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MUT/06/14

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

CONCLUSIONS ON BENZIMIDAZOLE ANEUGENICITY: COMBINED ASSESSMENT

Introduction

1. At the May 2006 meeting Members commented that it may be possible to develop a scoring system to determine whether a chemical could be included in a benzimidazole common mechanism group. Establishing a common mechanism group is a critical first step towards enabling risk assessments to be performed for exposures to multiple tubulin-active compounds. We have considered this further and would appreciate Members' advice on the below information and questions.

Demonstration of additivity

2. Members considered that it was necessary to demonstrate that any chemicals to be included in a benzimidazole common mechanism group bind to mammalian β-tubulin at the same binding site, and that they act additively. Members also commented on the structure-activity relationship for benzimidazoles and aneugenicity. The current state of knowledge is summarised below:

Tubulin binding site

3. There are data for some of the benzimidazoles to indicate that they bind to the colchicine binding site of mammalian tubulin. Mebendazole and oncodazole (a benzimidazole developed as a chemotherapeutic agent) have been shown to competitively inhibit the binding of cochicine to purified rat brain tubulin in competition binding assays using radiolabelled colchicine (Laclette *et al.*, 1980; Ireland *et al.*, 1979; Tahir *et al.*, 2000; Russell and Lacey, 1995; Hoebeke *et al.*, 1976). Oxibendazole and fenbendazole have been shown to competitively inhibit the binding of colchicine to bovine brain tubulin (Friedman and Platzer, 1978). Colchicine has also been shown to competitively inhibit mebendazole binding to rat brain tubulin, providing further evidence that these two compounds share a common binding site (Russell and Lacey, 1995).

4. In contrast, a recent study reported that while benomyl inhibited the polymerisation of purified goat brain tubulin, it did not inhibit colchicine binding to tubulin, as tested using an assay based on the principle that colchicine fluoresces when bound to tubulin (Gupta *et al.*, 2004). Benomyl also did not inhibit binding of a fluorescent analogue of vinblastine to tubulin, indicating that benomyl binds at a novel site. The implications of this are unclear since

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benomyl is metabolised to carbendazim, which is generally considered to be the biologically active form (EC Scientific Committee on Plants, 2001).

5. Nguyen *et al.* (2005) employed docking studies for a range of different chemicals all able to bind within the colchicine binding domain of β-tubulin, including the benzimidazoles nocodazole and mebendazole. They constructed binding models for all these compounds and proposed a common pharmacophore model that linked these diverse chemicals. This included seven points: three hydrogen bond acceptors, one hydrogen bond donor, two hydrophobic centres and one planar group. None of the compounds were characterised by all seven points, which it was suggested may explain some of the differences in the activity of these compounds.

6. Robinson *et al.* (2004) proposed a site in helminth tubulin at which benzimidazoles may bind to β-tubulin based on clues from existing data, including correlation of benzimidazole resistance in the helminth *Haemonchus contortus* with a phenylalanine to tyrosine substitution at a particular position. The authors also modelled docking of albendazole oxide into *H. contortus* β-tubulin. It is not clear from this paper how the binding site might compare to that of other compounds. It is also not clear how binding to mammalian tubulin would compare.

Role of the benzimidazole moeity

7. Presence of the benzimidazole ring appears to be important for the pesticidal/veterinary mode of action of the benzimidazoles. Since most of these compounds have been shown to inhibit the polymerisation of mammalian tubulin, the benzimidazole moiety appears to be important for this effect also.

8. Lacey and Watson (1985) studied the effect of the differences in the R_1 substituent (see Figure 1) on the concentrations of benzimidazoles required to produce 50% inhibition of polymerisation of sheep brain tubulin (IC_{50}). Carbendazim, which contains simply a hydrogen at this position, had weak activity, but replacing the hydrogen with one of the larger halides (Cl or Br) resulted in a progressive increase in activity. The presence of polar groups (OH, NH_2 or NO_2) resulted in loss of activity. An increase in alkoxy chain length from methyl to propyl resulted in increased activity but further increasing the chain length had little effect; a similar relationship was shown for alkyl chain length. The presence of branching in the substituent group at the α or β positions of R_1 reduced potency. The molecular geometry and the polarity in the α or β regions of the substituent also appeared important to the tubulin binding potency of the molecule.

9. Jayasekhar and Kasture (1999) tested a number of benzimidazoles with an ethoxy group at the R_1 position and various alkyl groups at the R_2 position and showed that increased pK_a was associated with increased antifungal activity.

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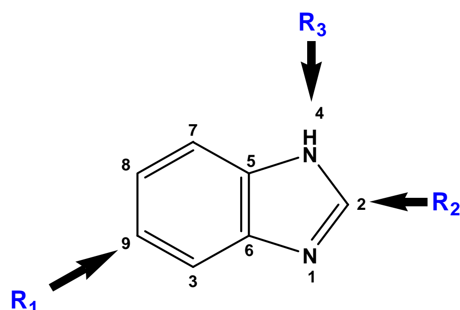
Figure 1: Molecular structure of the benzimidazole ring



Additivity

10. No studies of the potential additive effects of benzimidazoles in relation to tubulin depolymerisation or aneugenicity have been identified.

11. A study has been identified in which two aneugens which bind to tubulin at different binding sites (paclitaxel and the synthetic vinca alkaloid vinorelbine) were tested individually and in combination at various doses for their ability to inhibit cell proliferation of human StM11 1a and G361 melanoma cells (Photiou *et al*., 1997). Dose-response data indicated combined effects which the authors interpreted as being synergy at most, predominantly low, concentrations, with additivity or sub-additivity at some concentrations. This paper is attached as Annex A. In another study, phenytoin and vinblastine were reported to have additive effects on porcine brain tubulin polymerisation, despite binding to tubulin at different sites (phenytoin binds at the colchicine binding site, vinblastine at the vinca alkaloid binding site) (Lobert *et al*., 1999). The authors suggest that phenytoin sequesters free tubulin heterodimers whereas vinblastine affects forming microtubules. This paper is attached as Annex B.

Inclusion in a common mechanism group

12. Figure 2 contains a proposed decision tree by which it could be decided whether to include a benzimidazole in a common mechanism group. This takes into account Members' comments that non-mammalian data (i.e. aneugenicity in fungi, or helminth or fungal tubulin binding) were poor predictors of *in vivo* aneugenicity and that there were good correlations between mammalian tubulin binding and *in vivo* aneugenicity and aneugenicity *in vitro* in mammalian cells and *in vivo* aneugenicity.

13. It is suggested that other tubulin acting compounds could subsequently be added to the common mechanism group, providing they are:

1) aneugenic in vivo; and
2) additivity with at least one compound in the benzimidazoles CMG is demonstrated experimentally.

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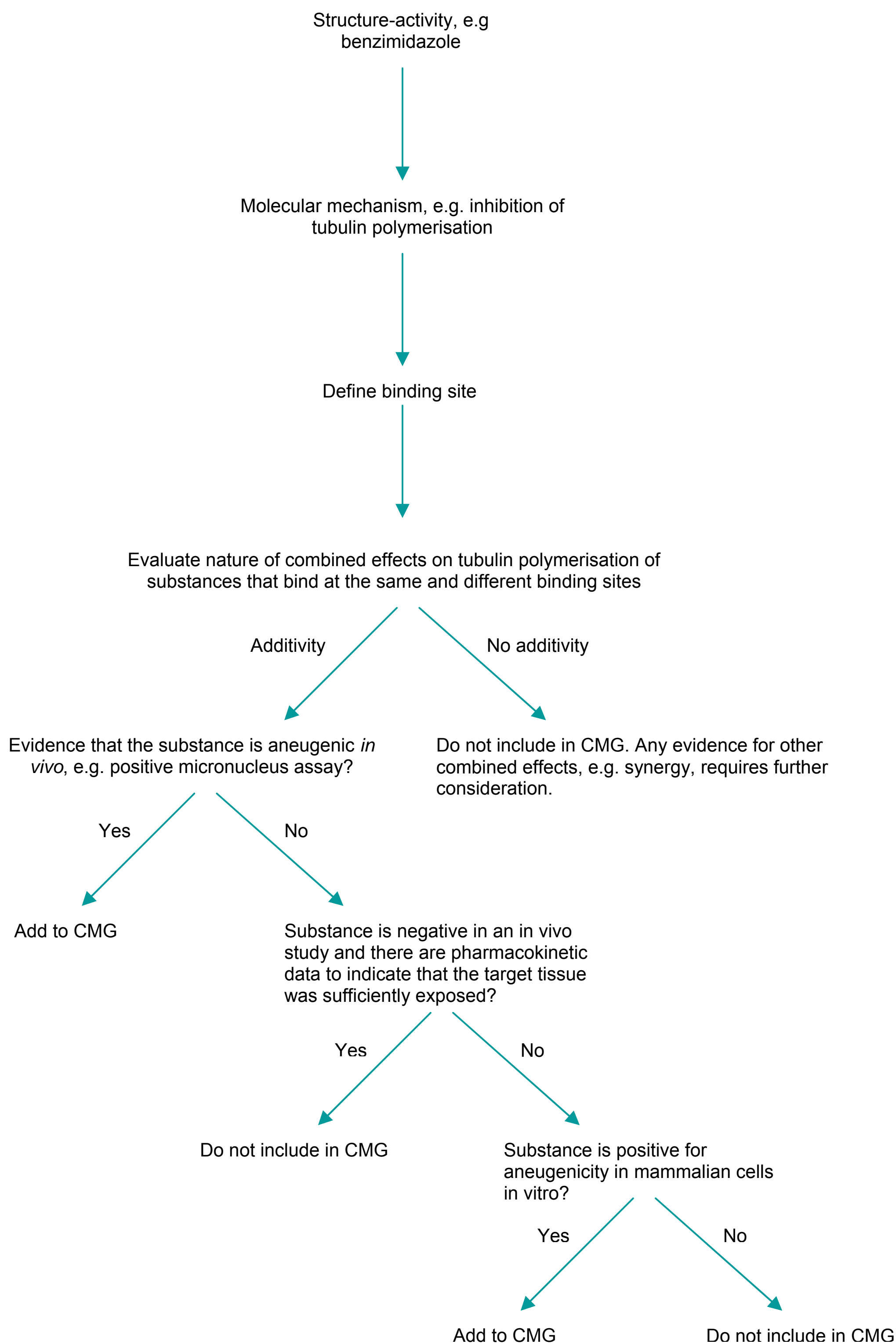
Figure 2: Possible decision tree for including a substance in a benzimidazole common mechanism group



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Questions

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

i) Do Member's agree with, or have any comments on, the suggested decision tree in Figure 2?

ii) Is there a need to consider not only aneugens which act by binding to tubulin at the same binding site but also those that bind to tubulin at different binding sites? What types of combined effect may be anticipated from mixtures of substances which bind to tubulin at different binding sites?

iii) Do all combinations of benzimidazole have to be tested to demonstrate additivity or could selected example compounds be tested?

iv) Could other aneugens be added to the common mechanism group if, for example they are aneugenic *in vivo* and additivity with at least one compound in the common mechanism group is demonstrated?

Secretariat
September 2006

References

EC Scientific Committee on Plants (2001). Opinion of the Scientific Committee on Plants regarding the evaluation of Benomyl, Carbendazim and Thiophanate-methyl in the context of council directive 91/414/EEC concerning the placing of plant protection products on the market (opinion adopted by the Scientific Committee on Plants on 7 March 2001). Available at http://ec.europa.eu/comm/food/fs/sc/scp/out98_ppp_en.html.

Friedman PA, Platzer EG (1978). Interaction of enthelmintic benzimidazoles and benzimidazole derivatives with bovine brain tubulin. *Biochimica et Biophysica Acta* **544**: 605-614.

Gupta K, Bishop J, Peck A, Brown J, Wilson L, Panda D (2004). Antimitotic antifungal compound benomyl inhibits brain microtubule polymerization and dynamics and cancer cell proliferation at mitosis, by binding to a novel site in tubulin. *Biochemistry* **43**: 6645-6655.

Hoebeke J, Van Nijen G, De Brabander M (1976). Interaction of oncodazole (R 17934), a new anti-tumoral drug, with rat brain tubulin. *Biochem. Biophys. Res. Commun.* **69**: 319-324.

This is a draft paper for discussion. It should not be quoted, cited or reproduced.
Ireland CM, Gull K, Gutteridge WE, Pogson CI (1979). The interaction of benzimidazole carbamates with mammalian microtubule protein. *Biochem. Pharmacol.* **28**: 2680-2682.

Jayasekhar P, Kastrure AV (1999). Physico-chemical relationship of some substituted benzimidazoles. *Boll. Chim. Farm.* **138**: 489-492.

Lacey E, Watson TR (1985). Structure-activity relationships of benzimidazole carbamates as inhibitors of mammalian tubulin, in vitro. *Biochem. Pharmacol.* **34**: 1073-1077.

Laclette JP, Guerra G, Zetina C (1980). Inhibition of tubulin polymerization by mebendazole. *Biochem. Biophys. Res. Commun.* **92**: 417-423.

Lobert S, Ingram JW, Correia JJ (1999). Additivity of dilantin and vinblastine inhibitory effects on microtubule assembly. *Cancer Res.* **59**: 4816-4822.

Photiou A, Shah P, Leong LK, Moss J, Retsas S (1997). In vitro synergy of Paclitaxel (Taxol) and Vinorelbine (Navelbine) against human melanoma cell lines. *Eur. J. Cancer* **33**: 463-470.

Robinson MW, McFerran N, Trudgett A, Hoey L, Fairweather I (2004). A possible model of benzimidazole binding to β-tubulin disclosed by invoking an inter-domain movement. *J. Mol. Graph. Model.* **23**: 275-284.

Russell GJ, Gill JH, Lacey E (1992). Binding of [^{3}H]benzimidazole carbamates to mammalian brain tubulin and the mechanism of selective toxicity of the benzimidazole anthelmintics. Biochem. Pharmacol. **43**: 1095-1100.

Tahir SK, Kovar P, Rosenberg SH, Ng S-C (2000). Rapid colchicine competition-binding scintillation proximity assay using biotin-labeled tubulin. *BioTechniques* **29**: 156-160.