterinary mechanism of action of the benzimidazoles, or are pro-drugs which are metabolised to compounds containing the benzimidazole ring. Propyzamide (a substituted amide), pendimethalin and trifluralin (dinitroaniline compounds) and chlorpropham (an N-phenylcarbamate) have been included because there are some data to indicate that these compounds also have anti-tubulin effects. The structures of all 20 substances are shown in Annex A.

Figure 1: Molecular structure of the benzimidazole ring



9. The primary mechanism of action of benzimidazoles as anthelmintics is considered to be disruption of energy metabolism by binding to free β-tubulin, inhibiting its polymerisation and thereby preventing intracellular movements of the cellular cytoskeleton required for the transportation of glucose from the cell surface to the mitochondria. The mechanism of benzimidazole compounds as fungicides is widely considered to be inhibition of mitosis, resulting from binding to free tubulin, particularly β-tubulin, and thereby disrupting microtubule formation (Hollomon *et al.*, 1998; Hess and Nakai, 2000; Davidse, 1986).

10. It has been reported that binding of benzimidazoles to mammalian tubulin is significantly weaker than to helminth tubulin, and is reversible, and this is believed to be the reason for the differential susceptibility of mammals and helminths to these compounds (Davidse, 1986). Even within helminths and fungi, differential affinities are seen. For example, carbendazim is highly effective against Ascomycetous fungi, but is less active against Basidomycetes and inactive against Oomycetes and Zygomycetes (Davidse, 1986). Therefore anti-tubulin effects in helminths or tubulin data from fungi or helminths cannot be directly extrapolated to mammalian cells. However, for a number of the compounds data are available on effects on mammalian tubulin.

This is a draft paper for discussion. It should not be quoted, cited or reproduced.

11. A difficulty the Science Group has identified is in the consistency of the data between compounds. For many of the compounds specific studies investigating the potential for aneugenicity are not available, although some of the regulatory studies may provide an indication of the potential for aneugenicity *in vitro* or *in vivo*. Thus for the majority of the compounds there are data gaps. There is not one specific endpoint which has been investigated for all the compounds, which raises a potential difficulty if compounds assigned to a CMG are to be ranked or compared in potency for the purposes of a combined risk assessment.

Advice requested from COM

12. The Committee is asked to consider and advise on whether it is possible to complete stage 1 and other stages of the process for establishing a CMG for the benzimidazoles and additional tubulin-binding compounds based on the available data. If so, the Committee is asked to advise on what the critical endpoint should be to assign a compound to the group. Members will wish to consider whether demonstration of aneugenicity *in vivo* is required or whether demonstration of aneugenicity *in vitro*, indications of the potential for aneugenicity *in vitro* (e.g. inhibition of mitosis of mammalian cells) or effects on tubulin polymerisation *in vitro* are sufficient to include a chemical. If the latter the Committee will wish to consider whether it is possible to extrapolate from data on effects on non-mammalian tubulin (particularly helminth tubulin binding) to likely effects in mammalian cells.

13. If it is not possible to group these compounds into a CMG, we would like to seek the Committee's advice on the key reasons for this. If there are data limitations, we would welcome advice on what the data requirements are to enable establishment of a CMG.

14. If it is considered possible to group a number of compounds into a CMG, we would welcome the Committee's advice on what data should be used or aquired in order to enable the determination of toxic equivalency factors (TEFs).

Review of data on benzimidazoles and other tubulin-binding compounds

15. Considering the studies which are commonly performed as part of regulatory packages, compounds to be included in a common mechanism group might be expected to show polyploidy or inhibition of mitosis of mammalian cells *in vitro*, positive results in *in vitro* micronucleus assays if performed (preferably with staining techniques to show the presence of whole chromosomes) and positive results in *in vivo* micronucleus assays (preferably with staining techniques to show the presence of whole chromosomes if performed).

16. The genotoxicity data for each compound, focusing on data relevant to aneugenicity evaluation and including data on tubulin binding, are described in Annex A. These data are mostly based on summaries of studies considered

This is a draft paper for discussion. It should not be quoted, cited or reproduced.
as part of the various national and European approval processes for these active substances. Some of the data provided in the summaries are limited; however, where data are particularly relevant to this area of discussion we have provided as much detail as possible. Additional studies identified from literature searches (PubMed and TOXNET, all years, using terms "[substance] and tubulin" and "[substance] and mutagenicity") have also been included and these are referenced. The paper of Bentley *et al*. (2000) evaluating thresholds for aneugenicity for carbendazim and benomyl is attached at Annex B for Members' interest.

17. The results of tubulin-binding studies and the genotoxicity studies which may indicate aneugenicity are summarised in Table 2. Eleven of the 20 substances have produced positive results in *in vivo* micronucleus assays (one of these is listed as equivocal since a positive result was not reproduced in further tests). Seven have produced negative results in *in vivo* micronucleus assays. Two have not been assessed *in vivo*.

18. Analysis of the data in Table 2 shows that while a positive result for helminth tubulin binding may indicate a need for study of potential aneugenicity, it is not alone a good predictor of *in vivo* aneugenicity. Of 7 compounds for which there are data on both helminth tubulin binding and *in vivo* aneugenicity, data on helminth tubulin binding for 4 substances correctly predict a positive or negative result in the *in vivo* micronucleus test.

19. Results for mammalian tubulin binding show a good correlation with *in vivo* aneugenicity. Of 9 compounds for which there are data on both mammalian tubulin binding and *in vivo* aneugenicity, data on mammalian tubulin binding for 7 compounds correctly predicts the result for *in vivo* aneugenicity.

20. Results for *in vitro* aneugenicity in non-mammalian cells show a very good correlation with *in vivo* aneugenicity. Of 7 compounds for which there are data on anti-tubulin effects in non-mammalian cells (aneugenicity or inhibition of mitosis), data on effects in non-mammalian cells correctly predicts the result for *in vivo* aneugenicity for all 7 compounds.

21. Results for *in vitro* aneugenicity in mammalian cells shows a very good correlation with *in vivo* mutagenicity. For 11 compounds for which there are *in vitro* data to indicate aneugenicity or an effect on mitosis *in vitro*, data *in vitro* correctly predicts the result *in vivo* for 8 compounds. However, if only *in vitro* positive results in micronucleus studies are considered, all eight substances which are positive *in vitro* are also positive *in vivo*.

22. Aneugens may have been expected to produce positive results in the *in vitro* mouse lymphoma L5178 cell forward mutation assay. Interestingly, results in the mouse lymphoma assay were not a good predictor of *in vivo* aneugenicity (data not included in Table 2). For 7 compounds which were positive in the *in vivo* micronucleus assay and were studied in the mouse lymphoma assay only two were positive in the mouse lymphoma assay.

This is a draft paper for discussion. It should not be quoted, cited or reproduced.

Table 2: Summary of positive and negative results indicating action on tubulin and *in vitro* and *in vivo* aneugenicity

Chemical	**Helminth tubulin binding**	**Inhibition of mammalian tubulin polymerisation *in vitro***	**Aneugenicity in non-mammalian cells**[†]	**In vitro aneugenicity in mammalian cells**[‡]	**In vivo aneugenicity (e.g. bone marrow micronucleus assay)**
Albendazole[a]	Yes	Yes	ND	Positive in micronucleus assay. No kinetochore staining	Positive in bone marrow micronucleus assay. No kinetochore staining
Albendazole oxide[a]	Yes	ND	Yes	Positive in micronucleus assay. No kinetochore staining	Positive in bone marrow micronucleus assay. No kinetochore staining
Benomyl	ND	Yes	Yes	Yes	Yes
Carbendazim[b]	ND	Yes	Yes	Yes	Yes
Chlorpropham	**ND**	**Yes**	**Yes**	**Yes**	**Equivocal in micronucleus assay**
Febantel[c]	ND	ND	ND	ND	Negative
Fenbendazole[c]	Yes	ND	ND	ND	Negative
Flubendazole	Yes	Yes	ND	Polyploidy and cell transformation	Negative
Fuberidazole	ND	ND	ND	Inhibition of mitosis	Negative
Mebendazole	Yes	Yes	Yes	Yes	Yes
Netobimin[a]	ND	ND	ND	ND	Positive in bone marrow micronucleus assay (no kinetochore staining)
Omeprazole	ND	ND	ND	Positive in micronucleus assay. No kinetochore staining	Positive in micronucleus study in hepatocytes. No kinetochore staining
Oxfendazole[c]	Yes	Yes	ND	ND	ND

This is a draft paper for discussion. It should not be quoted, cited or reproduced.

Oxibendazole	Yes	Yes	ND	Polyploidy and metaphases of abnormal morphology	Negative
Pendimethalin	*ND*	*ND*	*ND*	*ND*	*Negative*
Propyzamide	ND	ND	Antimitotic in plants	ND	ND
Thiabendazole	Yes	Yes	Yes	Yes	Yes
Thiophanate methyl[b]	ND	ND	ND	ND	Yes
Triclabendazole	No	ND	ND	ND	Negative
Trifluralin	*ND*	*ND*	*Antimitotic in plants*	*ND*	*Positive in bone marrow micronucleus assay (no kinetochore staining)*

ND: No data identified
[a]Netobimin is metabolised to albendazole which is metabolised to albendazole oxide
[b]Thiophanate-methyl is metabolised to carbendazim
[c]Febantel is metabolised to fenbendazole which metabolically interconvertible with oxfendazole

[†]Tests for mitotic aneuploidy in yeast and *Aspergillus nidulans* unless otherwise stated
[‡]Includes studies in human lymphocytes, human/mouse hybrid cell line R3-5, human ovarian granulosa cells, Chinese hamster LUC2 and DON:Wg3h cells, Chinese hamster primary cells, rat primary heptocytes, with additional data from CHO cells and mouse embryo fibroblast C3H/10T1/2 clone 8 cells.

NB: Compounds which are all in normal text, underlined, in bold or in italics are structurally related.

This is a draft paper for discussion. It should not be quoted, cited or reproduced.

Discussion

Process for inclusion in a common mechanism group

23. Table 1 described the process for assessing substances for inclusion in a common mechanism group (CMG). The benzimidazoles and other tubulin-binding compounds are unique in that they have been initially grouped based on a molecular effect rather than a broad toxicological effect.

24. If effects on tubulin polymerisation are critical to the toxicity of the benzimidazoles and other compounds then they would be expected to produce similar effects in toxicological studies in animals. The non-mutagenicity data are not described in detail in this paper. However, effects that have been commonly reported for the compounds under consideration in animal studies include bone marrow hypocellularity or other haematological effects (anaemia, leucopaenia, splenic extramedullary haematopoesis, increased spleen weights), testicular atrophy or hypo- or aspermatogenesis, teratogenicity, fetotoxicity (mostly fetal growth retardation), thyrotoxicity (morphological changes, increased thyroid weight) and/or hepatotoxicity (mostly hepatocellular vacuolation). Table 3 summarises the toxicological hazards commonly identified for the benzimidazoles.

25. A number of these effects may plausibly result from aneuploidy or inhibition of mitosis (e.g. haematotoxicity, testicular toxicity, teratogenicity, fetotoxicity). There is no clear indication of carcinogenicity. Most of the compounds have produced negative results in carcinogenicity studies. Five compounds have not been tested for carcinogenicity. Malignant tumours in the kidneys, bladder, thyroid and Leydig cells were produced by trifluralin in a study in F344 rats. There were also increased hepatocellular adenomas/carcinomas combined in Charles River CD rats with fenbendazole, hepatocellular carcinomas and carcinomas plus adenomas in B6C3F1 mice with propyzamide, follicular cell adenomas in male Sprague-Dawley rats with thiabendazole, and hepatocellular adenomas in albino Tif:MAGf mice with triclabendazole. These tumours have generally been considered to be due to non-genotoxic mechanisms.

26. As the relationship between aneuploidy and toxicological effects is not entirely clear it is suggested that aneuploidy itself should be considered to be the common toxic effect for inclusion into the CMG, with the mechanism being binding to α- or β-tubulin and inhibiting polymerisation.

27. Paragraph 5 suggested how the process for inclusion in a CMG could be interpreted for the benzimidazole and other tublin-acting compounds. However, based on this interpretation a compound would need clear evidence for aneugenicity *in vivo* in addition to evidence for binding to mammalian tubulin in order to complete all five stages. Few compounds meet this criteria. It is suggested that the results of the analysis of the genotoxicity data indicate

This is a draft paper for discussion. It should not be quoted, cited or reproduced.
that it may be possible to include compounds in a CMG on a more pragmatic basis, i.e. without requiring *in vivo* data.

This is a draft paper for discussion. It should not be quoted, cited or reproduced.

Table 3: Summary of toxicological hazards commonly identified for benzimidazoles and/or other tubulin-acting compounds.

Compound	Identified hazard					
	Teratogenicity	Fetotoxicity (e.g fetal gowth retardation)	Hepatotoxicity (e.g. hepatocellular vacuolation)	Thyrotoxicity (e.g elevated thyroid weight, tumour induction)	Testicular atrophy/aspermatogenesis	Haematotoxicity (e.g. bone marrow hypocellularity, anaemia, leucopaenia and/or splenic extramedullary haematopoesis)
Albendazole	✓	✓	✓		✓	✓
Albendazole oxide	✓	✓	✓		✓	✓
Benomyl	✓	✓ (post-implantation death)	✓		✓	
Carbendazim	✓	✓	✓	✓	✓	✓
Chlorpropham	✓ (dark spleens only)			✓		✓
Febantel		✓	✓		✓	✓
Fenbendazole	✓		✓			
Flubendazole		✓			✓	
Fuberidazole			✓	✓		
Mebendazole	✓	✓	✓		✓	✓
Netobimin	✓				✓	✓
Omeprazole				✓		
Oxfendazole	✓	✓	✓		✓	✓
Oxibendazole						✓
Pendimethalin		✓ (slight decrease in offspring nos. and birthweights in 3-generation study)				
Propyzamide			✓	✓	✓	
Thiabendazole		✓ (reduced implantation but		✓		✓

This is a draft paper for discussion. It should not be quoted, cited or reproduced.

		related to maternal bodyweight decrease)				
Thiophanate methyl		✓		✓		
Triclabendazole		✓	✓			
Trifluralin				✓		

This is a draft paper for discussion. It should not be quoted, cited or reproduced.

Defining a common mechanism group

28. Possible options for defining a CMG could include one of the following:

1. Structure-activity relationship + evidence for binding to mammalian tubulin + indication for aneugenicity *in vitro* + positive *in vivo* micronucleus assay.

2. Structure-activity relationship + any positive micronucleus study either *in vivo* or *in vitro*.

3. Structure-activity relationship or inhibition of tubulin polymerisation in any system (e.g. helminth, fungal, plant or mammalian) + positive *in vivo* micronucleus study.

29. The first option would provide the greatest evidence for a common toxicological mechanism. However, only a very small CMG could be defined at this time due to data limitations. Research would be required for most compounds before they could be included in a CMG, including *in vivo* studies.

30. The rationale for the second option is the observed 100% correlation between results in *in vitro* micronucleus tests in mammalian cells and results in *in vivo* micronucleus tests. This may be a reasonable approach for the benzimidazoles, enabling a larger CMG to be defined at this time and minimising the data requirements to enable more compounds to be added to the CMG. However, this approach could exclude the non-benzimidazoles since they are not considered to be structurally related to known *in vivo* aneugens and it is not clear that it may reasonably be assumed that the non-benzimidazoles which are aneugenic *in vitro* will also be aneugenic *in vivo* since the evaluation of correlations was mostly based on benzimidazoles.

31. The third option enables data showing anti-tubulin potential to be used in place of structure-activity relationship but would require a positive *in vivo* micronucleus study to include a chemical into the CMG.

32. A fourth option could be a mixture of option 2 for benzimidazoles (positive *in vitro* or *in vivo* micronucleus study) and option 3 for non-benzimidazoles (any data to indicate a potential effect on tubulin polymerisation + positive *in vivo* micronucleus test). This pragmatic approach would enable the largest CMG to be established at this time (11 compounds) with minimal data requirements for further additions.

33. It is suggested that compounds which are metabolised *in vivo* to other compounds which meet the criteria for inclusion should automatically be included in the CMG. Compounds which have produced negative results in *in vivo* micronucleus studies which are considered acceptable could be excluded (no compounds which are negative in *in vivo* micronucleus assays have been positive in *in vitro* micronucleus assays).

This is a draft paper for discussion. It should not be quoted, cited or reproduced.

34. Prioritisation for research to fill data gaps will need consideration of potential exposure.

Possibilities for ranking by potency

35. In order to ascertain whether a ranking is possible for these compounds some comparative data are presented in Table 4.

36. There are uncertainties relating to all three sets of data. Different studies have produced varying results for mammalian tubulin polymerisation for some compounds. The *in vitro* studies do not take into account factors such as absorption, distribution, metabolism and excretion. The *in vitro* aneugenicity studies have also investigated different endpoints, i.e. many have only investigated micronucleus induction whereas others have also examined chromosome non-disjunction. The *in vivo* studies have used different routes of exposure (oral gavage versus i.p.), different dosing periods (single or multiple doses) or have produced conflicting results. In Table 4 only data from single oral dose *in vivo* studies have been included.

Table 4: Comparisons of IC_{50}s for inhibition of mammalian tubulin polymerisation, LOELs for data indicative of aneugenic potential *in vitro* and LOELs for induction of micronuclei *in vivo* following single oral dosing

Compound	IC_{50} for inhibition of mammalian tubulin polymerisation *in vitro*	LOEL for *in vitro* aneugenicity in mammalian cells	LOEL for induction of micronuclei *in vivo* (oral dosing only)
Albendazole	6.9 μM	10 μM	(3117 mg/kg – single dose)
Albendazole oxide		100 μM	(10000 mg/kg – single dose)
Benomyl	70-75 μM	0.344 μM	50 mg/kg
Carbendazim	5-71 μM	1.72 μM	100 mg/kg
Chlorpropham	IC_{30} = 2000 μM	(4.68 μM –clear effect level)	
Febantel			
Fenbendazole	5.4-6.3 μM		
Flubendazole	3.5 μM		
Fuberidazole			
Mebendazole	5.2-9 μM	0.389 μM	40 mg/kg
Netobimin			(6.8 mg/kg - single dose)
Omeprazole			
Oxfendazole	>150 μM		
Oxibendazole	2.4-7 μM		
Pendimethalin			
Propyzamide			
Thiabendazole	549 μM	(4.97 μM – clear effect level)	
Thiophanate methyl			(1000 mg/kg – single dose)
Triclabendazole			(1680 mg/kg – clear effect level)

This is a draft paper for discussion. It should not be quoted, cited or reproduced.

Trifluralin			

37. There are more data for effects on mammalian tubulin polymerisation *in vitro* than for other endpoints. However, there is no clear correlation between the reported IC_{50}s for inhibition of mammalian tubulin polymerisation and LOELs for *in vitro* or *in vivo* aneugenicity. Therefore this endpoint does not appear to be useful for ranking *in vivo* aneugenic potential.

38. There are few comparable data from *in vivo* studies. Furthermore detailed pharmacokinetic data are not available to indicate the relationship between the administered oral dose and the exposure of the bone marrow.

39. It is suggested that the *in vitro* data are the most useful for ranking aneugenic potency. These indicate the following limited potency ranking:

Mebendazole ≈ benomyl > carbendazim > albendazole > albendazole oxide.

40. Further data are required to rank additional compounds which may be included in a CMG. The establishment of toxic equivalency factors (TEFs) would also require consideration of the toxicological and pharmacokinetic data and is beyond the scope of this study.

<u>Questions on which the views of the Committee are sought</u>

41. The Committee is invited to consider the following questions and raise any other matters that arise from the data in this paper:

i) Is it possible to group any of the benzimidazoles and other compounds into a common mechanism group (CMG), based on aneuploidy resulting from inhibition of tubulin polymerisation? If not, what further data would be required to enable this?

ii) What data are required to include a compound into the CMG? Are *in vivo* data required (such as an *in vivo* micronucleus assay) or can a compound be included on the basis of *in vitro* data?

iii) Would an acceptable negative *in vivo* micronucleus assay be sufficient to exclude a compound from the CMG?

iv) If a CMG can be established, what data would permit ranking by potency?

42. A short working paper will be drafted based on the Committee's discussion.

Secretariat
May 2006