COMMENTS TO CLH REPORT FOR

PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

BASED ON REGULATION (EC) Nº 1272/2008 (CLP REGULATION)

SUBSTANCE NAME: ANTHRAQUINONE

EC Nº: 201-549-0

CAS Nº: 84-65-1

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Dossier prepared by BAuA reviews all available data on mutagenicity and carcinogenicity studies for the substance and its main metabolites (1-hydroxyanthraquinone and 2-hydroxyanthraquinone) and a known impurity of certain quality of anthraquinone, 9-nitroanthracene.

BAuA report states that purified AQ was clearly negative for bacterial gene mutation assays (Pag. 35 of BAuA report) and mammalian cell cultures (pag. 37 of BAuA report). AQ also showed negative results for a large battery of genotoxicity studies *in vitro* and *in vivo*, in bacterial, mammal cells mutagenicity and chromosomal aberration test. There is just one positive Ames study where the test item is known to contain the impurity 9-nitroanthracene (9-NA).

Some hypothesis on the influence of metabolites (1-OH AQ and 2-OH AQ) have been done by different authors and BAuA as well, not coming up with a robust conclusion in the light on genotoxicity data on these chemicals.

9-nitroanthracene has shown positive output for Ames test with S9 (Butterworth 2004) and with and without S9 (NTP 2005); equivocal in OECD 476 with S9 (Butterworth 2004 and weakly positive (Durant et al 1996).

All available data for AQ and metabolites taking into account the manufacturing origin is summarised in DOC1.

The decision on classification of anthraquinone as carcinogen 1B is solely supported by two carcinogenicity studies conducted by NTP in rat and mice (pag. 7 of BAuA report). Those studies have been challenged by the nature of the test item that has been demonstrated to contain not just 9-NA but also other relevant impurities.

This report comments upon BAuA report and would like to highlight the following facts:

1. Lack of data on 9-NA:

The potential hazard of 9-NA was solely assessed by *in vitro* mutagenicity test (OECD 471 and 476).

No data is available for *in vivo* test for 9-nitroanthracene. Fu et al found out different metabolism of 9-NA depending on test conditions (aerobic or anaerobic). It may impact in different outputs for *in vitro* and *in vivo* test.

No data is available for chromosome aberration for 9-nitroanthracene in mammal cells (neither *in vitro* nor *in vivo*). This mechanism of action was not investigated in mammals.

Furthermore Dihl et al (2008) investigated 9-NA for genotoxicity in the wing somatic mutation and recombination test (SMART) of Drosophila. Results demonstrated that 9-NA induced genetic toxicity causing increased incidence (75%) of homologous somatic

recombinations. The evidence indicating that the major effect observed in this study is an increased frequency of mitotic recombination emphasizes another hazard that could be associated to NPAHs--the increment in homologous recombination (HR).

2. Metabolites 1-OH AQ and 2-OH AQ

BAuA report postulates that these metabolites may be responsible for the carcinogenetic effects seen in NTP studies. These are certainly main metabolites of AQ. However Graves et al.(2005) found out quantitative differences on the production of these metabolites depending on the origin of AQ. A metabolism study was conducted using male Fischer 344 rats in which they were fed formulations of 4 lots of anthraquinone, produced by three different synthetic routes: (AQ-OX, AQ-DA and AQ-FC). 1 and 2-hydroxyanthraquinone and anthraquinone were found in all samples from the dosed animals, with 2- hydroxyanthraquinone ten folds the concentration of 1-hydroxyanthraquinone. **Both metabolites were found to be 65% less concentrated in AQ-FC than in AQ.OX**. Original report is attached to this comment (DOC 2).

3. Purity of NTP samples

It is clearly stated that AQ impurities are different depending on the manufacture origin. Some of these impurities may be relevant to genotoxicity effect.
Butterworth et al. demonstrated that NTP studies were carried out with AQ containing impurities. BAuA report refers to 9-NA which has been reported to be at 0.1%.

Furthermore Chemical Products Corporation send a comments letter to NTP in 2004 (DOC 3) concerned by the fact that *Technical Report 494 of NTP itself recognized the presence of 0.1% mutagenic 9-Nitroanthracene in the test material, but also states on page 31 that high performance liquid chromatography/ultraviolet detection analysis indicates that the TR494* ***test material may contain up to 0.5% contaminants****.*

An analysis performed by Arkion Life Sciences ***indicates 0.6% contaminants*** *in an aliquot of the TR494 test material; all of the contaminants detected in the Arkion Life Sciences analysis could be expected to confound the results of the TR494 studies. Draft TR494 does not identify any contaminants other than 0.1% 9-Nitroanthracene. The quantity and identity of the remaining contaminants in the TR494 test material is uncertain.* (DOC 3)

Chemical Products Corporation' letter to NTP reads*: "CPC dissolved an aliquot of TR494 test material in concentrated sulfuric acid, and then reprecipitated it (the long-standing gravimetric technique for determining Anthraquinone purity).* ***The reprecipitated material contained no detectable 9-Nitroanthracene****, yet this refined TR494* ***test material still retained mutagenic activity****. CPC provided this information to NTP in October 2000. CPC found evidence that there were impurities present in the TR494 test material as discrete particulates. These may or may not be uniformly distributed in each*

aliquot; CPC reported to NTP in late 2000 that the refined aliquot of the NTP test material exhibited a dark gray color as opposed to the pale yellow color of refined Anthraquinone. NTP does not present a comprehensive characterization of the identities and distributions of impurities in the TR494 test material.

BAuA report states "The low exposure level, the bioavailability, and the weak mutagenicity make it unlikely and implausible that 9-NA was solely and totally responsible for the carcinogenic response" (page 55). We would add that metabolites 1-OH AQ and 2-OH AQ would also not be solely responsible too in the light of genotoxicity available test. CPC analytics demonstrated the mutagenicity activity of the test item after extraction of 9-NA but other impurities still remained.

This is extremely important point taking into account that proposal of classification is just supported by theses test, which has been demonstrated to be done with contaminated test item.

4. Some controversial affirmations

In the report there are some points which are controversial by being contradictory and sometimes biased explanation of the facts.

a) Genotoxicity test for AQ are negative both *in vitro* and *in vivo* (with purified item). However in page 55 the report reads: *"Hence, AQ has the potential to act through a mechanism involving mutagenicity, and 9-NA is not a necessary component of this action."*

b) There is any data on carcinogenicity for either 9-Na nor *2-OH-AQ. However in* page 55 reads: *"The NTP concluded that 2-OH-AQ is an in situ metabolite of AQ that, based on experimental mutagenicity data, is as likely to be of comparable carcinogenicity or (with higher probability) more carcinogenic than 9-NA.* " Furthermore 2-OH-AQ is mutagenicity is only based in Ames test.

c) Page 54: *"Moreover, measurements in the male rat urine showed, that 2-OH-AQ is systemically present at several-fold higher amounts than it is theoretically possible for the 0.1% 9-NA contamination, even if the latter was 100% bioavailable (NTP 2005, p. 92 table 23)."* It is assumed that 2-OH-AQ is a metabolite of 9-NA which is quite surprising (see Fu et al 1985 for 9-NA metabolites identification).

   However it links with Graves et al (2005) findings with respect to a difference of 65% less 2-OH-AQ in AQ FC than in AQ OX which seems to be the AQ quality used in NTP test. This seems to be one more piece of evidence of diverse effects of different AQ origins.

d) BAuA recognises that no carcinogenicity test is available for pure AQ in page 54: "In fact, it should be taken into account further that carcinogenicity has not been examined for 100% pure AQ or AQ-DA or AQ-FC. Thus, it is to conclude

that AQ as tested in the NTP studies (containing 0.1% 9-NA) was carcinogenic. A possible contribution of the impurity cannot be assessed."
This last may be true since no data for 9-NA is available, as stated in point 1 of this report; following this approach it is also true that a possible contribution of AQ cannot be assessed as well, due to the demonstrated composition of the tested sample with plausible confounding effects from co-exposure to other chemicals.
Therefore the principle for classification *"a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms"* is not meet.

e) Comparison with CLP criteria.
First point is not meet: "*two or more species of animals <u>or in two or more independent studies</u> in one species carried out at different times <u>or in different laboratories</u> or under different protocols*"; Studies are not independent since the sample is the same and also the laboratory.
However it seems to mach pretty much with some criteria for category 2, if any: "*(b) there are unresolved questions regarding the adequacy of the design, conduct or interpretation of the studies;* "

5. Mode of action

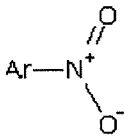
As a result of the output of carcinogenicity studies conducted by NTP, different authors have been trying to find out a plausible explanation and elucidate the mode of action of AQ in carcinogenesis. BAuA report also intends to do so.

Genotoxicity test are negative both *in vitro* and *in vivo* (with purified item). Therefore no genotoxic mode of action can be postulated. However in page 55 the report reads: *"Hence, AQ has the potential to act through a mechanism involving mutagenicity, and 9-NA is not a necessary component of this action."*

Page 55 reads. *"In addition, the lack of activity in mutagenicity assays does not give proof of non-carcinogenicity of AQ, because <u>carcinogenicity of AQ could also be mediated by other mechanisms</u>."*. It is well known that carcinogenicity may be due to epigenetics. However there is a piece of information to take into account that is contradictory with this hypothesis: *a negative output for* A mammalian cell transformation test in Chang (human liver) cells and BHK-21 C13 (baby Syrian hamster kidney)

6. **Analogues.**

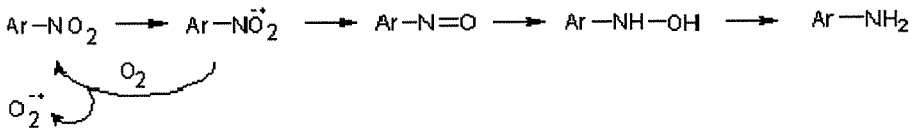
BAuA report mentions in page 55 results for so-called analogues reported in NTP 2005 report. Those chemicals are halogen, amino or nitro substituted which are known alert structures. Nelson et al (1982) explained the structure-activity relationship for nitro-aromatic compounds.



Ar = Any aromatic/heteroaromatic ring

- Chemicals with ortho-disubstitution, or with an ortho carboxylic acid substituent are excluded.
- Chemicals with a sulfonic acid group (-SO3H) on the same ring of the nitro group are excluded.

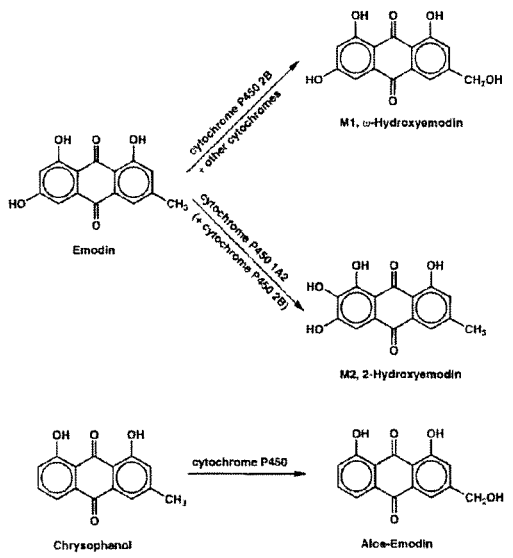
Metabolic activation of nitroaromatic compounds to toxic metabolites, involve initial one-electron reduction of the nitro group to yield a resonance-stabilized nitro anion radical. Under aerobic conditions, the radical can reduce molecular oxygen to form superoxide anion, which can generate various toxic and DNA-reactive oxygen species. In a more anaerobic environment, the nitro anion radical can be reduced further to nitroso, hydroxylamine, and amine (Nelson 1982). Further activation of hydroxylamine produces the reactive nitrenium ions (see SA25 and SA28).



Therefore should not be considered valid analogues. However these is used as a rationale for third criteria of CLP by BAuA: "*In additional considerations there are structurally related substances (from the NTP data base) with shown carcinogenic potential as well as a possible contribution of mutagenicity (in particular of metabolites) as a mode of action.* "

However the BAuA report also mentions emodin ((1,3,8-trihydroxy-6-methylanthraquinone) and we would also add chrysophanol (1,8-dihydroxy-3-methylanthraquinone) as analogues.

Metabolites of emodin are v-hydroxyemodin and 2-hydroxyemodin (Mueller 1998; Murakami 1987; Masuda, 1984) and chrysophanol is transformed, in a cytochrome P450-dependent oxidation, to aloe-emodin (1,8-dihydroxy-3-hydroxymethylanthraquinone) as the major product formed (Stephan et al. 1998).



Metabolic pathway of emodin and chrysophanol (from Stephan et al. 1998)

Summary of Avaliable data for Emodin (see all details in DOC 1. Point 4)

- Genetic toxicity in vitro: Chromosomal aberrations in mammalian cells:

NTP (National Toxicology Programme) (1991). Test method equivalent to OECD guideline 473.

Chromosome aberrations were induced in cultured CHO cells in the absence of S9 activation and in the presence of S9; the response observed without S9 was stronger than with S9.

- Genetic toxicity in vitro: Mammalian cell micronucleus test:

Kevekordes S, Spielberger J, Burghaus CM, Birkenkamp P, Zietz B, (2001): Test method equivalent to OECD guideline 487.

The test substance did not reveal any micronuclei inducing activity in either human lymphocytes or in Hep-G2.

- Genetic toxicity in vitro: Gene mutation in mammalian cells:

1. - Müller SO, Eckert I, Lutz WK and Stopper H (1996): Test method equivalent to OECD guideline 476.

The substance induced a moderate increase in mutant fraction (it was only tested without metabolic activation).

2. - Westendorf J, Marquardt H, Poginsky B, Dominiak M, Schmidt J and Marquardt (1990): Test method equivalent to OECD guideline 476.

Treatment with the test substance did not result in an increase in the number of colonies resistant to 6 -TG. Exposure in suspension in the presence of liver homogenate was also negative.

3. - Bruggeman IM and van der Hoeven JCM (1984): Test method equivalent to OECD guideline 476.

In the V79-HGPRT mutation assay, the test substance emodin was highly mutagenic without metabolic activation. However, the increase in the induction of mutation was observed at highly toxic concentrations. Furthermore, a low plating efficiency was observed in the negative and positive controls.

- Genetic toxicity in vivo: Mammalian erythrocyte Micronucleus test:

1. - Mengs U, Krumbiegel G and Völkner W (1997): Test according to OECD guideline 474.

There was no statistically significant enhancement in the frequency of micronucleated PCEs in comparison to the negative controls at both preparation intervals.

2. - NTP (National Toxicology Programme) (1992): Test method equivalent to OECD guideline 474.

In peripheral blood samples from mice in the 14-week feed study, an increase in the frequency of micronucleated NCEs was seen in females, but not in males. The small increase in NCEs observed in the female mice was statistically significant (P=0.001), but no individual exposed group value differed significantly from the control value; the result in female mice was concluded to be weakly positive.

- Genetic toxicity in vivo: Mammalian bone marrow chromosome aberration test:

1. - NTP (National Toxicology Programme) (1993a): Test method equivalent to OECD guideline 475.

No increases in the frequencies of micronucleated erythrocytes were observed in any of the treatment groups (only males were used).

2. - NTP (National Toxicology Programme) (1993b); Witt KL, Knapton A, Wehr CM, Hook GJ, Mirsalis J, Shelby MD and MacGregor (2000): Test method equivalent to OECD guideline 475.

No increases in the frequencies of micronucleated erythrocytes were observed in any of the treatment groups (males and females were used).

- Carcinogenicity

NTP (National Toxicology Programme) (2001): Test method equivalent to OECD guideline 451. GLP study. Tested in rats and mice.

Rats: There was no evidence of carcinogenic activity of emodin in male rats. There was equivocal evidence of carcinogenic activity in female rats.

Mice: There was equivocal evidence of carcinogenic activity in male mice. There was no evidence of carcinogenic activity in female mice.

CONCLUSIONS

- Proposal for classification is solely based on NTP carcinogenicity test. It has been demonstrated that NTP test item was not pure AQ and therefore tests results are not matching with all previous mutagenicity test. No evidence for mutagenicity was found for AQ in *vitro* or *in vivo*.
- Those tests should be withdrawn and considered not valid for AQ assessment.
- Mechanism for carcinogenicity has not been elucidated; this is the result of intending create a link between all available data on AQ with NTP test. Pattern of effects seen in NTP studies seems to be done by a direct acting mutagen that AQ demonstrated no potential for.
- Further investigation is required before proceeding with decision on classification of AQ.

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