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DRAFT

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

HORIZON SCANNING: ASESMENT OF MUTAGENIC IMPURITIES USING THE AMES TEST AND GENERIC GUIDANCE PUBLISHED ON PHARMACEUTICALS.

Background to COM consideration

1. The COM were made aware of a published literature survey to evaluate lowest detectable level of response in the Ames test for mutagens during the Horizon scanning exercise for 2007. The approach adopted by the authors has a potential wider generic use which could be valuable for the review of the COM strategy and also for generic advice to Government Departments. (Kenyon MO et al *Regulatory Toxicol, Pharmacol*, 48, 75-86, 2007.) Members agreed to review this publication and also noted some recent publications which had considered a rationale for determining, testing, controlling specific impurities in pharmaceuticals that possess potential for genotoxicity. Evaluation of sensitivity of Ames test to detect low level impurities in pharmaceutical ingredients.

Overview of approach proposed by Kenyon et al 2007 (Annex 1)

2. Briefly, a literature survey of 454 mutagens tested in the Ames test was undertaken to estimate the lowest effective concentrations for a variety of classes of mutagens and to develop an understanding of the sensitivity of the test system. In addition, Ames tests were conducted using known mutagens in the presence of excess mannitol (considered to represent an excess of active pharmaceutical ingredient (API)). In addition a number of Ames tests were undertaken using promutagens (i.e chemicals requiring metabolic activation by S-9) in the presence of 'excess' verapamil and diltiazem, two highly metabolised medicines. The Lowest Effective Concentration (LEC µg/plate) was determined as the concentration expected to give a two-fold increases in revertants. Estimated LECs were converted to Theoretical Assay Detection Limits (TDLs). This is essentially the ratio of the LEC compared to the maximum applied dose/plate in the Ames tests of 5000 µg/plate expressed as a percentage. Structural classes were defined using DEREK (with a minimum of 5 chemicals in each class). The Lower and Upper limit for LECs for each class were determined (LL, UL).

3. LECs varied between 0.0003 to 4860 µg/plate. 87% were below 250 µg/plate, with 11% between 250-2500 µg/plate and 2% between 2500-5000 µg/plate. The authors report that most classes of mutagen had a median LEC of 250 µg/plate or less. Only one class had a median LEC greater than 250 µg/plate (mono-di-aklyhydrazines). Overall for most representative classes, all compounds were detected at 2500 µg/plate. In a further analysis by class,

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the authors reported that only a small number of compounds had LECs that were greater than 250 µg/plate. Overall, the authors estimated that 85% of mutagenic impurities in an API should be detected in Ames tests if present at ≥5% assuming the API is tested up to 5000 µg/plate.

4. This suggests there will be approximately 15% of mutagenic impurities present at ≥5% which are unlikely to be detected in the Ames test. For mutagenic impurities where the LEC is above 250 µg/plate, the probability of detection in the Ames test would reduce (below 85%), particularly for mutagens at the lower end of the potency range for each class. It is noted that for some classes of mutagen there is a wide variation of LEC within the class (e.g. aromatic nitro compounds, c.f. figure 2 where the LEC appears to vary over 6 orders of magnitude). Thus for example, an unknown impurity in this class (aromatic nitro compounds), the evaluator would not be able, in the absence of compound specific mutagenicity data, to gauge the mutagenic potency of the impurity within the range for the structural class with any certainty inferring that mutagenic hazard identification for the impurity would involve isolating the impurity and testing to assess mutagenic potential. The authors reach conclusions on classes of mutagen which should be detected in the Ames assay at 250 µg/plate. This is briefly summarised below

Should be detected at 250 µg/plate;

Bifuranoid mycotoxins, hydroxylated anthraquinones, polycyclic aromatic hydrocarbons, aromatic hydroxamic acid, quinolines, aromatic azos, azirine or aziridines and isocyanate or isothiocyanates.

Majority of compounds in class should be detected at 250 µg/plate

N-nitro or N-nitroso, glycidyl ether, amine, ester or amides, chromium compounds, aromatic nitros, halogenated alkenes, alkyl ester of phosphoric acid or phosphonic acids, aromatic amines, epoxides, alkylating agents and halogenated methanes.

Unlikely to be detected at 250 µg/plate

Mono-di-alkylhydrazines

5. The authors noted there were insufficient data to draw conclusions on a number of classes of impurity which might be present in pharmaceuticals such as aromatic amides and halogenated heterocyclic compounds.

6. Spiking experiments using six well characterised mutagens showed that the presence of an excess of mannitol in the Ames test system did not affect the shape of the dose-response curve for these mutagens.

7. To investigate the effect of excess compounds requiring metabolic activation, the authors investigated the effect of verapamil and diltiazem on the dose-response for benzo(a)pyrene (BaP) and aflatoxin B1 (AFB1). Dose-

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response data for *Salmonella typhimurium* TA100 (72h incubation) were presented which indicated a reduced mutagenic response at the higher end of the dose-response curve for both BaP and AFB1. The authors noted that there were limitations in the study (e.g the extent of substrate competition was unknown and both verapamil and diltiazem have been reported to enhance the mutagenicity of some compounds in the Ames test). The interpretation placed on these results by the authors, was that cytotoxicity (presumably due to the metabolism of verapamil or diltiazem) was present at concentrations where reduced mutagenic response was observed and this represented an interaction within the tests system between the impurity under tests and the surrogate API.

7. What are members views of the results published by Kenyon et al. Can these data be used to draft generic guidance for the mutagenicity testing of impurities?

An example (potential) application: Impurity X in compound Y.

8. Compound Y is being evaluated through a regulatory scheme. It is very cytotoxic to *Salmonella*. An impurity is present with unique structure. The evaluator has some concerns with regard to potential mutagenicity of impurity X. It is noted that only ca 50 ng/plate of the impurity would have been present in the Ames test at the levels of compound Y that can be adequately tested in the Ames test. Hence the way forward has been to consider isolation of the impurity and testing as a single entity.

9. However if Y had not been toxic the maximum concentration in the Ames test would have been around 50 µg/plate (assuming 5000 µg/plate of compound y was used in the Ames test). The structural class of impurity X can not be assigned according to the evaluation by Kenyon et al 2007. Thus unless impurity X was a very potent mutagen, it wouldn't be detected in the Ames test. Hence the key factor requiring isolation and separate testing of the impurity is the concentration of impurity X in compound Y. The use of a predetermined limit of for acceptable testing of impurity X of 250 µg/plate would not have been sufficient as the mutagenic activity and the range of potency in the Ames test of impurity X and structurally related compounds are unknown. [This observation may have some generic importance with regard to the assessment of mutagenic impurities in pharmaceuticals as discussed below]

Short overview of published mutagenicity studies of impurities

10. A brief overview of published studies is given at the end of this paper in tabular form. (This is based on a single PUBMED search for mutagenic impurities dating back to around 1979) The information in the table is largely based on abstracts and is intended to give members some background information on the aspects of impurity mutagenicity testing that have been investigated to date. Most investigators have used a battery of *in vitro* and *in vivo* mutagenicity tests to assess the adequacy of purification procedures at

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removing mutagenic impurities. More detailed work involves isolating impurities and testing for mutagenicity. LECs can be set when a compound is isolated and tested separately. In one instance (carbendazim), investigators subsequently confirmed the LEC values for impurities by testing commercial batches of carbendazim.

Overview of published approaches suggested for the identification and regulation of mutagenic impurities in pharmaceuticals (Annex 2)

11. Four relevant references were identified and are appended as Annex 2. Of these references, the key publication is Müller et al Reg Tox Pharm, 44, 198-211, 2007. This publication has been summarised below and no specific comments have been made on the other references included in Annex 2.

Müller L et al 2007. A rationale for determining, testing, and controlling specific impurities in pharmaceuticals that possess potential for genotoxicity. *Regulatory Toxicology and Pharmacology*, 44, 198-211.

12. The basic approach is outlined in three steps listed on page 203;
a) classification into one of five classes based on structural aspects, mutagenicity data (published or generated for the impurity), carcinogenicity data (if available).

b) establish a qualification for impurities based upon classification.

c) establish acceptable limits of the impurity in the API, based on ADI (Allowable Daily Intake) and the TTC concept.

13. The five category classification includes both carcinogenicity and mutagenicity considerations. For this COM discussion paper, class 1 (genotoxic carcinogen impurities) have not been considered any further.

14. In deriving the strategy, the authors have incorporated the TTC (Threshold of Toxicological Concern) concept as a key element in the strategy for compounds where a non-threshold mechanism is considered unlikely. The COC considered TTC during its consideration of a revised guidance for risk assessment of carcinogens. The relevant extract from the COC guidance is appended at the end of this covering paper. The COC considered the main use of TTC would be for hazard ranking and prioritisation particularly for chemicals not subject to regulatory schemes.

15. The authors use the TTC (1.5 µg/person/day) to derive an Allowable Daily Intake (ADI) based on 12 months use of a pharmaceutical as being equivalent to life-time intake. [This term should not be confused with Acceptable Daily Intake which is an internationally agreed reference dose defined as 'Estimated maximum amount of an agent, expressed on a body mass basis, to which an individual in a (sub) population may be exposed daily over its lifetime without appreciable health risk',

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http://www.who.int/ipcs/publications/methods/harmonization/en/terminol_part-II.pdf). A definition of Allowable Daily Intake was not provided in the paper] The authors applied a stochastic mode of action approach (based on total cumulative dose) to derive Allowable Daily Intakes for ≤ 1 month (120 μg), >1-3 months (40 μg), >3-6 months (20 μg) and >6-12 month (10 μg) (table 1 page 202 of paper). This is then scaled according to concentration of contaminant in the API and the dose of API administered on a daily basis (c.f table 2 of the published paper).

16. A number of comments are made on the individual classes of compound as summarised below; (Summary in figure 2 of the published paper by Müller et al 2007, page 204.)

Class 2 Genotoxic but with unknown carcinogenic potential; *Class 2a* (sufficient evidence of a threshold mechanism) use NOAEL approach. *Class 2b* (without sufficient evidence of a threshold-mechanism) control using TTC approach.

Class 3 Alerting structure (unrelated to parent API) but with unknown mutagenic potential. If not tested control as per TTC approach (This proposal suggests that the TTC can be used as a guide as to whether to initiate consideration of the potential genotoxicity of an impurity). If mutagenicity data are to be generated, spiking tests can also be undertaken. Authors suggest 250 $\mu\text{g}/\text{plate}$ as an adequate upper dose to test (see comment above on this proposal in paragraph 9). If the impurity is isolated and tested, positive results would suggest regulation according to TTC. If not positive then the impurity could be controlled as an ordinary impurity.

Class 4 Alerting structure related to API. Authors suggest thorough testing of parent should be sufficient. Regulate as an ordinary impurity. (This proposal suggests that for this class there is no trigger to initiate the genotoxicity assessment of the impurity)

Class 5 No alerting structure or sufficient evidence of absence of genotoxicity. Regulate as an ordinary impurity. (This proposal suggests that for this class there is no trigger to initiate the genotoxicity assessment of the impurity)

17. The proposals outlined by Müller et al makes a short reference to the principles underlying ALARP in the introduction section to the paper (page 199), but the overriding principle of ALARP over the use of TTC is not stated. In the discussion section 4, page 208, the authors note that the staged approach to setting the Allowable Daily Intake with regard to *in vivo* mutagens, could allow concentrations of up to 1.2% genotoxic impurities in an API. This is inconsistent with an ALARP approach and is of potential public health concern (see arrangements introduced by EMEA para 17 and Annex 3). A number of comments are made in the discussion section of the Müller et al paper which indicate the need to balance regulatory control with progressing the development of medicines regulatory approval.

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EMA guideline on genotoxic impurities (Annex 3)

18. The European Medicines Agency published a guideline on genotoxic impurities which came into force on 1 January 2007. CPMP/SWP/5199/02 EMEA/CHMP/QWP/251344/2006. A copy is appended for members use as Annex 3. The EMEA guidance uses the TTC concept to guide decisions on acceptability of concentrations of genotoxic impurities in pharmaceuticals and in this regard reflects some of the rationale set out by Müller et al 2007. The EMEA guideline does make specific reference to the principle of ALARP but this is overridden by the TTC concept. The EMEA guidance allows for short term excursions above the TTC e.g. for treatment of life-threatening illnesses.

COM Questions

19. What are members views of the results published by Kenyon et al 2007 on the development of the LEC for mutagens in the Ames test? Can this analysis be used to draft generic guidance for the mutagenicity testing of impurities?

20. What are members views on the approach suggested by Müller et al 2007?

21. What are members views on the approach published by the EMEA?

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Brief overview of mutagenicity studies investigating impurities.

Compound	Impurity	Comment on Approach taken	Reference
1,3 Dichloropropene (Two stereo isomers, Z and E)	Oxidative impurities and secondary products. Pathway and identity of impurities known	Effect of stabilisers on mutagenicity of impurities formed by oxidation when used as nematocide investigated. Tests included ST TA100 and SOS repair in <i>E.coli</i> . Storage of isomers at 20°C for 10 weeks resulted in significantly increased mutagenicity in TA100. Overall authors question whether stabilisers work.	Eder Chem Res Tox, 2006, 19, 952-9. (paper reviewed)
Capsaicin	Unknown	Purification resolves mutagenicity in in-vitro tests (Ames CAs) and in rodent BM MN assay (six daily s.c injections and sampling 24h 48h post dose).	Proudlock Env Mol Mutagen, 2004, 44, 441-7. (Paper reviewed)
Cefotaxime	Specific impurity dimeric cefotaxime isolated	Not mutagenic in package of in-vitro in Salmonella and cytogenicity in CHO cells. .	Agarwal SK Int J of Tox, 2004, 23, 41-5. (Paper reviewed)
Benomyl/Carbendazim	Aminophenazine impurities; 2,3 diaminophenazine and 2-Amino-3-hydroxyphenazine.	The varying batch to batch positive with in Ames tests with carbendazim, only occurring at high dose levels (ca 5000 µg/plate) suggested a mutagenic impurity was present. Impurities isolated and tested in Ames tests. Detection limits in Ames tests (plate incorporation) 5-10 µg –S9. 0.025-0.05 µg +S-9. Authors undertook Ames tests with commercial batches with different levels of these two impurities to validate the LEC data. Authors undertook studies with benomyl, and metabolites of carbendazim, which were all negative.	Sarrif AM, Mut Res, 1994, 321, 43-56. (Paper reviewed)
Sodium dichloroacetate	Unknown	Pharmaceutical grade material tested giving negative results. Superseded earlier tests using preliminary grade material that gave positive results. (tests Ames, MLA, CAs, rodent BMMN)	Fox AW. Fund Appl Tox, 1996, 32, 87-95.
Triphenylmethane dyes	Unknown	Purification resolves mutagenicity (tests Ames, MLA, rodent BMMN and yeast D5 recombination)	Lin GH, J Appl Tox, 1992, 12, 267-74.
Hair dye HC Blue1	Unknown	Use of Ames tests and ³² P-postlabelling assays to direct HPLC separation of impurities.	Abu Shakra A, Mutat Res, 1991, 260, 377-85.
Malondialdehyde (MDA)	Unknown	Three methods of preparation were used to produce highly	Basu AK Carcinogenesis, 1983,

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		purified MDA which gave consistent but reportedly 'weak' mutagenicity in <i>Salmonella</i> . The authors conclude that previous suggestions that mutagenicity was due to an impurity were incorrect.	4, 331-3.
3-nitro-9-fluorene	Preliminary identification	HPLC/MS directed separation of mutagenic impurities using TA100.	Jin ZL, Cancer Lett, 15, 209-14.
Saccharin	Unknown. Impurities isolated (three organic solvent procedures) from one material prepared by manufacturing process and also boiled saccharin tested. 9 possible impurities.	Negative when tested at up to 2500 µg in <i>Salmonella typhimurium</i> strain.	Herbold B, Mutat Res, 1981, 90, 365-72.
Carbon black	Nitropyrene	Mutagenic activity independent in samples derived from a range of xerographic hardware, but was dependent on manufacturing process used to produce carbon black. Extraction procedures used to identify nitropyrenes	Rosenberg HS, Science, 1980, 1039-43.
Beta-Adrenergic blocking agents Zani 1305, Zani 1327.	Epoxides	Positive in TA 1535, TA 100. (Process of identifying the epoxides not given).	Quinto I, Tox Lett, 1980, 109-14.
Rhodamine Dyes 6G and 6B	Unknown	Purification of rhodamine 6G resulted in loss of mutagenicity, whereas reduction did not occur with rhodamine 6b. Mutagenicity dependent on exogenous metabolic activation. Test used included Ames and ssDNA formation in CHO cells.	Nestmann ER, Cancer Res, 1979, 4412-7

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Extract from COC guidance 2004. Threshold of Regulation (TTC).

Threshold of Regulation

66. A 'threshold of regulation' has been proposed as a method for setting a regulatory exposure level, which will be associated with a minimal risk for carcinogenic substances. The FDA originally introduced this approach in order to reduce toxicological data requirements for indirect food additives (US FDA, 1995). More recently, JECFA have used a similar method to evaluate the safety of flavouring substances, excluding genotoxic carcinogens (Munro *et al* 1999). Recent workshops have attempted to develop the concept that a *de minimus* risk value (Threshold of Toxicological Concern (TTC)) could be identified for any chemical, including those of unknown toxicity, taking chemical structure into consideration (Barlow *et al* 2001; Kroes R *et al* 2004).

67. It has been proposed to further extend the TTC, by defining a common exposure level for any unstudied chemical (regardless of its chemical class) that will not pose a risk of significant carcinogenicity (Barlow *et al* 2001, Kroes *et al* 2000, 2004, Cheeseman *et al* 1999). One proposal was derived from an evaluation (involving linear extrapolation from the TD50 down to 10⁻⁶ excess cancer risk) of substances in the Gold Carcinogen Potency Database (Gold *et al* 1984; 1999).

68. The threshold of regulation concept for regulatory purposes is a relatively new approach and the Committee felt that, careful consideration was needed of the biological, analytical and mathematical issues as well as a much wider database for validation. The Committee consider that it should not currently be used as a generic approach, as the proposed exclusions covered some important classes of genotoxic carcinogens (such as aflatoxin-like compounds, azoxy compounds and *N*-nitroso compounds) and a number of classes of other carcinogens, such as heavy metals and TCDD (Kroes *et al* 2004). However, as it is based on ranking by theoretical risk and exposure the Committee agree that it could be used, along with hazard identification and characterisation data, for prioritisation of chemicals, particularly for chemicals that are not subject to regulatory approval schemes.