Expert Review: **Comments on the conclusion regarding a mutagenic mode-of-action (MoA) for the liver tumorigenicity of methyl ethyl ketoxime (butanone oxime) in rats**

Reviewer: Wolfgang Dekant, Dr. rer. nat.; Professor of Toxicology

Rhönstrasse 9, 97080 Würzburg

e-mail: dekant@toxi.uni-wuerzburg.de

Sponsor: **Honeywell International Inc.**
101 Columbia Road
Morristown, NJ 07960
USA

Aug. 8, 2012

W. Dekant, Professor of Toxicology Date

Table of contents

1. Summary 2
2. Introduction 2
3. Comments 3
4. Conclusions 6
5. References 6

1. Summary

Butanone oxime (methyl ethyl ketoxime, MEKO) causes liver tumors in rats and mice after longterm inhalation exposures. In a position paper titled “Positionspapier des AK CM im UAIII”, it is postulated that genotoxicity is involved in the tumorigenicity of MEKO to the liver based on a mode-of-action (MoA) derived for the liver carcinogenicity of 2-nitropropane. The MoA for 2-nitropropane is thought to involve formation of a reactive sulfate conjugate which aminates DNA. In agreement with this MoA, 2-nitropropane is genotoxic in mammalian cells expressing sulfotransferases. MEKO has been tested regarding genotoxicity in a variety of systems including rat hepatocytes and an *in vivo* peripheral blood micronucleus test with an administration period of 90 days with consistently negative results. The “Positionspapier des AK CM im UAIII” proposes that the negative genotoxicity of MEKO is due to the absent capacity of the *in vitro* systems to simulate the specific metabolic activation pathway developed for 2-nitropropane and the short half-life of the reactive sulfate intermediates formed in the liver, which may not reach other target organs. 2-Nitropropane also does not consistently cause DNA-damage in bone marrow after single administration. However, the MoA developed for 2-nitroproane has a number of inconsistencies. Since primary rat hepatocytes have the capacity to simulate the necessary bioactivation reactions postulated for MEKO, MEKO would be expected to induce DNA-damage in this system. Moreover, the postulated reactive MEKO-metabolite, 2-butanone oxime sulfate, is not mutagenic. The negative *in vivo* micronucleus study administered MEKO in high doses for up to 90 days also supports absence of genotoxicity since 2-butanone oxime sulfate is chemically stable in aqueous solution and should reach the bone marrow when formed in the liver. Therefore, a potential genotoxic and DNA-damaging activity of MEKO should have been revealed by genotoxicity testing in primary rat hepatocytes and by the administration scheme selected for the *in vivo* micronucleus test. Therefore, the negative genotoxicity studies with MEKO do not support the postulated genotoxic MoA and support a major contribution of repeated liver injury in the tumorigenicity of MEKO to rodent liver.

2. Introduction

The draft of the “Positionspapier des AK CM im UAIII” titled “AGW-Ableitung und ERB-Begründung zu Butanonoxim” (dated May 30, 2012) describes the toxicity of butanone oxime (methyl ethyl ketoxime, MEKO) and derives an occupational exposure limit for MEKO (Anonymous, 2012). Lifetime inhalation exposure of rats and mice to MEKO by inhalation to concentrations up to 0, 15, 75, and 375 ppm induced hepatocelluar adenoma and carcinoma with males being more sensitive as compared to females in both species (Newton *et al.*, 2001). The draft document concludes that MEKO induced liver tumors in rodents are initiated by an interaction of (a) MEKO-metabolite(s) with nucleic acids and thus a genotoxic mode-of-action (MoA); cytotoxic effects of MEKO enhance tumor susceptibility due to the non-tumor associated pathologic changes seen in the liver of MEKO exposed rodents (Anonymous, 2012).

Since, as correctly described in the document, MEKO is not genotoxic in most of the standard genotoxicity assays including bacterial mutagenicity, unscheduled DNA-synthesis in primary rat

hepatocytes, and micronucleus tests in rats and in mice, the draft document supports the argument of a genotoxic MoA with observations made regarding the MoA postulated for structurally related compounds (2-nitropropane and other sec. nitroalkanes). These compounds cause DNA-damage in rat liver *in vivo* and have genotoxic properties in several *in vitro* systems (Fiala *et al.*, 1987; Fiala *et al.*, 1989; George *et al.*, 1989; Conaway *et al.*, 1991a; Conaway *et al.*, 1991b; Haas-Jobelius *et al.*, 1991; Fiala *et al.*, 1993; Fiala *et al.*, 1995; Deng *et al.*, 1997).

However, the database on the MoA derived for 2-nitropropane and the extrapolation of this MoA to MEKO has several inconsistencies. Therefore, Honeywell has asked me to provide comments regarding the conclusions of a mutagenic MoA for MEKO based on the MoA information and the available genotoxicity data on MEKO.

3. Comments

The “Positionspapier” bases its conclusions on a mutagenic mode of action for MEKO mainly on proposed mechanisms of biotransformation and DNA-interaction for 2-nitropropane and some data on the MEKO-analogue 2-propanone oxime. As MEKO, these compounds induce tumors in rodent liver with higher incidences in male animals as compared to female animals at identical exposure conditions (Mirvish *et al.*, 1982; NTP, 2002). For 2-nitropropane, an activation pathway involving sulfotransferases to give acetoxime sulfate is proposed based on the genotoxicity of 2-nitropropane in mammalian cells containing sulfotransferases and the structure of DNA-modifications observed in the liver of animals administered 2-nitropropane (Sodum *et al.*, 1993; Sodum *et al.*, 1994; Sodum and Fiala, 1997; Kreis *et al.*, 1998; Sodum and Fiala, 1998; Kreis *et al.*, 2000). The ultimate reactive metabolite postulated in the pathway is hydroxylamine O-sulfate, which aminates/oxidizes nucleosides through a nitrenium ion intermediate. Hydroxylamine O-sulfate is a presumed product of the hydrolysis of acetoxime O-sulfate formed postulated to be formed from 2-nitropropane/propane 2-nitronate by sulfate conjugation. However, the need for a reduction of an initial sulfate conjugate of propane 2-nitronate to acetoxime O-sulfate or the chemistry of a reaction of a sulfate conjugate of propane 2-nitronate with DNA-bases to aminated DNA-bases are not mechanistically explained and thus are questionable.

Extrapolation of this information to MEKO requires either oxidation of MEKO to the corresponding 2-nitrobutane which is in equilibrium with butane 2-nitronate. Following this oxidation, a conjugation of 2-nitrobutane/butane 2-nitronate to give MEKO-sulfate and DNA-amination by MEKO sulfate should occur. Alternatively, MEKO could be directly conjugated with sulfate (Fig. 1). Oxidation of MEKO to 2-nitrobutane has been demonstrated to occur in liver microsomal preparation (Volkel *et al.*, 1999), but likely is only a minor pathway of MEKO-biotransformation in rats *in vivo*. In rats, hydrolysis of MEKO to 2-butanone is the major pathway of biotransformation, and 2-butanone is further catabolized to CO_2 (Burka *et al.*, 1998).

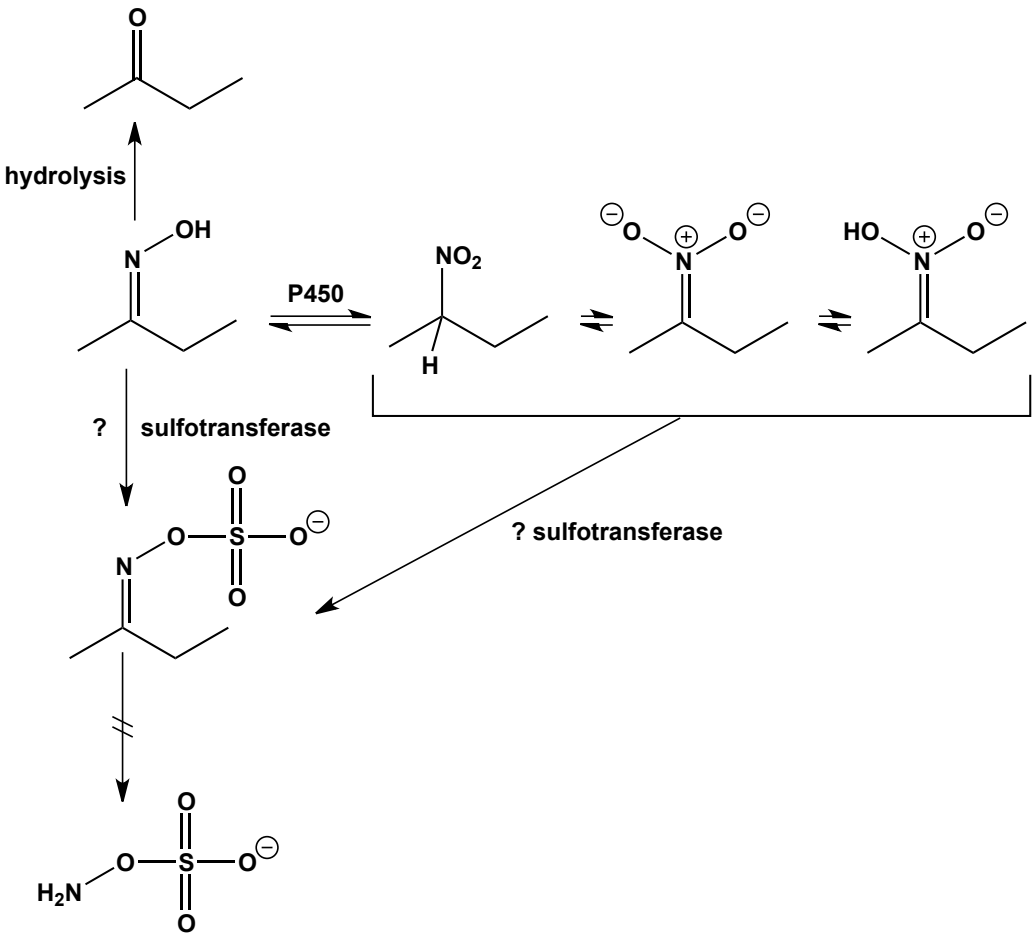

Fig. 1: Conceivable pathways of biotransformation of MEKO in analogy to scheme 1 presented by AKCM.

As outlined in chapter 9 “Vorherrschendes Wirkprinzip der Kanzerogenität” of the “Positionspapier des AK CM im UAIII” (Anonymous, 2012), there is support for this pathway to occur with 2-nitropropane based on

- the selective DNA-damage by 2-nitropropane/propane 2-nitronate in V79 cells expressing sulfotransferases (Kreis *et al.*, 1998; Kreis *et al.*, 2000),
- formation of DNA-modifications (8-amino- and 8-oxo-guanosine) from 2-nitropropane in the presence of sulfotransferases *in vitro* (Sodum *et al.*, 1993; Sodum *et al.*, 1994; Sodum and Fiala, 1998),
- identification of 8-amino-guanosine, N_2-aminoguanosine, and increased concentrations of 8-oxo-guanosine in liver DNA of rats given 2-nitropropane/acetoxime by intraperitoneal injection (Hussain *et al.*, 1990; Sodum and Fiala, 1998).
- Oxidation of MEKO and acetoxime to 2-nitrobutane/2-nitropropane by rat liver microsomes at low rates (Kohl *et al.*, 1992; Volkel *et al.*, 1999)

However, a number of other observations question the relevance of this pathway for MEKO:

- MEKO and acetoxime are not substrates for sulfotransferases from rat liver (Honeywell, 2000)
- MEKO sulfate and acetoxime sulfates are chemically stable in aqueous solution (Honeywell, 2000)
- MEKO and acetoxime sulfate have little reactivity with DNA *in vitro* as compared to hydroxylamine O-sulfate. Indeed, the observed modifications of DNA with MEKO sulfate

and acetoxime sulfate may be due to residual hydroxylamine O-sulfate in the synthetic products used for the study (Honeywell, 2000)

- Single MEKO inhalation exposures of rats at concentrations up to 1,000 ppm for 6 h does not form 8-amino-deoxyguanosine or 8-oxo-deoxyguanosine in liver DNA of male or female rats.
- 2-Nitropropane/propane 2-nitronate is mutagenic in bacteria without metabolic activation suggesting that other pathways of biotransformation than sulfate conjugation may contribute to genotoxicity (Kohl *et al.*, 1995; Kohl and Gescher, 1996; Kohl and Gescher, 1997).
- The genotoxicity of 2-nitropropane is increased by oxidative biotransformation possibly involving radical metabolites and acetoxime-induced DNA-damage may be mediated by metal ions (Sakano *et al.*, 2001)
- Nitroreduction apparently is not involved in the genotoxicity of 2-nitropropane (Haas-Jobelius *et al.*, 1991)

Considering these contradictory observations, it remains unclear if the MoA postulated for MEKO in the “Positionspapier des AK CM im UAIII” actually occurs. Moreover, when considering all the available data from MEKO mutagenicity and genotoxicity testing summarized in the “Positionspapier des AK CM im UAIII”, the information is insufficient to conclude that MEKO has a mutagenic MoA regarding liver tumor induction.

As correctly outlined in the “Positionspapier des AK CM im UAIII”, a weight of evidence approach regarding MEKO-mutagenicity results in the conclusion that MEKO is not mutagenic. The available test results show that MEKO does not induce mutations in Salomonella (neither with nor without metabolic activation), other changes in endpoints indicative of genotoxicity in mammalian cells, or bone marrow micronuclei or chromosome aberrations in rodents.

The “Positionspapier des AK CM im UAIII” argues that the structurally related 2-nitropropane also does not show mutagenic responses in many of these systems. It is also argued that the capacity for performing the metabolic reactions resulting in MEKO bioactivation to DNA-aminating species are not effectively simulated by the *in vitro* systems applied and that reactive metabolites formed from MEKO in the liver *in vivo* may not reach the bone marrow.

However, a closer examination of the MEKO-responses observed, the metabolic capacities of the systems used, and the postulated bioactivation mechanisms does not support these conclusions:

- 2-Nitropropane is mutagenic in Salmonella (Conaway *et al.*, 1991a; Kohl *et al.*, 1994) while MEKO is negative. However, MEKO is oxididized to the analog 2-nitrobutane in rat liver microsomes at low rates suggesting that the metabolic capacity for MEKO-oxidation is present in the S9-fractions used in the bacterial mutagenicity testing (Volkel *et al.*, 1999). Apparently, the oxidative biotransformation of MEKO is of little relevance regarding mutagenicity in the presence of S9. Either the formed 2-nitrobutane is rapidly reduced back to MEKO by cytosolic enzymes present or rapidly oxidized to give 2-butanone and nitric oxide (Caro *et al.*, 2001).
- MEKO and acetoxime (Haas-Jobelius *et al.*, 1991) do not induce DNA-damage in primary rat hepatocytes. However, primary rat hepatocytes have the metabolic capacities to bioactivate MEKO and acetoxime by oxidation and sulfotransferease along the pathways postulated; moreover, 2-nitropropane induces DNA-repair in rat hepatocytes (George *et al.*, 1989; Davies *et al.*, 1993; Fiala *et al.*, 1995). The negative results regarding MEKO-induced DNA-damage in primary rat hepatocytes strongly argue against the MoA outlined above.
- The “Positionspapier des AK CM im UAIII” proposes that 2-nitropropane is negative in the rodent bone marrow micronucleus test since the target tissue does not have the capacity to

bioactivate 2-nitropropane and reactive metabolites formed from 2-nitropropane in the liver may not reach the bone marrow. Therefore, the negative bone marrow and peripheral blood micronucleus test with MEKO in mice using a period of administration for 13 weeks with drinking water (doses up to several hundred mg/kg bw/day) does not support the conclusion that MEKO is not genotoxic. In addition, negative bone marrow micronucleus results are also observed with 2-nitropropane supporting the conclusion that reactive metabolites formed in the liver may not reach the bone marrow. However, it needs to be considered that the studies available for 2-nitropropane only applied single doses and that doses applied were limited by toxicity and lethality (George *et al.*, 1989). Moreover, the US EPA concludes that an slightly increased frequency of bone marrow micronuclei was present in the highest dose in the pivotal study for 2-nitropropane and that the results are "ambiguous" (EPA, 2011), 2-nitropropane was also reported to induce DNA-damage in bone marrow (Deng *et al.*, 1997; Loft *et al.*, 1998). In addition, the discussed reactive sulfate intermediates formed from MEKO by the bioactivation sequences (Fig. 1) are chemically stable and have a low molecular weight (Honeywell, 2000). Therefore, they likely are translocated from the liver to the systemic circulation and may reach the bone marrow. Since the available study on bone marrow and peripheral blood used longterm administration of high MEKO doses in a sensitive species, based on the arguments made above, a genotoxic activity of MEKO should have been observable.

4. Conclusions

Based on the consistently negative genotoxicity testing with MEKO, the metabolic capacities of the systems used to assess MEKO (primary rat hepatocytes and intact animals after longterm MEKO-administration), and the inconsistent results with sec. nitroalkanes regarding MoA, a weight-of-evidence approach does not support the conclusion that MEKO is genotoxic and that genotoxicity contributes to the carcinogenicity of MEKO in rodents. Essentially, the MoA for MEKO regarding induction of liver tumors is unknown, but the results of the genotoxicity testing including many different endpoints support the absence of a direct interaction of MEKO and its metabolites with DNA.

5. References

Anonymous, 2012. AGW Ableitung und ERB-Begründung zu Butanonoxim (Stand 30.5.2012 - Entwurf).

Burka, L.T., Black, S.R., Mathews, J.M., 1998. Disposition of methyl ethyl ketoxime in the rat after oral, intravenous and dermal administration. Xenobiotica **28**, 1005-1015.

Caro, A.A., Cederbaum, A.I., Stoyanovsky, D.A., 2001. Oxidation of the ketoxime acetoxime to nitric oxide by oxygen radical-generating systems. Nitric Oxide **5**, 413-424.

Conaway, C.C., Hussain, N.S., Way, B.M., Fiala, E.S., 1991a. Evaluation of secondary nitroalkanes, their nitronates, primary nitroalkanes, nitrocarbinols, and other aliphatic nitro compounds in the Ames Salmonella assay. Mutation Research **261**, 197-207.

Conaway, C.C., Nie, G., Hussain, N.S., Fiala, E.S., 1991b. Comparison of oxidative damage to rat liver DNA and RNA by primary nitroalkanes, secondary nitroalkanes, cyclopentanone oxime, and related compounds. Cancer Research **51**, 3143-3147.

Davies, J.E., Mynett, K., Gescher, A., Chipman, J.K., 1993. DNA modification and repair by 2-nitropropane is extensive in hepatocytes of rats compared to those of humans and mice. Mutation Research **287**, 157-164.

Deng, X.S., Tuo, J., Poulsen, H.E., Loft, S., 1997. 2-Nitropropane-induced DNA damage in rat bone marrow. Mutation Research **391**, 165-169.

EPA, 2011. Screening Level Hazard Characterization 2-Nitropropane. Hazard Characterization Document.

Fiala, E.S., Conaway, C.C., Biles, W.T., Johnson, B., 1987. Enhanced mutagenicity of 2-nitropropane nitronate with respect to 2-nitropropane--possible involvement of free radical species. Mutation Research **179**, 15-22.

Fiala, E.S., Conaway, C.C., Mathis, J.E., 1989. Oxidative DNA and RNA damage in the livers of Sprague-Dawley rats treated with the hepatocarcinogen 2-nitropropane. Cancer Research **49**, 5518-5522.

Fiala, E.S., Nie, G., Sodum, R., Conaway, C.C., Sohn, O.S., 1993. 2-Nitropropane-induced liver DNA and RNA base modifications: differences between Sprague-Dawley rats and New Zealand white rabbits. Cancer Letters **74**, 9-14.

Fiala, E.S., Sodum, R.S., Hussain, N.S., Rivenson, A., Dolan, L., 1995. Secondary nitroalkanes: induction of DNA repair in rat hepatocytes, activation by aryl sulfotransferase and hepatocarcinogenicity of 2-nitrobutane and 3-nitropentane in male F344 rats. Toxicology **99**, 89-97.

George, E., Burlinson, B., Gatehouse, D., 1989. Genotoxicity of 1- and 2-nitropropane in the rat. Carcinogenesis **10**, 2329-2334.

Haas-Jobelius, M., Ziegler-Skylakakis, K., Andrae, U., 1991. Nitroreduction is not involved in the genotoxicity of 2-nitropropane in cultured mammalian cells. Mutagenesis **6**, 87-91.

Honeywell, 2000. Biotransformation, toxicokinetics and DNA-binding of methyl ethyl ketoxime and its metabolites. Summary of final report, Project: TOX-011F/MEKO MA-RR-00-2602.

Hussain, N.S., Conaway, C.C., Guo, N., Asaad, W., Fiala, E.S., 1990. Oxidative DNA and RNA damage in rat liver due to acetoxime: similarity to effects of 2-nitropropane. Carcinogenesis **11**, 1013-1016.

Kohl, C., Gescher, A., 1996. Catalysis of nitro-aci tautomerism of the genotoxicant 2-nitropropane by cytosol from rodent and human liver. Chemico-biological Interactions **99**, 219-226.

Kohl, C., Gescher, A., 1997. Denitrification of the genotoxicant 2-nitropropane: relationship to its mechanism of toxicity. Xenobiotica **27**, 843-852.

Kohl, C., Morgan, P., Gescher, A., 1995. Metabolism of the genotoxicant 2-nitropropane to a nitric oxide species. Chemico-biological Interactions **97**, 175-184.

Kohl, C., Mynett, K., Davies, J.E., Gescher, A., Chipman, J.K., 1994. Propane 2-nitronate is the major genotoxic form of 2-nitropropane. Mutation Research **321**, 65-72.

Kohl, C., Schiller, C.D., Gescher, A., Farmer, P.B., Bailey, E., 1992. Acetoxime is metabolized by human and rodent hepatic cytochrome P450 enzymes to the genotoxicant and carcinogen propane 2-nitronate. Carcinogenesis **13**, 1091-1094.

Kreis, P., Brandner, S., Coughtrie, M.W., Pabel, U., Meinl, W., Glatt, H., Andrae, U., 2000. Human phenol sulfotransferases hP-PST and hM-PST activate propane 2-nitronate to a genotoxicant. Carcinogenesis **21**, 295-299.

Kreis, P., Degen, G.H., Andrae, U., 1998. Sulfotransferase-mediated genotoxicity of propane 2-nitronate in cultured ovine seminal vesicle cells. Mutation research **413**, 69-81.

Loft, S., Deng, X.S., Tuo, J., Wellejus, A., Sorensen, M., Poulsen, H.E., 1998. Experimental study of oxidative DNA damage. Free Radic Res **29**, 525-539.

Mirvish, S.S., Salmasi, S., Runge, R.G., 1982. Carcinogenicity test of acetoxime in MRC-Wistar rats. Journal of the National Cancer Institute **69**, 961-962.

Newton, P.E., Wooding, W.L., Bolte, H.F., Derelanko, M.J., Hardisty, J.F., Rinehart, W.E., 2001. A chronic inhalation toxicity/oncogenicity study of methylethylketoxime in rats and mice. Inhal Toxicol **13**, 1093-1116.

NTP, 2002. 2-Nitropropane. Rep Carcinog **10**, 174-175.

Sakano, K., Oikawa, S., Murata, M., Hiraku, Y., Kojima, N., Kawanishi, S., 2001. Mechanism of metal-mediated DNA damage induced by metabolites of carcinogenic 2-nitropropane. Mutation Research **479**, 101-111.

Sodum, R.S., Fiala, E.S., 1997. Amination of tyrosine in liver cytosol protein of male F344 rats treated with 2-nitropropane, 2-nitrobutane, 3-nitropentane, or acetoxime. Chemical Research in Toxicology **10**, 1420-1426.

Sodum, R.S., Fiala, E.S., 1998. N2-amination of guanine to 2-hydrazinohypoxanthine, a novel in vivo nucleic acid modification produced by the hepatocarcinogen 2-nitropropane. Chemical Research in Toxicology **11**, 1453-1459.

Sodum, R.S., Nie, G., Fiala, E.S., 1993. 8-Aminoguanine: a base modification produced in rat liver nucleic acids by the hepatocarcinogen 2-nitropropane. Chemical Research in Toxicology **6**, 269-276.

Sodum, R.S., Sohn, O.S., Nie, G., Fiala, E.S., 1994. Activation of the liver carcinogen 2-nitropropane by aryl sulfotransferase. Chemical Research in Toxicology **7**, 344-351.

Volkel, W., Wolf, N., Derelanko, M., Dekant, W., 1999. Slow oxidation of acetoxime and methylethyl ketoxime to the corresponding nitronates and hydroxy nitronates by liver microsomes from rats, mice, and humans. Toxicol Sci **47**, 144-150.