STUDIES ON THE MUTAGENICITY AND TUMOR INDUCING POTENCY OF ARTIFICIAL FRAGRANCES

V Mersch-Sundermann[1*], M Emig[2], WQ Lu[3], H Hoelzke[2], R Gminski[4] and S Boehmer[2]

[1]Dept. of Indoor & Environmental Toxicology, Justus-Liebig-University, Giessen, Germany
[2]Institute of Microbiology and Hygiene, Faculty of Clinical Medicine Mannheim, University of Heidelberg, Mannheim, Germany
[3]Dept. of Environmental Health, Tongji Medical College of Huazhong University of Science and Technology, Wuhan, P. R. China
[4]Dept. of Ecotoxicology and Toxicology, University of Trier, Trier, Germany

ABSTRACT
5 Nitro musks and 6 polycyclic musks were studied for genotoxicity using Salmonella assay (*S.typhimurium* TA97, TA98, TA100, TA102), sister chromatid exchange (human lymphocytes), *in vitro* (human lymphocytes, human HepG2 cells) and *in vivo* micronucleus test (polychromatic erythrocytes in CD1 mouse bone marrow), single cell gel electrophoresis (HepG2 cells), cogenotoxicity assays with CD1 mice, Sprague Dawley (SD) rats and HepG2 cells, cytochrome P450 ELISA (CYP1A, 2B, 3A, 4A) and mouse skin I/P protocol for tumor promoting and co-initiating effects. Except musk ambrette none of the nitro musks exhibited direct mutagenicity. As the most important result we identified co-mutagenic and co-initiating effects of musk ketone in combination with benzo[a]pyrene (Bap) in SD rats (*ex vivo*), 7,12-DMBA in CD1 mice (*in vivo*), and Bap in HepG2 cells (*in vitro*) indicating that the combination of MK and polycyclic aromates (PAH) could cause a genetic risk. Polycyclic musks exhibited no genotoxic effects.

INDEX TERMS
Fragrances, Nitro musks, Musk ketone, Polycyclic musks, Genotoxicity

INTRODUCTION
At the end of the 19th century A.Baur (1891) discovered the group of nitro musks (NM) which consists of dinitro- and trinitro-substituted benzene derivatives (Liebl and Ehrenstorfer, 1993)(Figure 1). Although structurally different from compounds occurring in natural musk both the synthetic nitro musks and polycyclic musks possess fragrance properties similar to those of the natural compounds. Because of their low production costs and easy preparation artifical musks have been used in large amounts in the production of soaps, cosmetics, scents for indoor use, Indian joss sticks, laundry detergents etc. In 1996 the worldwide production of NM has been estimated to be approximately 2,000 t (Gebauer and Bouter, 1997). Among the group of NM musk xylene (MX) and musk ketone (MK) were the most important compounds [Use in the European Community (1996): 50 t musk xylene, 110 t musk ketone (Ford, 1998)]. During the last years a number of nitro musks, i.e. MX and MK, were banned in industrial countries because of their bioaccumulation and questionable (geno)toxic potency. Additionally, musk ambrette has been withdrawn by the fragrance industry due to its proven photo allergic activity in humans. Thus, since the 1990s nitro musks were replaced by polycyclic musks but nitro musks are still used in numerous products. Because of the fact that only a few toxicological data of these important compounds were available we studied the

* Contact author email: mersch@rumms.uni-mannheim.de

genotoxicity of nitro musks and polycyclic musks (Figure 1). In view of the fact that MK showed enzyme inducing activity further toxicological investigations about the combined effects of MK with promutagens/procarcinogens were carried out.

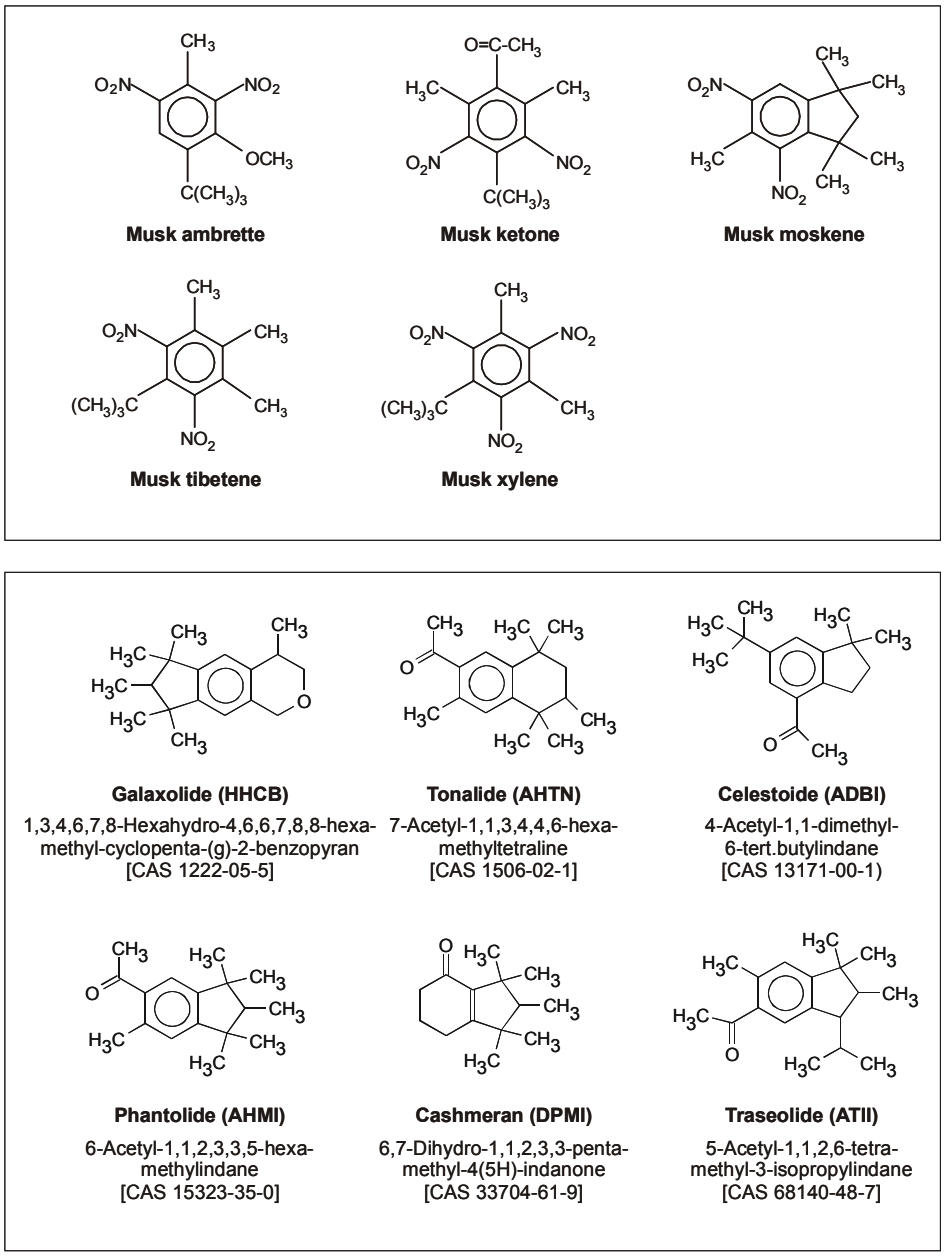

Figure 1. Chemical structures of the 5 nitro musks and 6 polycyclic musks tested for mutagenicity, genotoxicity and tumerogenicity

METHODS

The Amestest using *S.typhimurium* TA97, TA98, TA100 and TA102 with (+S9) and without (-S9) an exogenous metabolizing system obtained from liver fractions of Aroclor-1254 treated Spraque Dawley (SD) rats was carried out as described by Emig *et al.* (1996) and Mersch-Sundermann *et al.* (1998a). Additionally, the nitroreductases deficient strain TA 100 NR (+/-S9) was used to illuminate the mechanisms of mutagenicity of musk ambrette (Mersch-Sundermann and Emig, 1998). The SOS chromotest (DNA repair assay) was carried out with *Escherichia coli PQ37* (+/-S9) as described by Emig *et al.* (1996) and Mersch-Sundermann *et*

al. (1998b). The procedure of the sister chromatid exchange test with primary human lymphocytes (+/-S9) was described by Kevekordes *et al.* (1996, 1998). The *in vitro* micronucleus test with human lymphocytes and HepG2 cells was described by Kevekordes *et al.* (1997a+b) and Mersch-Sundermann *et al.* (2001). The *in vivo* micronucleus test using polychromatic erythrocytes in bone marrow of CD1 mice was carried out according to OECD guideline for testing of chemicals No.474. The single cell gel electrophoresis (comet assay) with HepG2 cells was carried out as recommended by Mersch-Sundermann *et al.* (2000). For the studies about the co-genotoxic and co-initiating potency in HepG2 cells, Sprague Dawley rats and CD1 mice we used with the laboratory protocols described by Mersch-Sundermann *et al.* (1996b, 2001). Cytochrome P450 enzyme linked immunosorbent assays (ELISA) were carried out according to Mersch-Sundermann *et al.* (2001 and unpublished). The tumorigenic, tumor promoting and co-initiating potential of musk ketone was evaluated in CD1 mice using the mouse skin two-stage (induction/promotion) protocol.

RESULTS AND DISCUSSION

The results of the genotoxicity studies of nitro musks and polycyclic musks are shown in Table 1. Only musk ambrette exhibited mutagenicity in the Ames test with *S. typhimurium* TA100 (base pair exchange) in the presence of an exogenous metabolizing system (+S9)(Emig *et al.* 1996). The lack of mutagenicity in the nitroreductases deficient tester strain TA100 NR indicated that mammalian and bacterial enzymes are necessary for musk ambrette genotoxicity (Mersch-Sundermann and Emig, 1998). None of the polycyclic musks tested showed genotoxic potential in the assays used (Table 1).

As the most interesting results we found co-genotoxic effects of musk xylene (MX) and musk ketone (MK) in SD rats (Mersch-Sundermann *et al.* 1996b) and human Hep G2 hepatoma cells (Mersch-Sundermann *et al.* 2001). Especially the pretreatment with MK showed a significant increase of the genotoxic potency of benzo[a]pyrene (Bap) in both animals and human cells. As shown in the studies with Cytochrome P450 ELISA the cogenotoxic potency of MK and MX was caused by an induction of CYP 1A enzymes which are responsible for the toxification of various promutagens and procarcinogens to DNA reactive metabolites, for instance the metabolisation of Bap to mutagenic and carcinogenic diol epoxides.

In contrast the *in vivo* micronucleus test with mice using MK alone or in combination with Bap showed no mutagenic and co-mutagenic effect of MK, whereas Bap significantly increased the frequency of micronucleated erythrocytes in bone marrow of mice. The lack of co-mutagenicity in the *in vivo* micronucleus assay is probably caused by a first-pass effect of MK in the liver of MK treated animal after oral application.

The results of the two-stage I/P protocol with mice revealed that MK alone possess no tumor promoting potency on mouse skin and failed to induce tumors at a significant level. MK-pretreated groups of CD1 mice developed about 3-fold more 7,12-DMBA induced tumors as compared to the unpretreated 7,12-DMBA control groups after a period of 11 weeks. For 7,12-DMBA alone 10 tumors/group were developed, whereas the animals in the MK pretreated groups showed 32 tumors/group. These results were in accordance with the results of the *in vitro* assay with HepG2 cells and the *ex vivo* assay using microsomal fractions of livers of MK pretreated SD rats for the toxification of promutagens and confirmed the local co-genotoxic and co-initiating potency of MK in combination with PAH.

Table 1. Results of genotoxicity assays with artificial fragrances

	TA97[1]	TA98[2]	TA100[3]	TA100 NR[4]	TA102[5]	EC PQ37[6]	SCE (HI)[7]	MN (HI)[8]	MN (HH)[9]	SCGE[10]	Co (r)[11]	Co (HH)[12]	CYP[13]	MN (PCE)[14]	I/P (skin)[15]
Musk xylene	N[A]	N[A]	N[A]	∅	N[A]	N[C]	N[F]	N[O]	N[O]	N[K]	P[L]	P[M]	P[N]	∅	∅
Musk ketone	N[A]	N[A]	N[A]	∅	N[A]	N[C]	N[F]	N[O]	N[O]	N[K]	P[L]	P[M]	P[N]	N[P]	P[Q]
Musk ambrette	N[A]	N[A]	P[A]	N[B]	N[A]	N[C]	N[F]	N[O]	N[O]	N[K]	∅	∅	∅	∅	∅
Musk moskene	N[A]	N[A]	N[A]	∅	N[A]	N[C]	N[F]	N[O]	N[O]	N[K]	∅	∅	∅	∅	∅
Musk tibetene	N[A]	N[A]	N[A]	∅	N[A]	N[C]	N[F]	N[O]	N[O]	N[K]	∅	∅	∅	∅	∅
Galaxolide	N[D]	N[D]	N[D]	∅	N[D]	N[E]	N[G]	N[H]	N[H]	∅	∅	∅	∅	∅	∅
Tonalide	N[D]	N[D]	N[D]	∅	N[D]	N[E]	N[G]	N[H]	N[H]	∅	∅	∅	∅	∅	∅
Celestoide	N[D]	N[D]	N[D]	∅	N[D]	N[E]	N[G]	N[H]	N[H]	∅	∅	∅	∅	∅	∅
Phantolide	N[D]	N[D]	N[D]	∅	N[D]	N[E]	N[G]	N[H]	N[H]	∅	∅	∅	∅	∅	∅
Cashmeran	N[D]	N[D]	N[D]	∅	N[D]	N[E]	N[G]	N[H]	N[H]	∅	∅	∅	∅	∅	∅
Traseolide	N[D]	N[D]	N[D]	∅	N[D]	N[E]	N[G]	N[H]	N[H]	∅	∅	∅	∅	∅	∅

Explanations to table 1:

N: negative outcome; P: positive outcome; ∅ no data availabe

1-5) Salmonella assay with *S.typhimurium* TA97, TA98, TA100, TA100NR, TA102 with and without an exogenous metabolizing system (+/-S9); 6) SOS chromotest with *E.coli* PQ37 (+/-S9); 7) Sister chromatid exchange test with primary human lymphocytes (+/-S9); 8) *in vitro* micronucleus assay with primary human lymphocytes; 9) *in vitro* micronucleus assay with HepG2 cells; 10) SCGE (Comet assay) with HepG2 cells; 11) *ex vivo* comutagenicity assay with SD rats; 12) *in vitro* cogenotoxicity assay with HepG2 cells; 13) CYP 1A1 measurement in SD rats and CYP ELISA; 14) *in vivo* micronucleus assay with CD1 mice (OECD 474); 15) I/P protocol (tumerogenicity) in CD1 mice

A) Mersch-Sundermann V *et al.* (1996a); B) Mersch-Sundermann V and Emig M (1998); C) Emig M and Mersch-Sundermann V (1996); D) Mersch-Sundermann V *et al.* (1998a); E) Mersch-Sundermann V *et al.* (1998b); F) Kevekordes S *et al.* (1996); G) Kevekordes S *et al.* (1998); H) Kevekordes S *et al.* (1997a); K) Mersch-Sundermann V *et al.* (unpublished); L) Mersch-Sundermann V *et al.* (1996b); M) Mersch-Sundermann V *et al.* (2001); N) Mersch-Sundermann V *et al.* (unpublished); O) Kevekordes S *et al.* (1997b); P) Mersch-Sundermann V *et al.* (unpublished); Q) Gminski R *et al.* (unpublished)

Despite the decision of the cosmetic and detergent industry of the developed countries to stop or reduce the use of NM various products can still contain MX and MK. Additionally, not only food contaminations (oral) and cosmetic products (transdermal) are responsible for NM exposure but also the indoor use of NM containing scents (inhalative). In fact, it is assumed that the world wide production of NM may further decrease in future whereas the production

of polycyclic musk fragrances may increase. However, the data available on the environmental behavior and mammalian toxicity of polycyclic musks are also fragmentary and do not allow an appropriate risk estimation of this group of compounds.

Among the nitro musks MX and MK are the major environmental contaminants. Although MX and MK are structurally similar and possess nearly identical physico-chemical properties they differ significantly in their biological properties, especially with respect to their enzyme induction and/or inhibition. Therefore results derived in studies with MX can not be applied to MK. Both MX and MK have not shown genotoxic activity in the assays used in the present study. Nevertheless, in view of the fact that MX appears to have a much longer half-live in humans than in rodents, showed haemoglobin adducting activity, and seemd to produce mouse liver tumors in high concentrations further toxicological investigations on the chronic effects in humans appear necessary.

In contrast to MX only a few toxicological studies of MK are available. However MK showed a significant induction of enzyme activities in rodents, which were species specific and distinct from MX. As a result of its enzyme inducing potency MK acts as a cogenotoxicant in combination with several human carcinogens not only in *E.coli* but also in the metabolically competent human hepatoma cell line HepG2 at rather low doses. This finding suggests an additional genotoxic risk for humans by exposure to MK which effectively induce CYP1A in liver and possibly other organs. From the present point of view MK can be classified as toxicologically of more concern than MX.

CONCLUSION

Musk ambrette act as a mutagen in the presence of (bacterial) nitroreductases and mammalian (microsomes) enzymes using *S. typhimurium* TA 100 (Ames test). Because of the fact that musk ambrette exhibited no genotoxicity in the other toxicity assays used (especially in assays with human cell cultures) a genetic risk caused by the use of musk ambrette seems to be improbable. Except musk ambrette none of the nitro containing musks exhibited direct mutagenicity or genotoxicity in the assays used. Therefore, a risk caused by direct DNA damaging or cancer initiation seems to be inlikely.

None of the polycyclic musks showed mutagenicity or genotoxicity in the assays used. Therefore, a genetic risk caused by polycyclic musks seems to be improbable.

Musk ketone acts as a co-mutagen in animals and human cell cultures. Additionally, MK exhibited co-initiating potency with respect to the development of tumors in mouse skin. Therefore, musk ketone seems to possess an indirect-acting and dose-dependent genetic risk. Additionally, musk ketone showed bioaccumulating properties in animals and humans. On the basis of these results the use of MK as well as its emission into urban and indoor environment should be avoided. Additionally, the presence of MK in breast milk is not acceptable as babies are exposed during nursing and have presumably an increased susceptibility to DNA damaging and co-mutagenic agents.

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