DSM Nutritional Products

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Global Regulatory Affairs Manager

May 30th, 2008

Dr Barbara Shane
Executive Secretary for the NTP BSC
NTP Office of Liaison, Policy and Review
NIEHS
P.O. 12233
MD A3-01
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RE   Request for Additional Information on NTP Testing Program – 2-Ethylhexyl-p-Methoxycinnamate Proposed Research Project – 73 Federal Register 20289

Dear Dr Shane,

DSM Nutritional Products Ltd, appreciates the opportunity to help providing additional information on 2-Ethylhexyl-p-Methoxycinnamate.

Enclosed are detailed summary of studies on development toxicity, subchronic toxicity and ADME including dermal penetration which was reviewed by the European Scientific Committee of Cosmetics (SCC) in 1996. In addition, we would like to share our comments with regard to estrogenic activity, reprotoxicity and carcinogenicity.

We would like to thanks NTP for the opportunity to provide information and comment some existing results relevant for the evaluation of 2-Ethylhexyl-p-Methoxycinnamate.

Sincerely,

Anne-Gaël Glevarec
Global Regulatory Affairs Manager

2-Ethylhexyl Methoxycinnamate

[CAS No : 5466-77-3]

Comments to NTP Proposal

DSM Nutritional Products Ltd.

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1. Introduction

The UV-filter Ethylhexyl Methoxycinnamate (CAS 5466-77-3) was nominated by the National Cancer Institute for evaluation by National Toxicology Program (NTP) and recommended for comprehensive toxicological characterization including carcinogenicity and developmental toxicity studies, and characterization of photodecomposition products (nomination background document NTP 2006).
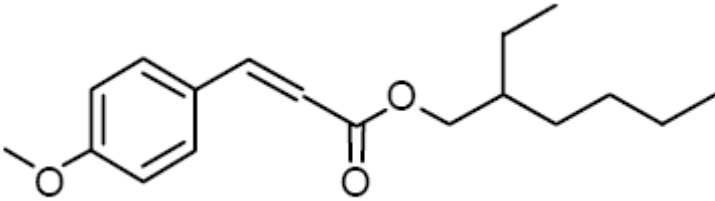
The intention of the present document is to share toxicological information on Ethylhexyl Methoxycinnamate with the National Toxicology Program (NTP). Additional respective studies and evaluations were not referenced in NTP documentation.

DSM Nutritional Products Ltd. would like to inform that DSM owns a comprehensive toxicological data package for Ethylhexyl Methoxycinnamate which was evaluated in 1996 by the European Scientific Committee Cosmetology (SCC).

DSM Nutritional Products Ltd. identified the following topics in the Draft NTP research concept on Ethylhexyl Methoxycinnamate (NTP, 2008) for which we would like to comment and supply information. In this document a summary of the conclusions by the SCC (1996) is given followed by detailed summary of studies on developmental toxicity, subchronic toxicity, and ADME including dermal penetration. In addition, we would like to give our comments with regard to estrogenic activity, reprotoxicity, and carcinogenicity.

2. Identity

2.1. Chemical Identity

INCI name	Ethylhexyl Methoxycinnamate
USAN name	Octinoxate
Chemical names	3-(4-methoxy-phenyl)-propionic acid 2-ethyl-hexyl ester; 2-ethylhexyl 3-(4-methoxyphenyl)-2-propenoate; 2-ethylhexyl-4-methoxycinnamate; 2-ethylhexyl-p-methoxycinnamate
Trade names and abbreviations	Parsol® MCX; OMC, EHMC
CAS / EINECS number	5466-77-3
Structural formula	
Empirical formula	$C_{18}H_{26}O_3$
Physical form	Liquid, oily
Molecular weight	290.4 g/mol
Solubility	< 0.75 mg/l, water (21°C, OECD No.105) Miscible, ethanol and commonly used lipids and fats miscible with isopropanol but does not mix with water
UV specific extinction (E1%, 1cm at 310nm)	835-865
Partition coefficient (Log Pow)	> 6.0 (octanol/water)

2.2. Function and Use

UV-filters are used in sunscreen formulation with the purpose to protect the skin against harmful UV-radiation. UV-filters efficacy is linked with a good distribution on the skin surface and a remaining layer on the skin. Therefore UV-filters are aimed to stay on skin surface and not to penetrate to ensure efficacy.

Ethylhexyl Methoxycinnamate is a globally allowed oil-soluble UV-B filter in sun-care and cosmetic preparations with a maximum use concentration of 7.5% in US and 10% in Europe.

3. Toxicology of Ethylhexyl Methoxycinnamate

3.1. Evaluation of Ethylhexyl Methoxycinnamate by SCC

During its plenary session held on 24th May 1996 the Scientific Committee Cosmetology (SCC) adopted the SCC opinion on Ethylhexyl Methoxycinnamate (SCC, 1996):

SCC concluded that Ethylhexyl Methoxycinnamate is of low acute toxicity. The overall NOAEL resulting from a subchronic oral toxicity study in rats is 450 mg/kg bw/day. 13-week dermal application to rats results in an NOAEL of 555 mg/kg bw/day (the highest dose administered). Ethylhexyl Methoxycinnamate is not irritating to skin and eyes and was not skin sensitizing. A standard carcinogenicity study is not available but Ethylhexyl Methoxycinnamate is neither genotoxic / mutagenic nor photomutagenic / photoclastogenic. Phototoxicity and / or photosensitization were also not noted. Animal studies for teratogenic activity in rats and rabbits showed no evidence for embryotoxicity, developmental toxicity, or teratogenicity. Percutaneous absorption was extensively studied in naked rat, minipig, and human skin in vitro and experiments in man were carried out in vivo. Overall evidence from these experiments suggests that dermal penetration of Ethylhexyl Methoxycinnamate is low (1 to 2% of applied dose, NTP 2008a). Experiments with radioactive a.i. in man indicate that only about 0.3% of the applied amount appears in the urine. In a detailed study in man, which compared oral and percutaneous absorption indicated that about one fifth of an oral dose (100 mg) was found in urine but when 200 mg were applied dermally none at all was found in plasma and urine.

3.2. Developmental Toxicity / Teratogenicity

DSM Nutritional Products Ltd. owns two developmental toxicity studies in rats and rabbits which are summarized in the following. The respective studies were evaluated by SCC in 1996. Overall, the data show that Ethylhexyl Methoxycinnamate is not embryotoxic, not developmental toxic, nor teratogenic in two species i.e. rats and rabbits up to the limit dose of 1000 mg/kg bw/day and up to the highest feasible concentration, respectively.

DSM / External + Ref.	Kistler A (1983) Embryotoxicity and teratogenicity study in rats with oral administration of Ro 5-8640, parsol MXC. Segment II-teratological study, Hofmann-LaRoche Ltd, Basle, Switzerland, Internal Memoradum IM 104958 Please refer to SCC opinion (1996)
Type	Teratological study in rats
Guideline + deviations	FDA (1966) and CSM (segment II-teratological study of 1974)
GLP	Yes, Memorandum of Understanding between FDA and Swiss Federal Office of Foreign Economic Affairs of 5th March 1980
Test substance / Batch	2-ethylhexyl-p-methoxy cinnamate (Ro 5-8640) / Lot 565542
Species / sex	Rat / female
Strain	Fü-albino (outbred)
Route of administration	Oral by gavage
Period of administration	Day 7 to 16 of gestation (presence of vaginal plug day 1 of gestation)
Frequency of administration	Daily
Doses	0 (vehicle), 250, 500, 1000 mg/kg bw/day
Control group	Yes
Remark	none
Date	26th September 1983
Result	no embryotoxicity, no teratogenicity, no developmental toxicity
NO(A)EL maternal	500 mg/kg bw/day
LOEL maternal	1000 mg/kg bw/day
NO(A)EL developmental	1000 mg/kg bw/day
LOEL developmental	> 1000 mg/kg bw/day

Materials and Methods

Ethylhexyl Methoxycinnamate was tested in a developmental toxicity study which included a rearing experiment. Therefore 36 mated female rats (Fü-albino) per group were treated by oral gavage using SSV (0.5% Carboxymethylcellulose, 0.5% Benzyl-EtOH, 0.4% Tween 80, 0.9% NaCl) as vehicle at dose levels of 0, 250, 500, and 1000 mg/kg bw/day from day 7 to 16 of gestation. Dose levels were selected based on a pilot study. About half of the animals were assigned for subgroup I (investigations for visceral and skeletal changes, teratology part). The other females were allowed to litter and to rear their offspring to day 23 of lactation. Due to an human error the foetuses for skeletal examination of subgroup I were too extensive macerated and were thus replaced by a new experiment using 20 mated females per group.

Body weights were determined on day 1, 7, 17, and 21 of gestation for all females, on the day of parturition, and on days 4, 12, and 23 of lactation (subgroup II).

Subgroup I animals were killed on day 21 of gestation, uteri were removed and examined (including determination of corpora lutea). Half of the foetuses per litter were examined for visceral changes; the other half was examined for skeletal changes. Foetal body weights were determined.

Subgroup II animals were allowed to litter and rear their offspring. On day 1, 4, 12, and 23 of lactation litter size was determined. Maternal and pup body weights were determined. Females were necropsied on day 23 of lactation. Offspring was observed for anomalies and discarded.

Results

Overall, Ethylhexyl Methoxycinnamate was well tolerated by dams. Body weight development was slightly impaired in the highest dose group. Reproduction parameters were not changed when compared to historical control database. There were no indications of malformations. The rearing experiment showed no indication of any functional anomaly. The weight development and surviving rate were not affected. Gestation period was slightly increased in the two highest dose groups but this had no influence on pup development and was therefore not considered adverse.

Conclusion

Ethylhexyl Methoxycinnamate was not embryotoxic and not teratogenic up to the limit dose of 1000 mg/kg bw/day.

DSM / External + Ref.	Bürgin H (1983) Embryotoxicity Study in Rabbits with Oral Administration of Ro 05-8640. Segment II-Teratological Study, Hofmann-LaRoche Ltd, Basle, Switzerland, Internal Memorandum 104752 Please refer to SCC opinion (1996)
Type	Teratological study in rabbits
Guideline + deviations	FDA (1966) and CSM (segment II-teratological study of 1974)
GLP	Yes, Memorandum of Understanding between FDA and Swiss Federal Office of Foreign Economic Affairs of 5th March 1980
Test substance / Batch	2-ethylhexyl-p-methoxy cinnamate (Ro 05-8640) / Lot 559684
Species / sex	Rabbit / female
Strain	Swiss hare rabbits
Route of administration	Oral by gavage
Period of administration	Day 7 to 20 (inclusive) of gestation (day of copulation day 1 of gestation)
Frequency of administration	Daily
Doses	0 (vehicle), 80, 200, and 500 mg/kg bw/day
Control group	Yes
Remark	none
Date	14th October 1983
Result	no embryotoxicity, no teratogenicity
NO(A)EL maternal	200 mg/kg bw/day
LOEL maternal	500 mg/kg bw/day
NO(A)EL developmental	200 mg/kg bw/day
LOEL developmental	500 mg/kg bw/day

Materials and Methods

Ethylhexyl Methoxycinnamate was tested in a developmental toxicity study. Therefore 20 mated female rabbits per group were treated by oral gavage using SSV as vehicle at dose levels of 0, 80, 200, and 500 mg/kg bw/day from day 7 to 20 of gestation. Dose levels were selected based on a pilot study.

Body weights were determined on day 1, 7, 20, and 30 of gestation. Animals were observed daily for clinical changes. All rabbits were sacrificed on day 30 of gestation. Uteri were removed and examined (determination of corpora lutea, implantations, resorptions). The foetuses were examined macroscopically, weighed and the crown-rump length was measured. All young were

tested for their 24h viability. Afterwards, they were killed and examined viscerally. For skeletal examination, foetuses were X-rayed and macerated if necessary. After being x-rayed, the young were decapitated and the heads were fixed and examined.

Results

No obvious intoxication could be observed at any dosage except a slight impairment of body weight gain and a slightly increased frequency of constipated and anorectic dams in the high dose group. No test substance related mortality was noted.

Reproduction parameters i.e. pregnancy rate, number of implantations, resorptions, and Corpora lutea were not changed.

Foetal body weights of the highest dose group was lower compared to controls and other dose group which is considered to be the result of impaired body weight gain of the does at the highest dose group. Neither survival rate nor sex ratio was affected.

Examination of foetuses at necropsy or following skeletal processing did not reveal drug-related deviations.

Conclusion

Ethylhexyl Methoxycinnamate was not embryotoxic and not teratogenic up to the limit dose of 500 mg/kg bw/day. However, 500 mg/kg bw/day decreased body weight gain of does and foetuses at this dose group.

3.3. Subchronic Toxicity

Two subchronic toxicity studies are available which are summarized below. The NOAEL for oral administration is 450 mg/kg bw/day. For dermal application using mineral oil as vehicle (i.e. worst case scenario with regard to systemic exposure) the highest applied dose i.e. 555 mg/kg bw/day did not result in adverse reactions.

DSM / External + Ref.	Camponovo F (1984) Ro 05-8640/000: 13-Week oral dietary study in the rat, Hofmann – LaRoche, Basle, Switzerland, Internal Memorandum 104812 Please refer to SCC opinion (1996)
Type	90-day feeding study including 5 week recovery period
Guideline + deviations	Not indicated, comparable to OECD 407, 6 animals per sex and group for main study and 6 animals per sex of control and high dose for recovery
GLP	Yes, Memorandum of Understanding between the FDA and the Swiss Federal Office for Foreign Economic Affairs of 5th May 1980
Test substance / Batch	Ethylhexyl Methoxycinnamate, Ro 05-8640/000 / Batch 565 542 / purity: 99%
Species / sex	Rat / both sexes
Strain	Füllinsdorf Albino SPF outbred
Route of administration	Oral via feed
Period of administration	At least 90 days
Frequency of administration	Ad libitum
Post-exposure period	Yes, 5 weeks
Doses males	0, 200, 450, 1000 mg/kg bw/day (12 per group)
Doses females	0, 200, 450, 1000 mg/kg bw/day (12 per group)
Control group	Yes, plain diet
Remark	
Date	23rd July 1984
Result	

DSM / External + Ref.	Camponovo F (1984) Ro 05-8640/000: 13-Week oral dietary study in the rat, Hofmann – LaRoche, Basle, Switzerland, Internal Memorandum 104812 Please refer to SCC opinion (1996)
NO(A)EL	450 mg/kg bw/day
LOEL	1000 mg/kg bw/day

Materials and Methods

Ro 05-8640/000 was orally administered to rats (12/sex/group) at the doses 0 (control), 200, 450 and 1000 mg/kg/day, 7 days per week for at least 13 weeks. At the termination of the treatment, half of the controls and of the high-dose rats were allowed 5 additionally weeks on normal diet prior to sacrifice. Body weight, feed consumption and signs of toxicity were recorded weekly. Ophthalmoscopy and urine analysis were performed twice during the study. Blood chemical and hematological investigations were carried out at the beginning, during and at the end of the treatment period. An additional blood chemical investigation was performed after a recovery period. Post mortem investigations comprised full autopsy, organ weight determinations and histological examination.

Results

The feed intake and the body weight development of treated animals were similar to those of controls. There was good agreement between nominal and actual amount of daily ingested compound. No symptoms indicative of pathologic conditions, ophthalmological abnormalities or mortalities as consequence of the treatment with the test compound were recorded during the study. Laboratory investigations in high-dose females (1000 mg/kg/day) revealed an increase of the plasma activity of GLDH which was reversed after the recovery period. The absolute as well as the allometrically adjusted weights of the kidneys were slightly increased in males. No deviations of the weights were found after the recovery period, thus indicating an adaptive change. The glycogen content of the livers was reduced and in 5 of 12 animals it was accompanied by slight shrinkage of the hepatocytes. In females the amount of the iron positive material phagocytized by Kupffer cells was slightly increased. These conditions were reversed after the recovery period.

Conclusion

There was no obvious effect related to the treatment, which was detectable by the hematological, blood chemical and urine parameters at the mid- (450 mg/kg bw/day) and low-dose (200 mg/kg bw/day) levels.

It is concluded that the treatment with Ro 05-8640/000 was well tolerated at any dose-level and that under the conditions of this study only minor and reversible changes occur at the dose-level of 1000 mg/kg/day, whereas the dose of 450 mg/kg/day does not induce any adverse effect in the rat.

External + Ref.	SCC (1996) SCC Opinion Concerning: 2-ethyl-4-methoxycinnamate (S28), adopted by the plenary session of the SCC on 24th May 1996
Type	13-week dermal study
Guideline + deviations	Not indicated / not applicable
GLP	Not indicated
Test substance / Batch	Not indicated
Species / sex	Rat / both sexes
Strain	SD
Route of administration	Dermal
Period of administration	5 days per week for 13 weeks
Frequency of administration	Once daily
Post-exposure period	No
Doses males	0, 55.5, 277, 555 mg/kg bw/day (10 animals per group)
Doses females	0, 55.5, 277, 555 mg/kg bw/day (10 animals per group)
Control group	Yes
Remark	Not proprietary of DSM Nutritional Products Ltd. Summary available from SCC evaluation
Date	Not applicable
Result	
NO(A)EL	555 mg/kg bw/day
LOEL	> 555 mg/kg bw/day

Materials and Methods

Four groups of 10 male and 10 female SD rats were treated by an application of various concentrations of a.i. in light mineral oil. The doses were 0, 55.5, 277 and 555 mg/kg bw/day applied to shaved skin 5 days a week for 13 weeks. (The top dose is believed to be about 135 times the amount which would be used daily by the average consumer). Various laboratory and 'clinical tests were carried out during the experiment.

Results

All animals survived. All animals showed a slight scaliness at the site of application, which was attributed to the vehicle. Body weight gain was greatest at the low dose. Haematological investigations showed no significant change. SAP was elevated in high dose animals, but not significantly. The relative liver weight in high dose animals was elevated, but appeared normal on microscopical examination.

Conclusion

The NOAEL is 555 mg/kg bw/day.

3.4. ADME including dermal penetration

Standard ADME studies in laboratory animal via oral or dermal route are not available to DSM Nutritional Products Ltd. In one study, in vitro experiment on metabolism in human plasma was performed. In this study male volunteers received a single oral dose of 100 mg and a single dermal dose of 200 mg Ethylhexyl Methoxycinnamate. This study is summarized in detail below.

3.4.1. ADME

DSM / External + Ref.	Kemper FH (1989) Investigations on the Cutaneous Absorption of 4-methoxy-cinnamonic-acid-2-ethylhexylester (Parsol ® MCX) Translation from German Report, University of Münster, Germany Please refer to SCC opinion (1996)
Type	Absorption, human study
Guideline + deviations	Not applicable
GLP	No
Test substance / Batch	Ethylhexyl Methoxycinnamate / not indicated
Species / sex	Human volunteer
Remark	The present investigation was done to evaluate cutaneous absorption in human volunteers thereby examining urine and blood samples after oral and dermal application to healthy male volunteers for the presence of Ethylhexyl Methoxycinnamate and potential metabolites. During this investigation pre-experiments were done to investigate hydrolysis of Ethylhexyl Methoxycinnamate in human plasma.
Date	20th September 1989
Result	Ethylhexyl Methoxycinnamate is cleaved slowly **in vitro** by esterases present in human blood plasma. 13.2 mg 4-methoxy-cinnamonic-acid (21.5% of the expected figure for complete absorption and renal elimination) was determined after 24 h. Urinary excretion is largely completed within 6 h after oral dose. Dermal application experiments showed no relevant increase of Ethylhexyl Methoxycinnamate in plasma and 4-methoxy-cinnamonic acid equivalents in urine.

Materials and Methods

The purpose of the study was to investigate cutaneous penetration of Ethylhexyl Methoxycinnamate in an o/w sunscreen formulation containing 10% of Ethylhexyl Methoxycinnamate.

In preliminary experiments in vitro hydrolysis of Ethylhexyl Methoxycinnamate in human blood plasma was investigated at concentration of 10 µg/mL.

The oral absorption of Ethylhexyl Methoxycinnamate was investigated in one human male volunteer who received one single dose of 100 mg (1.6 mg/kg bw/day). Urine was collected 6, 12, and 24 h after application. Urine was analysed without pre-treatment for Ethylhexyl Methoxycinnamate and for hydrolysis product 4-methoxy-cinnamonic acid. The same samples were then treated under alkaline conditions and analysed for 4-methoxy-cinnamonic acid equivalent.

Dermal penetration was investigated using the above mentioned sunscreen formulation thereby applying 2 g of cream (equivalent to 200 mg Ethylhexyl Methoxycinnamate) to 25 x 30 cm in the dorsocapular area under occluded conditions. Blood and urine were obtained pre-treatment and at regular intervals up to 96 h after application.

Results

Ethylhexyl Methoxycinnamate is cleaved slowly in vitro by esterases present in human blood plasma: Half-life-time of Ethylhexyl Methoxycinnamate at 35°C is approx. 10 h. After 120 h at 20°C approx. 17.8% Ethylhexyl Methoxycinnamate and 83.3% 4-methoxy-cinnamonic-acid were found.

Ethylhexyl Methoxycinnamate and potential hydrolysis product 4-methoxy-cinnamonic-acid were not detectable in native urine after a single oral dose of 100 mg (1.6 mg/kg bw) to one male

healthy volunteer. After alkaline hydrolysis of urine, 13.2 mg 4-methoxy-cinnamonic-acid (21.5% of the expected figure for complete absorption and renal elimination) was determined after 24 h. Excretion is largely completed within 6 h after oral dose.

An o/w Sunscreen Cream containing 10% Ethylhexyl Methoxycinnamate (200 mg active ingredient) was applied to skin to 5 healthy male volunteers. Results of this investigation showed no relevant increase (when compared to pre-test values) of Ethylhexyl Methoxycinnamate in plasma and of 4-methoxy-cinnamonic-acid equivalents in urine (after alkaline treatment).

Conclusion

Absorption after oral application takes place but not after dermal application using representative sunscreen formulation.

Hydrolysis of Ethylhexyl Methoxycinnamate at the ester bond to yield 4-methoxy-cinnamonic acid and the consequent alcohol i.e. 2-ethylhexanol may take place in human blood plasma in vivo to a very limited extent (half-life in vitro at 35°C: 10 h).

One can therefore assume that Ethylhexyl Methoxycinnamate is hydrolysed also after application in toxicological studies in laboratory animal to 4-methoxycinnamonic acid as well as 2-ethylhexanol which may be further oxidized to yield 2-ethylhexanoic acid. It is concluded that the potential teratogenic effect of the latter compound is investigated in the available teratology studies in rats as well as in rabbits.

3.4.2. Dermal Penetration

DSM / External + Ref.	Darragh A & Lambe R (1980) Percutaneous Absorption and Excretion of Radioactivity Following Topical Administration of Labelled Parsol MCX and Parsol 1789 to Healthy Volunteers Study I, Biological Medical Research Institute, Dublin Please refer to SCC opinion (1996)
Type	Absorption, human study
Guideline + deviations	Not applicable
GLP	No
Test substance / Batch	Ethylhexyl Methoxycinnamate, Parsol MCX in carbitol 10% (w/v) unlabelled material and 125 µCi/mL ^{14}C
Species / sex	Human volunteer
Remark	Despite Ethylhexyl Methoxycinnamate another UV-filter was investigated. Results for this are not included in this summary
Date	29th July 1980
Result	0.3% urinary excretion

Materials and Methods

The purpose of the study was to investigate cutaneous penetration of Ethylhexyl Methoxycinnamate thereby using radiolabelled material (^{14}C).

In total 4 healthy volunteers were treated either under occluded or unoccluded conditions with the radiolabelled test substance. The composition of the test substance was as follows: 10% (w/v) unlabelled material and 125 µCi/mL ^{14}C.

Volunteers were treated with 25 µCi ^{14}C which was applied to areas of about 10 cm^2 to the skin between the shoulder blades for 8 h. Afterwards, the substances were removed by washing procedures. Blood, urine, and faecal samples as well as skin strippings were collected at regular

intervals up to 120 h post-treatment and analysed for total radioactivity. The health of volunteers was monitored prior to onset and after termination of the study by means of complete physical examination including blood and urine analysis.

Results

There were no adverse reactions of volunteers.

Radioactivity was neither detectable in plasma nor in faeces for both preparations. Recovery of radioactivity was high (97.7 +/- 3%). It was concluded that there was very low percutaneous penetration due to high recovery from the application area (97.0 +/- 3.4%), low amounts in skin strippings (0.3 +/- 0.2%), undetectable radioactivity in plasma and faeces and due to low amount of radioactivity excreted in urine (0.3 +/- 0.3%).

Conclusion

Only 0.3% of applied dose were excreted via urine.

3.5. Further information

The following chapter gives information on other sections of toxicology.

3.5.1. Reprotoxicity

In 2005, a robust multigeneration reprotoxicity study was published (Schneider et al., 2005) feeding Ethylhexyl Methoxycinnamate to Wistar rats over two consecutive generations at dose levels of 0, 150, 450, and 1000 mg/kg bw/day.

The authors concluded that the NOAEL for fertility, reproduction, parental and developmental toxicity was the mid dose i.e. 450 mg/kg bw/day. There was no evidence from this publication that potential but extremely low estrogenic activity would negatively affect individuals or their progeny.

3.5.2. Estrogenic Activity

Potential estrogenic activity of Ethylhexyl Methoxycinnamate was reviewed in 2001 by the European Scientific Committee for cosmetic products (SCCNFP, 2001).

Reference is made to that evaluation: A standard uterotrophic assay for Ethylhexyl Methoxycinnamate showed no evidence for estrogenic activity in vivo. It was concluded that the organic UV-filters used in cosmetic sunscreen products allowed in the EU market today (this included Ethylhexyl Methoxycinnamate) have no estrogenic effect that could potentially affect human health (SCCNFP, 2001)

3.5.3. Carcinogenicity

Although no chronic / carcinogenic evaluation of Ethylhexyl Methoxycinnamate is available, we would again like to make reference to the evaluation of SCC (1996). There a study in hairless mice is summarized which gave no evidence for tumour initiation and tumour promoter activity. In addition, we would like to emphasis that a comprehensive mutagenicity / genotoxicity data package has shown the absence of mutagenicity and genotoxicity in vivo and in vitro (in the presence and absence of UV-exposure). Please refer to SCC (1996) for a summary.

DSM

4. Conclusion

A comprehensive data package of toxicological data for Ethylhexyl Methoxycinnamate is available which has been thoroughly evaluated by European Competent Authority (SCC, 1996).

Overall the data show that Ethylhexyl Methoxycinnamate is not acute toxic, not skin or eye irritant, not skin sensitizing, as well as not photo-reactive as evidenced by negative results form phototoxicity and photosensitization studies.

It was suggested that Ethylhexyl Methoxycinnamate may be cleaved to 4-methoxycinnamonic acid and 2-ethylhexanol plus 2-ethylhexanoic acid the latter one being considered as developmental toxicants (NTP 2008). In vitro experiments on metabolism indicate that a slow hydrolysis of Ethylhexyl Methoxycinnamate to 2-ethylhexanol and 4-methoxycinnamonic acid might be possible. Thus, the toxicity of these metabolites was also investigated in comprehensive pre-clinical studies with Ethylhexyl Methoxycinnamate.

Ethylhexyl Methoxycinnamate is not genotoxic / mutagenic with or without exposure to UV-light.

The overall NOAEL for oral and dermal toxicity subchronic studies as well as from teratogenicity and multigeneration reprotoxicity studies is 450 mg/kg bw/day.

The substance is not embryotoxic, not developmental toxic, not reprotoxic, nor teratogenic.

Dermal penetration of Ethylhexyl Methoxycinnamate is low as already indicated. Less than 3% are expected to penetrate (NTP 2008).

In the light of the above summarized information and in the context of animal welfare, NTP may reconsider its testing proposal (comprehensive toxicological characterization including carcinogenicity and developmental toxicity studies, and characterization of photodecomposition products) for Ethylhexyl Methoxycinnamate.

5. References

NTP (2008) Draft NTP Research Concept: 2-Ethylhexyl p-methoxycinnamate. Draft: NTP Board of Scientific Counselors Meeting, June 11-12 2008, available at http://ntp.niehs.nih.gov/go/165

NTP (2006) Background Document for Nomination: Update of Sunscreen Ingredients Nomination to NTP, available at http://ntp.niehs.nih.gov/go/nom

SCC (1996) SCC Opinion Concerning: 2-ethyl-4-methoxycinnamate (S28), adopted by the plenary session of the SCC on 24th May 1996, SPC/1037/93 rev. 4/96.

SCCNFP (2001) Opinion of the Evaluation of Potentially Estrogenic Effects of UV-filters adopted by the SCCNFP during the 17th Plenary meeting of 12 June 2001 available at http://ec.europa.eu/health/ph_risk/committees/sccp/docshtml/sccp_out145_en.htm accessed on 28th May 2008

Schneider S, Deckardt K, Hellwig J, Küttler K, Mellert W, Schulte S, van Ravenzwaay B (2005) Octyl methoxycinnamate: Two generation reproduction toxicity in Wistar rats by dietary administration, Food Chem Toxicol 43: 1083-1092.
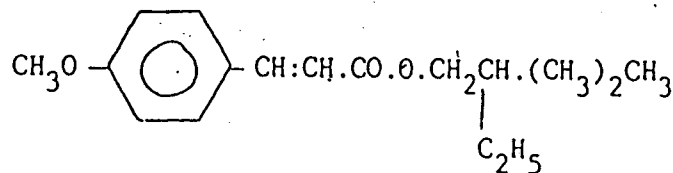
SPC/1037/93 rev. 4/96

SCC OPINION CONCERNING :

2 - ethylhexyl -4- methoxcinnamate (S28)

Adopted by the plenary session of the SCC
on 24.05.96

EC 2.13. Colipa S28.
2-ethylhexyl-4-methoxycinnamate. "Parsol MCX".

$$CH_3O - \langle\bigcirc\rangle - CH:CH.CO.O.CH_2CH.(CH_3)_2CH_3$$
$$\underset{C_2H_5}{|}$$

Colourless pale yellow slightly oily liquid. MW 290. Miscible with alcohols, propylene glycol, etc. Immiscible with water (1).
Use level up to 10%.

Acute toxicity.
Oral LD_{50}: Mouse, greater than 8 g/kg bw. (1, 2, 6). Rat, greater than 20 ml/kg bw. (3).

Subacute toxicity.
Rat. Three week oral study. Groups of 5 male and 5 female animals were given 0, 0·3, 0·9 and 2·7 ml/kg bw/day by gavage for 3 weeks. All animals of the top dose groups exhibited loss of body weight and a reduced relative and absolute weight of the thymus. Male rats showed a decrease in absolute weight of the left kidney and female rats showed a decrease in the absolute weight of the heart. At the two lower doses, the only significant alteration observed was an increased absolute weight of the pituitary gland in male rats receiving the lowest dose. As the number of animals was small, the investigators considered this not to be biologically significant. The NOAEL was put at 0·9 ml/kg bw/day. (4).

Subchronic toxicity.
Rat. Thirteen week oral study. Four groups of 12 male and 12 female SPF rats received the compound in the diet at levels of 0, 200, 450 and 1 000 mg/kg bw/day. During the experiment the usual clinical observations were carried out, as well as extensive haematological and biochemical studies. Full gross necropsy was carried out on all survivors. Histological investigations were carried out in half the animals of the control and top dose groups. The organs studied included the heart, lungs, liver, stomach, kidneys, spleen, thyroid and retina. In the remaining animals histological examination of the liver only was carried out. Six control animals and 6 top dose animals were allowed to recover over 5 weeks, and then examined.
The results of the experiment showed no dose related mortality. The kidney weights of top dose animals were increased, but were normal in the recovery animals; the increase was attributed to a physiological response to an increased

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excretion load. There was a diminution of glycogen in the liver, and a slight increase in iron in the Kupfer cells in the high dose animals. Two of these also showed minimal centrilobular necrosis of the liver with some infiltration; similar less marked findings were made in 2 of the control animals as well. These findings were attributed to infection. High dose females had increased GLDH which reversed during the recovery period. The NOAEL was put at 450 mg/kg bw/day (10).

Rat. Thirteen week dermal study. Four groups of 10 male and 10 female SD rats were treated by an application of various concentrations of a.i. in light mineral oil. The doses were 0, 55·5, 277 and 555 mg/kg bw/day applied to shaved skin 5 days a week for 13 weeks. (The top dose is believed to be about 135 times the amount which would be used daily by the average consumer). Various laboratory and clinical tests were carried out during the experiment.

All animals survived. All animals showed a slight scaliness at the site of application, which was attributed to the vehicle. Body weight gain was greatest at the low dose. Haematological investigations showed no significant change. SAP was elevated in high dose animals, but not significantly. The relative liver weight in high dose animals was elevated, but appeared normal on microscopical examination. The NOAEL is 555 mg/kg bw/day. (11).

Test for capacity to irritate mucous membranes.

Rabbit. Groups of 4 animals had 0.1 ml of a test preparation instilled into the conjunctival sac (concentration not stated). No further treatment in one group; in the other, the instillation was followed by washing out. There were no signs of irritation. (5).

A Draize test carried out with undiluted a.i. was found to be practically non-irritant. (1, 2, 6).

Test for capacity to cause irritation of the skin.

Guinea pig. The a.i. was applied undiluted twice daily to 20 animals for 16 days. There were no signs of irritation. (5).

Man. Occlusive applications of undiluted a.i. were made to 60 subjects, of whom 20 had sensitive skin. The applications were made for 24 hours. Observations at removal of the patches, and 24 and 48 hours later, showed no evidence of a reaction. (5).

In 51 male and female subjects, similar patch tests were carried out. The dilution of the a.i. (if any) was not stated. There was no irritation. (1).

A formulation (concentration not stated) tested on the skin of 50 subjects caused no adverse effect. (1, 2, 6).

In 53 subjects, a Draize repeated insult patch test at a concentration of 2% caused no irritation. (7)

In 54 subjects, a Draize repeated insult patch test of a 7·5% dilution of a.i. in petrolatum caused no irritation. (1).

A 10% solution of a.i. in dimethylphthalate was used. A

total of 58 subjects was recruited, 12 males and 46 females, aged 18-63. Of these, 6 subjects failed to complete the test for reasons unconnected with the experimental procedure.

Induction applications were made on the skin of the back, for 24 hours with occlusion, 3 times a week for 9 applications. Following a rest period of 2 weeks, a further patch was now applied to a new site on the back for 24 hours with occlusion. The area was inspected at 0, 24 and 48 hours after removal of the patch. No adverse reaction was noted at any stage of the experiment. (30).

Tests for capacity to cause sensitisation.
Guinea pig. Twenty animals received applications of undiluted a.i. twice daily for 16 days. After a 3 day interval without treatment, a daily challenge application was made for 3 days. There was no evidence of sensitisation (5).

Two groups of 4 animals were used. Animals of one group were exposed to 0.05 ml injections of undiluted a.i. daily for 5 days. In the other group, 0.025 ml of a 50% acetone solution of a.i. was applied to 2 cm^2 areas of shaved skin on either side. There was no evidence of sensitisation. (1).

Man. A Draize repeated insult patch test was carried out at a concentration of 2% in 53 subjects. There was no sensitisation. (7).

In 54 subjects, a formulation of 7.5% a.i. in petrolatum was applied for 48 hours under occlusion for 11 applications. After a 14 day rest, a challenge application of a single dose was made. There was no adverse reaction. (1, 8).

In an extensive series of patch tests carried out in man, the a.i. was found to be very rarely resposible for allergic contact effects. (28).

Tests for capacity to produce phototoxicity.
Man. In 10 subjects, patches were applied for 24 hours and the areas then exposed to a suberythematous dose of UV irradiation. There was no evidence of phototoxicity. (5).

Test for capacity to produce photosensitisation.
Tests which "showed that the product did not provoke photosensitisation." No details supplied. (1, 6).

Tests for percutaneous absorption.
(a) In *vitro* tests.
Rat. Naked rat skin. This was studied in a chamber experiment. The investigators used a 1% solution of a.i. in carbitol, and the amounts applied were 120, 360 and 1200 ug/cm^2. Most of the material was found in the stripped skin; there was less in the stratum corneum, and least in the the chamber. The approximate amounts found in the chamber were: after 6 hrs, 1·13%; after 16 hrs, 11·4%; and at 24 hrs 17·9%. The figures for the horny layer and

the strippings combined were, respectively, 31·4%, 44·4% and 45·7% (percentages of applied doses). The amount of a.i. applied did not seem to affect the results. (32).

In another set of experiments, various amounts of "Parsol 1789" (4-tert-butyl-4'-methoxydibenzoylmethane) were added to the a.i. in the formulation. There seemed to be no effect on the absorption of the a.i. (14).

Pig. A similar experiment using mini-pig skin was carried out in which "Parsol 1789" was used as well as the a.i. Using 3 sorts of formulation, about 3% of a.i. was found in the chamber in 6 hrs. Using the concentrations proposed for a particular commercial use (i.e., 2% of "Parsol 1789" and 7.5% of a.i.) about 2.2% of the amount of a.i. applied was found in the chamber. It is calculated by the authors that the total absorption for a 60 kg consumer would be about 56 mg, or 0.9 mg/kg bw. This figure may be too high; a different calculation gives a value of 0.2 mg/kg bw. (15).

Man. A test on human abdominal skin in a chamber was carried out. With 7·5% a.i., about 0·03% is found in the chamber in 2 hours, 0·26% in 6 hours, and 2·0% in 18 hours. Various combinations of a.i. and "Parsol 1789" were investigated. A calculation shows that these results might indicate an absorption of about 0.2 mg/kg bw. in use. (16).

(b) In vivo tests.

Man. Eight healthy volunteers had small amounts of radioactive a.i. applied to the interscapular region. One group of 4 had the material applied under a watch glass; the other 4 had it applied on gauze, with occlusion in one case. Tests for absorption of a.i. were negative except for about 0.2% in urine. The concentrations used were not stated. (17).

In a preliminary experiment, a capsule containing 100 mg of a.i. was taken orally. As a lipophilic substance, the a.i. is very likely to be metabolised; it is known in any case to be hydrolysed by plasma esterases, although slowly. The cumulative excretion of 4-methoxycinnamate in the urine over 24 hours was studied by GC/MS of the methyl ester derivative. (This method would also detect 4-hydroxycinnamic acid). Over 24 hours, an amount of cinnamate was found in the urine equivalent to about one-fifth of the amount that would have been expected if all the dose of a.i. had been absorbed. Nearly all of the metaolite was found in the first 6 hours.

In the main part of the experiment, an o/w cream containing 10% a.i. was used. Applications of 2 grams of this material (= 200 mg a.i.) were made to the interscapular area of each of 5 male subjects, aged 29 to 46. The area of skin covered was 750 cm^2. After application, the area was covered with 3 layers of gauze, left in place for 12 hours. Blood was taken at times 0, 0·5, 1, 2, 3, 5, 7, and 24 hours. Urine was collected at 0, 2, 3, 4, 4, 5, 6, 7, 12, 24, 48, 72 and 96 hours.

The control plasma samples showed a level equivalent to about 10 ng/ml before any application had been made. There

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was no evidence of any rise in plasma levels during the experiment. The urine showed a "physiological" level of 100 to 300 ng/ml. No significant increase in this amount was found in any sample. The experiment seems to have been carefully conducted. The authors conclude that very little, if any, of the compound was absorbed after application to the skin, compared with the reasonably well marked absorption after ingestion. (29).

Tests for mutagenic activity.
Salmonella mutagenesis assays were performed on the usual strains. There was a positive result with TA 1538 without metabolic activation. This was thought to have been a batch effect. (9). From another laboratory, a very weak positive was found with TA 1538 without activation, at 10 ul/plate; it was not found in 2 replicates, nor in a second Ames test. (18).
A test for mutagenesis and crossing over in S. cerevisiae was negative. (20).
A test using Chinese hamster V 79 cells showed a very slight increase in mutant colonies with dose. (19).
A test in human lymphocytes in vitro was negative. (21).
A test for cell transformation in Balb/c 3T3 cells was negative. (22).
A test for unscheduled DNA synthesis was negative. (23).
Feeding tests in Drosophila:
There was an increase in the frequency of sex-linked recessive lethals; this was attributed with fair certainty ot a batch effect. (9).
There was no evidence of mutagenicity in feeding tests (adults and larvae). (24).
Somatic mutation and combination tests using wing structure were negative. (26).
Mouse. A standard micronucleus test was carried out. No effect was found up to 5000 mg/kg bw. (25).

Tests for photomutagenic activity.
A test was carried out in cells of S. cerevisiae, which had previously been shown not to be affected by a.i. (supra). Evidence of mitotic gene conversion, gene mutation, and mitotic crossing-over was looked for. Doses of a.i., dissolved in DMSO, ranged from 0.05 to 625 µg/ml, and radiation up to 500000 J m^{-2} UVA and up to 12000 J m^{-2} UVB. Chlorpromazine was used as the positive control. Suitable negative controls were also employed. The experiment appears to have been well carried out. The results show that the a.i. is not photomutagenic under these conditions; that UVA and (more markedly) UVB are mutagenic; and that the a.i. protects against this effect in a dose dependent manner. (31, 34).
A test for the production of chromosomal aberrations was carried out in Chinese hamster ovary cells in culture. The test was carried out in accordance with GLP. The intensity of the ultraviolet radiation (mJ/cm^2) ranged from 200 to 2000 for UVA and from 4 to 25 for UVB. The positive

control was chlorpromazine; the negative controls consisted of cultures irradiated but without the addition of active ingredient, and cultures not irradiated but with the addition of the active ingredient. The doses of active ingredient used ranged from 5 to 25 µg/ml. It was noted that the top dose of UV irradiation was clastogenic, but that there was a protective effect with the active ingredient. The positive control showed satisfactory activity. There was no evidence of a photoclastogenic effect. (33, 34).

Test for inhibition of UV-induced tumours.
Hairless mouse. The animals were exposed to repeated doses of UV simulating the solar energy spectrum. After a rest period. 3 applications a week were made to an area of skin of 12-o-tetradecanoyl phorbol-13-acetate (at first at 10 µg/ml, but later at 2 µg/ml, as the higher concentration was found to be irritant). Suitable controls were used. The test group was completely protected by 50% a.i., and 7·5% gave an effect equivalent to reducing the insolation four-fold. It had been suggested that the a.i. could itself have been a promoter, but there was no evidence of this. (25).

Tests for teratogenic activity.
Rabbit. Groups of 20 female animals were mated and given a.i. in doses of 0, 80, 200 and 500 mg/kg bw/day by gavage during the period of organogenesis. Except for a slight reduction of maternal and foetal weight in the top dose animals, no abnormality was found. (12).
Rat. Following a pilot study, groups of 36 rats were mated and treated with 0, 250, 500 and 1000 mg/kg bw/day of a.i. (probably by gavage) during days 6-14 of pregnancy. Owing to an error, the preparation of the control foetuses led to their destruction, so this part of the test was repeated under identical conditions. Subgroups of each dose group were allowed to litter normally and rear the offspring. The percentage of resorptions in the high dose group was elevated by comparison with the other groups. The investigator records, however, that this relatively high rate is the usual one with this strain of rat in this laboratory, and he attributes the difference to an unusually low level of resorption in the other groups. No other abnormality was found. (13).

Evaluation.
The compound appears to have low acute toxicity. A subchronic oral toxicity study showed a NOAEL of 450 mg/kg bw/day. A subchronic dermal study showed a NOAEL of 550 mg/kg bw/day, which was the highest dose tested. The a.i. does not irritate the mucous membranes in conventional animal tests. The data presented suggest that the compound is not a skin irritant or sensitiser in animals; however, tests for sensitisation were carried out at levels below the proposed maximum use level. Clinical investigation shows that this compound is very rarely responsible for

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allergic contact dermatitis in man.
There is no carcinogenicity study, but an extensive range
of mutagenicity studies has been carried out; these show
no evidence of mutagenicity. A test for photomutagenicity
in _S_. _cerevisiae_ was negative. Photoclastogenicity tests
in CHO cells _in vitro_ were negative.
Animal studies for teratogenic activity showed a NOAEL
of more than 500 mg/kg bw/day (which was the highest dose
tested). Percutaneous absorption was studied in naked rat,
minipig, and human skin _in vitro_; and experiments in man
were carried out _in vivo_. The experiments show that there
is a decreasing amount of absorption as one goes from rat
skin to human skin; the last suggests that about 0.9 mg/kg
bw might be absorbed. Experiments with radioactive a.i.
in man indicate that only about 0.2% of the applied amount
appears in the urine. In a detailed sudy in man, which
compared oral and percutaneous absorption, using GC/MS,
although about one-fifth of 100 mg of ingested a.i. was
found in the urine, none at all was found when 200 mg of
active ingredient was applied to the skin in a concentration
of 10%.

Calculation of margin of safety.
Amount of formulation applied (mg) (F) = 18000 (assuming
an application rate of 1 mg/cm^2 over the entire body sur-
face).
Concentration of active ingredient (C) = 10%.
Total amount of active ingredient applied (I) = 18000 X
10/100 = 1800 mg.
Percutaneous absorption (A) = 2%.*
Total amount absorbed = I X A/100 = 36 mg.
Systemic exposure (SED) = 36/60 = 0·6 mg/kg bw.
NOAEL (13 week rat oral study) = 450 mg/kg bw/day.
Margin of safety = NOAEL/SED = 750.

(*This figure is derived from experiments in human and
animal skin _in vitro_. A carefully carried out study in
man showed absorption of about 20% following oral ingestion,
but none of the a.i. appeared in the plasma after dermal
application).

Suggested classification: 4.

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(1991-08-25 FN S28REFB D3 This p. rev.1993-04-29; 1996-04-02).

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CLASSIFICATION OF SUBSTANCES

Classification of substances as ingredients of cosmetic products is recommended by the Scientific Committee on Cosmetology on the basis of evaluations of data provided, pursuant to the Guidelines on the Safety Assessment of Cosmetic Ingredients. The overriding consideration is that the substances should be safe for consumer use under conditions of intended exposure and concentrations.

New substances for the positive lists must not be used until a final classification in Group 1 has been made. For substances already in use, the classification may be reconsidered if necessary.

Substances in provisional lists for which there are insufficient data for a final assessment of safety, additional information must be adequate and provided within a specified time limit. Otherwise it is concluded that no further use of the substance in cosmetic products should be allowed for the specified purpose.

Group 1 :   Substances for which data at the time of assessment support the conclusion that they do not pose a health hazard. They may be used in cosmetic products for the designated purposes and in concentrations not exceeding the limits indicated.

Group 2 :   Substances which must not be used in cosmetic products. These substances may be included in this group because either a) the available data support the conclusion that they constitute a health hazard or b) the available data do not justify the assumption that their use in cosmetic products can be considered safe.

Opinion on the Evaluation of Potentially Estrogenic Effects of UV-filters adopted by the SCCNFP during the 17th Plenary meeting of 12 June 2001

Exposure of the human body to sunlight and UV $_A$/UV $_B$-light and being tanned became fashionable, particularly during summer and holidays. However, with the rise in sunlight exposure an impressive increase in the number of skin cancer cases occurred (English et al., 1997, Whiteman and Green 1999). A direct link between the carcinogenic action and sunlight radiation has been made and the International Agency for Research on Cancer (IARC) has classified solar radiation, UV $_A$ and UV $_B$, as human carcinogens (IARC, 1992).

National and international health authorities have urged the public to take protective measures, among these to use sunscreens. IARC has recently evaluated the cancer-preventive activity of sunscreens (IARC, 2000). They conclude that the use of sunscreens reduces the risk of sunburn in humans and probably prevents squamous-cell carcinoma of the skin. No conclusion could be drawn about cancer preventive activity against basal-cell carcinoma and cutaneous melanoma. However, use of sunscreens can extend the duration of intentional sun exposure and such an extension may increase the risk for cutaneous melanoma.

Sunscreens today contain one or several UV-B filters, often enriched with UV-A filters. UV-filters usually are synthetic organic chemicals but may also be inorganic in nature. Their safety for human use is regulated by national and international bodies. In the EU, before a new UV-filter is allowed on the market, a stringent toxicological safety evaluation is carried out and only in the case of a safe toxicological profile and a margin of safety of at least 100, the molecule can be approved by the SCCNFP for human use (Notes of Guidance for testing of cosmetic ingredients for their safety evaluation SCCNFP/0321/00 Final).

Comparable safety approval procedures exist in the USA and Japan.

2. Terms of Reference

The SCCNFP has been asked to evaluate the possible estrogenic effects of organic UV filters used in cosmetic products and to respond to the following questions :

* Could the SCCNFP provide a critical analysis of the article " *In vitro* and *in vivo* estrogenicity of UV screens" by Margret Schlumpf et al?

* More generally, does the SCCNFP consider that organic UV filters used in cosmetic sunscreen products have any estrogenic effects which have the potential to affect human health?

3. Expert review

Introduction

There is growing concern regarding possible harmful consequences of exposure to xenobiotic compounds that are capable of modulating or disrupting the endocrine system. This concern for endocrine disrupting chemicals is directed at both wildlife and humans. It should be noted that although there are associations between endocrine disrupting chemicals, so far investigated, and human health disturbance, a causative role of these chemicals in diseases and abnormalities related to an endocrine disturbance has not been established. The Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) has recently discussed toxicological test guidelines and testing strategies and has concluded that reliance on *in vitro* assays for predicting *in vivo* endocrine disrupter effects may generate false-negative as well as false-positive results. Thus, the development of *in vitro* pre-screening test methods is not recommended. The current enhancement by OECD of the existing *Repeated Dose 28-day Oral Toxicity Study in Rodents* (406) and the *Two-Generation Reproduction Toxicity* (416) tests has high priority support [CSTEE, 1999]. Recently, an article by Schlumpf et al (2001) suggested that several UV screens show estrogenic activity. They used an *in vitro* test with the MCF-7 breast cancer cell line and an *in vivo* rat uterotrophic assay. The investigations of this Swiss group on the safety of UV-filters have alerted the popular press and the public. Controversial interpretations of the results initiated vigorous debates about the safety of sunscreens. More particularly estrogenic properties were attributed to a number of UV-filters including benzophenone-3, homosalate, 4-methyl-benzylidene camphor, octyl-methoxycinnamate and octyl-dimethyl-PABA, even suggestions towards carcinogenic effects were made.

On 27/4/2001, an ad hoc working group of the SCCNFP has discussed the issue and published a preliminary report on the scientific review of the investigations of Schlumpf et al. (2001). Serious concerns were expressed as to the validity of the results published.

As a follow-up, a final expert opinion of the SCCNFP is given below, consisting of :

(1) a scientific review of the investigations of Schlumpf et al. (2001),
(2) a scientific review of the investigations carried out by the industry concerned,
(3) a risk assessment and margin of safety according to the EU-procedure for UV-filters,
(4) data on human exposure to environmental and dietary estrogens,
(5) conclusions.

3.1. Scientific Review of the Results of Schlumpf et al. (2001)

3.1.1. Study protocol and results

The *in vitro* and *in vivo* estrogenicity of 5 UV $_B$-filters, benzophenone-3 (Bp-3), homosalate (HMS), 4-methyl-benzylidene camphor (4-MBC), octyl-methoxycinnamate (OMC) and octyl-dimethyl-PABA (OD-PABA) and 1 UVA-filter, butyl-methoxydibenzoylmethane (B-MDM) were studied.

3.1.1.1. *In vitro* study

A general screening assay (E-screen) with a human breast cancer cell line, MCF-7 cells, was carried out. A positive test was based upon the binding of the test compound with the estrogen receptor leading to cell proliferation.

As a positive control, 17 b -estradiol, was used and it was, as expected, positive in the assay. The 5 UV-B filters were found to be positive in the assay and caused cell proliferation. The UV-A filter gave a negative result. EC $_{50}$ values for 17 b -estradiol, Bp-3, 4-MBC, OMC, OD-PABA and HMS were found to be 1.22 pM, 3.73 μM, 3.02 μM, 2.37 μM, 2.63 μM and 1.56 μM, respectively .

The results were supported by the expression of the estrogen-dependent pS $_2$ protein and by an inhibition of effects with the anti-estrogen ICI 182,780.

3.1.1.2. *In vivo* tests

A uterotrophic assay was carried out using two different exposure routes, namely

- oral exposure of young Long-Evans rats to the 6 UV-filters from day 21 of life until day 24 of life, with ethinylestradiol serving as a positive control;

- dermal exposure of hairless Nu rats to 4-MBC from day 21 of life until day 26 of life by immersion of the animals in warm olive oil containing 2.5%, 5% and 7.5% of 4-MBC, respectively.

For the oral exposure, a dose-dependent increase of uterine weights was observed for 4-MBC and OMC, a slighter response was seen for Bp-3, but no maximal effect was seen as was the case for the positive control.

ED $_{50}$ values were found to be 0.818 μg/kg/day, 1000-1500 mg/kg/day, 309 mg/kg/day and 934 mg/kg/day, for the positive control, Bp-3, 4-MBC and OMC, respectively. OD-PABA, HMS and B-MDM were found to be inactive.

For the dermal exposure assay, 4-MBC exhibited a dose-dependent increase in uterine weight, with a significant effect at a concentration of 5% and 7.5% in olive oil.

3.1.2. Comments by SCCNFP

3.1.2.1. *In vitro* study

- The potency of the positive control is in the order of picomoles; the *in vitro* potency of the UV-filters tested lays in the range of micromoles, which means a difference of 1 million units. The *in vitro* potency of the UV-filters is thus importantly lower than the one observed for 17 b -estradiol. Probably a lot of industrial chemicals would show some *in vitro* estrogenic effects when this type of comparisons is taken seriously.

- It should be emphasized here that *in vitro* assays can only demonstrate whether UV-filters bind on the estrogen receptor or not, but they do not provide evidence whether the compounds have estrogenic activity or not. *In vitro* assays are therefore screening tests useful in setting priorities for further *in vivo* testing. The CSTEE committee clearly stated in its report on endocrine disrupters (1999) that utilising *in vitro* data for predicting *in vivo* endocrine disrupter effects may generate false negative as well as false positive results and that major emphasis should therefore be put on *in vivo* assays. Claiming that 5 UV-filters have estrogenic properties based on an *in vitro* test is premature.

The *in vitro* ranking for the UV-filters going from Bp3, 4-MBC, OMC, OD-PABA to HMS, did not correspond with the *in vivo* results. Indeed, in the latter test 4-MBC was most active, followed by OMC and Bp-3. The most active UV-filter *in vitro* displayed only a weak activity *in vivo*. In addition OD-PABA and HMS were found to be inactive.

Only precise toxicokinetic data can link the *in vitro* and *in vivo* data, a conclusion that was also reached by the authors.

3.1.2.2. *In vivo* studies

- The OECD *draft protocol* on the rodent uterotrophic assay, was issued on April 21, 2000. The protocol used by the Swiss group dates from before that time and therefore shows some important deviations. Moreover, GLP conditions have not been applied.

Deviations from the current OECD guideline proposal :

· the choice of the rat strains is unusual and not explained.

· the exposure period of the rats runs until the 26th day of life, which is too close to the onset of puberty.

· the dermal exposure conditions are inappropriate : dipping pups into olive oil is not a standard procedure and the galenic form to deliver the UV-filter, namely a solution in warm olive oil, is not reflecting in use conditions : indeed, today sunscreens are formulated as poorly penetrating o/w-emulsions.

· the calculation of the absorbed dose via dermal exposure is unclear and oral intake by the animals cannot be excluded.

- The potency of the positive control, ethinylestradiol, is in the order of 1 µg/kg/day; the potency of the UV-filters tested lays in the range of 100 to 1000 mg/kg/day; which means a difference of 100.000 to 1 million units. The *in vivo* potency of the UV-filters is thus importantly lower than the one observed for the control hormone. Furthermore, 3 of the 6 UV-filters have no measurable potency at all.

- The uterotrophic assay can only serve a limited function as a test for *in vivo* identification of chemicals with estrogenic activity. The uterotropic assay is a short-term high-dose test.

3.2. Scientific Review of the Investigations carried out by Industry

3.2.1. Submission of 4-methyl-benzylidene camphor (Colipa code S60)

Two uterotrophic assays have been carried out in immature female Sprague Dawley rats [Crl : CD (SD) BR rats] either by the subcutaneous route (subcutaneous injections) either by the oral route (gavage). Animals were dosed on 3 consecutive days, day 19-20-21 of life; the positive control was 17-ethinylestradiol. The protocol was according to the OECD guidelines proposal and the tests were carried out under GLP conditions.

Six groups were tested :

- 0, 10, 100,1000 mg/kg/day S60 in corn oil
- 0.3 µg/kg/day of control in ethanol
- 1 µg/kg/day of control in ethanol

Subcutaneous study : A statistically significant lower mean body weight gain was observed in the 1000 mg/kg/day S60-treated group during the interval day 19-day 20, compared to the vehicle control group.

Oral study : A statistically significant lower mean body weight gain was observed in the 100 mg/kg/day and the 1000 mg/kg/day S60-treated group during the interval days 19-20 and day 20-21, respectively, together with a lower mean daily food consumption.

For both studies, the positive control induced a significant dose-related increase of the uterus weight (both as wet uterus and blotted uterus). S60 did not induce a significant increase of the uterus weight at 10, 100 and 1000 mg/kg/day.

3.2.2. Submission of octylmethoxycinnamate (Colipa code S28)

A uterotrophic assay of OMC was carried out using female immature Wistar rats [CRL : WI(GLX/BRL/HAN) IGS BR] by the oral route (gavage) for 3 consecutive days. The positive control was DES-SP (diethylstilbestrol dipropionate).

Four groups were tested :

- 0, 250, 1000 mg/kg/day S28 in olive oil,
- 5 µg/kg/day of positive control in olive oil.

The protocol deviated from the OECD guideline proposal, but was carried out under GLP conditions. A statistically significant lower mean body weight gain was observed in the 250 mg/kg/day S28-treated group during the interval

day 2-day 3; the same was true for the 1000 mg/kg/day S28-treated group during the interval day 0-day 3.

The positive control induced a significant uterotrophic effect and showed histopathologic changes in the uterus.

S28 did not induce a uterotrophic effect and no histopathologic changes could be shown in the uteri concerned.

3.2.3. Submission of benzophenone-3 (Colipa code S38)

A uterotrophic assay of Bp-3 was carried out using female immature Wistar rats [Chbb : THOM, SPF] of 22 days old. The compound was given by the oral route (gavage) for 4 consecutive days. The positive control was DES-SP.

Four groups were tested :

- 0, 500, 1000 mg/kg/day S38 in sesame oil,
- 5 μg/kg/day of control in sesame oil.

Appropriate control groups were included. The protocol deviated from the OECD guideline proposal, but the test was performed under GLP-conditions. Dosing was carried out until day 26, which is too close to the onset of puberty. A statistically significant lower body weight gain was observed in the 1000 mg/kg/day S38-treated group during the interval day 0-day 1.

The positive control induced a significant increase in uterine weight (absolute and relative). S38 did not promote growth of the uterus and therefore does not exhibit estrogenic activity.

Industry made 4 uterotrophic studies available on UV-filters of which 2 were performed with 4-MBC (subcutaneous and oral administration), one with Bp-3 (oral route) and one with OMC (oral route). The results of the 4 studies show no evidence for any uterotrophic response and doses up to 1000 mg/kg/day were used for the 3 UV-filters concerned. The animals were female immature Sprague-Dawley or Wistar rats treated for 3 to 4 consecutive days.

Only the 4-MBC study is strictly carried out according to the OECD guideline proposal and no evidence for uterotrophic activity could be seen, although 4-MBC was found to be the most active UV-filter in the study of Schlumpf et al. (2001), showing a significant increase in uterine weight at a dose of 119 mg/kg/day. In the same study Bp-3 had a weak effect at a dose of 1.500 mg/kg/day but in the study conducted by industry no uterotrophic effect could be detected at dosing of 1000 mg/kg/day. The dosing at 1.500 mg/kg/day is higher than the top dose present in the OECD guideline proposal and should therefore be seen as a negative result.

Thus as far as Bp-3 is concerned, the results of Schlumpf et al and industry are in line. A negative finding for Bp-3 was also reported earlier by Baker et al (2000) during the poster session in the SOT meeting in Philadelphia. For Bp-3 it was already known that about 1% of the Bp-3 dose in rats is metabolised to p-hydroxy-benzophenone, which might exhibit an estrogenic effect (Hayden et al.1997, Felix et al.1998, Stocklinski et al.1980, Nakagawa et al 2001).

A clear discrepancy, however, exists between the negative results obtained by industry and the positive ones of Schlumpf et al. (2001), in particular for 4-MBC. Differences in strain of the animal may be an important factor. This was highlighted in the expert report of Bolt et. al [2001, in press], which discusses the significant differences in toxicokinetics of p-tert-octylphenol found in different rat strains (Certa et al. 1996, Upmeier et al. 2000).

Also dosing of the animals was different : oral administration of UV-filters was performed by gavage in the industrial studies and by mixing the chemicals in the food by the Swiss group.

3.3. Risk Assessment and Margin of Safety

3.3.1. Some general considerations

On a general basis, the MoS is calculated by dividing the lowest No Observable Adverse Effect Level (NOAEL) of a compound by its Systemic Exposure Dose (SED) during normal foreseeable use. If the MoS exceeds 100, the compound is regarded as safe for use.

The question can be raised whether an additional safety factor should be introduced for children with regard to the use of sunscreens.

Based on a number of previous publications [Schaefer and Riedelmayer 1996, Marzulli and Maibach 1984, Jiang et al. 1999 and Weltfriend et al. 1996], Nohynek and Schaefer [2001] recently concluded that there is no reason to assume that children should be more susceptible to potential adverse effects of topically applied sunscreens than adults. This conclusion was based upon the findings that

- there is no significant difference between the skin of children and adults regarding the penetration of topically

applied substances;

- Skin permeability to externally applied substances remains relatively constant throughout life; this particularly being confirmed for sunscreens;

- The skin of children is not more susceptible to local irritant effects of topically applied substances than adult skin.

Thus the major difference between adults and children, relevant for risk assessment of a topically applied substance, is the larger body surface / body weight ratio of children, when compared with that of adults. As a consequence, the relative systemic exposure of children to a topically applied substance may be somewhat (about 1.4 times) higher than that of a typical adult.

Based on this relatively small difference between the systemic exposure of adults and children, no additional safety factor is introduced for children.

3.3.2. Risk assessment and margin of safety for 4-MBC

3.3.2.1. According to SCCNFP notes of guidance

The safety of 4-MBC has been reviewed by the SCCNFP (XXIV/1377/96 rev.1/98) in 1998. It was concluded that tests for skin irritation, sensitisation, phototoxicity, photosensitisation, photocontact allergy, mutagenicity and photomutagenicity were negative. Percutaneous absorption was estimated to be 1.9%. Teratogenicity tests were negative and via dermal application no stimulating effect on the thyroid function could be seen as was suggested by subchronic oral tests.

The Margin of Safety [**MoS** = NOAEL / SED] was found to be **110**, which is acceptable.

3.3.2.2 According to data of Schlumpf et al. (2001)

The NOEL (estrogenic activity) of 4-MBC in the uterotrophic assay in immature Long-Evans rats published by Schlumpf et al (2001) is 66 mg/kg/day.

SED = 0.23 mg/kg/day

" **Screening MoS**" = 66 mg/kg/day / 0.23 mg/kg/day = **289**, which is higher than 100 and would consequently be acceptable.

Important to notice is the calculation of a *" Screening MoS"*, since :

· the exposure time can hardly be called long-term or chronic, but is clearly short-term, which is of great significance for the use of the deduced "NOEL"-value.
A 2-generation study would be able to generate the real NOEL value necessary for the calculation of the MoS related to reproduction toxicity. However, this particular type of study should only be considered as a last resort because of the large number of animals required to perform it correctly.

· as long as the relevance for humans of positive results in a uterotrophic assay is not known, it remains questionable whether it is correct to use a safety margin for hormonal activity.

3.3.3. Risk assessment and margin of safety for OMC

3.3.3.1. According to SCCNFP notes of guidance

The safety of OMC has been reviewed by the SCC (SPC/1037/93, S28) in 1993. It was concluded that the compound has a low acute toxicity. OMC is not irritating or sensitising in animals, but can be very rarely responsible for allergic contact dermatitis in man.

Mutagenicity, photomutagenicity and photoclastogenicity tests were negative. The teratogenic activity has a NOAEL of more than 500 mg/kg bw/day, which was the highest dose tested.

The percutaneous absorption was estimated to be 2%, a figure derived from experiments in human and animal skin *in vitro*, plus the results of an *in vivo* human study via oral uptake.

The **MoS** was calculated to be **750**, which is acceptable.

3.3.3.2. According to data of Schlumpf et al (2001)

The NOEL (estrogenic activity) of OMC in the uterotrophic assay in immature Long-Evans rats (Schlumpf et al. 2001) is 522 mg/kg/day.

SED = 0.6 mg/kg/day

" *Screening MoS*" = 522/0.6 = **870**, which would be acceptable (>100).

3.3.4. Risk assessment and margin of safety of Bp-3

3.3.4.1. According to SCCNFP notes of guidance

Bp-3 was taken up in the Annex of the UV-filters before the activities of the SCC started. Therefore, Bp-3 has not undergone the standard safety procedure review by the SCCNFP.
It is advised to ask the industry for a complete toxicological dossier on Bp-3 so that a full risk assessment and a calculation of the margin of safety can be performed according to the SCCNFP standards.

According to the *Final Report on the Safety Assessment of Benzophenones-1, -3, -4, -5, -9 and -11*) (Cosmetic Ingredient Review 2000), the risk assessment can provisionally be performed as follows :

LD_{50} oral rat > 2000 mg/kg
LD_{50} dermal rabbit > 16.000 mg/kg
NOEL (27d oral, rat) = 1% in diet
NOEL (90d oral, rat) = 0.1% in diet [effects (90d oral, rat) noticed at 0.5% to 1% in diet]

Bp-3 is not irritating to skin and eyes of rat, not photosensitising, not phototoxic in guinea pigs and rabbits, not sensitising in guinea pigs and not mutagenic in the Ames-test. Bp-3 is not irritating or sensitising on human skin, although some cases of positive patch tests have been seen in humans.

From these data a NOEL (90d, oral rat) can be estimated :

food uptake of adult rat is » 10g/100g bw
no effect when Bp-3 0.1% in diet » 1g/kg/day (diet)
» 100 mg/kg/day in rat
thus the estimated NOEL is 100 mg/kg/day.

The percutaneous absorption is not known; therefore the real SED cannot be calculated. However, assuming a percutaneous absorption in the order of 1% (Nohynek and Schaefer, 2001), a SED = 0.3 mg/kg can be calculated.

MoS = **333**, acceptable (>100)

Note that this is an estimation.

3.3.4.2. According to Schlumpf et al (2001)

NOEL (estrogenic activity) of Bp-3 in the uterotrophic assay in immature Long-Evans rats published by Schlumpf et al (2001) is 937 mg/kg/day. Since the SED is not known, the MoS cannot be calculated. Assuming the SED = 0.3 mg/kg/day,

" *Screening MoS*" = NOEL/SED = 937/0.3 = **3123** >100, would be acceptable.

3.4. Data on Human Exposure to Environmental and Dietary Estrogens

In table 1 recently published data are shown with respect to the potency of xenoestrogens in the rodent uterotrophic assay.

Table 1 : Potency of xenoestrogens in the rodent uterotropic assay.

[a] in alfalfa and leguminosae
[b] soybeans
[c] soybean-containing bread sold in UK health food stores (Ashby and Tinwell, 1998).
[d] from Casanova et al. 1999.

From these data it appears that UV-filters have an extremely small relative potency in comparison with ethinylestradiol (1 to 1 million) but also a low relative potency in comparison with dietary estrogens.

When the mass balance of human exposure to environmental and dietary estrogenic compounds was estimated, data as represented in table 2 were found (Safe 1995).

It is known that several food formulas contain soy products. Soy contains phytoestrogens including genistein and daidzein. The total phytoestrogen content of infant food formulas for instance, represents 135 ± 5 µg/g total genistein and daidzein (Irvine et al. 1998) and may go up as high as 600µg/g (Zimmerli et al. 1997).

From these data it seems that the estrogenic potency of UV-filters is several orders of magnitude lower as compared with that of natural dietary estrogens.

During the last years, it became evident that a variety of different mechanisms of endocrine disruption exist for different compounds [CSTEE, 1999]. For pragmatic reasons this will not be further discussed here.

Table 2 : Estimated mass balance of human exposure to environmental and dietary estrogens (Safe 1995).

3.5. Conclusions

(i) A number of important technical shortcomings in the study of Schlumpf et al. were detected. This was mentioned in the first part of this report as well as in a preliminary report of an ad hoc working Party.

(ii) Industry has performed a further uterotrophic assay in rats for 4-MBC according to the OECD guideline proposal and under GLP conditions; no evidence was found for an increase in uterus weight (part 2 of this report).

(iii) Industry has provided data on uterotrophic assays in rats for Bp-3 and OMC (not according to OECD guidelines, but under GLP conditions), in which no positive uterotrophic effect could be detected for both UV-filters (part 2 of this report).

(iv) The margins of safety for 4-MBC and OMC, calculated according to the SCCNFP, using NOAELs obtained from subchronic animal studies, are higher than 100.
The estimated margin of safety for Bp-3 is higher than 100 (part 3 of this report).

(v) The calculated *" Screening MoS"*, in which the experimental non-estrogenic-effect-level data, obtained by Schlumpf et al. (2001), are used in the official MoS calculations of the EU, are found to be higher than 100 for 4-MBC, OMC and Bp-3 (part 3 of this report).

However, the data presented by Schlumpf et al (2001) are unsuitable for long-term risk assessment. Use of the data of Schlumpf et al will only give a rough approximation of the possible risk. A two-generation reproduction toxicity test might possibly generate more accurate data.

(vi) The activity of the UV-filters found in the study of Schlumpf et al. (2001) is very low in comparison with exposure to "estrogenic" substances in food (flavonoids) and hormonal therapy (birth control pill, morning after pill, post-menopausal therapy) (part 4 of this report).

With the information available above, the SCCNFP concludes that there is no need for regulatory actions to protect the consumer with regard to potential estrogenic effects of the UV-filters studied :

- Although positive data were observed for the UV-filters HMS and OD-PABA in the *in vitro* assay (Schlumpf et al., 2001), this *in vitro* test with MCF-7 cells remains only a screening. The uterotrophic assay (*in vivo* test) was negative in the Schlumpf study.

- The UV-A filter B-MDM was negative in both the *in vitro* and *in vivo* assay.

- Bp-3 showed to be positive in the *in vitro* assay by Schlumpf et al. (2001) and weakly positive in the *in vivo* test. The SCCNFP recommends that industry is asked to submit a complete toxicological dossier on Bp-3 in order to perform a full risk assessment and to calculate a final MoS for Bp-3 according to the EU standards for UV-filters.

As UV-filters are an effective tool to protect humans from excessive exposure to sunlight, a known carcinogen, their use is recommended by the SCCNFP.

3.6. Opinion (answer to the questions)

Answer to the question ' *Could the SCCNFP provide a critical analysis of the article "In vitro and in vivo estrogenicity of UV screens" by Margret Schlumpf et al?*' :

The article of " *In vitro* and *in vivo* estrogenicity of the UV screens" by M. Schlumpf et al. has been critically analysed and the comments of the SCCNFP can be summarised as follows :

In vitro study :

- The potency of the positive control is in the order of picomoles; the *in vitro* potency of the UV-filters tested lays in the range of micromoles, which means a difference of 1 million units. The *in vitro* potency of the UV-filters is thus importantly lower than the one observed for 17 b -estradiol. Probably a lot of industrial chemicals would show some *in vitro* estrogenic effects when this type of comparisons is taken seriously.

- It should be emphasised here that *in vitro* assays can only demonstrate whether UV-filters bind on the estrogen receptor or not, do not provide evidence whether the compounds have estrogenic activity or not. *In vitro* assays

are therefore screening tests useful in setting priorities for further *in vivo* testing. The CSTEE committee clearly stated in its report on endocrine disrupters (1999) that utilising *in vitro* data for predicting *in vivo* endocrine disrupter effects may generate false negative as well as false positive results and that major emphasis should therefore be put on *in vivo* assays. Claiming that 5 UV-filters have estrogenic properties based on an *in vitro* test is premature.

The *in vitro* ranking for the UV-filters going from Bp3, 4-MBC, OMC, OD-PABA to HMS, did not correspond with the *in vivo* results. Indeed, in the latter test 4-MBC was most active, followed by OMC and Bp-3. The most active UV-filter *in vitro* displayed only a weak activity *in vivo*. In addition OD-PABA and HMS were found to be inactive. Only precise toxicokinetic data can link the *in vitro* and *in vivo* data, a conclusion that was also reached by the authors.

I *n vivo* study :

The OECD *draft protocol* on the rodent uterotrophic assay, was issued on April 21, 2000. The protocol used by the Swiss group dates from before that time and therefore shows some important deviations. Moreover, GLP conditions have not been applied.

Deviations from the current OECD guideline proposal :

* the choice of the rat strains is unusual and not explained

* the exposure period of the rats runs until the 26 [th] day of life, which is too close to the onset of puberty

* the dermal exposure conditions are inappropriate: dipping pups into olive oil is not a standard procedure and the galenic form to deliver the UV-filter, namely a solution in warm olive oil, is not reflecting in use conditions: indeed, today sunscreens are formulated as poorly penetrating o/w-emulsions.

* the calculation of the absorbed dose via dermal exposure is unclear and oral intake by the animals cannot be excluded.

* The potency of the positive control, ethinylestradiol, is in the order of 1 m g/kg/day; the potency of the UV-filters tested lays in the range of 100 to 1000 mg/kg/day; which means a difference of 100.000 to 1 million units. The in vivo potency of the UV-filters is thus importantly lower than the one observed for the control hormone. Furthermore, 3 of the 6 UV-filters have no measurable potency at all.

* The uterotrophic assay can only serve a limited function, as a test for *in vivo* identification of chemicals with estrogenic activity. The uterotrophic assay is a short- term high-dose test.

The SCCNFP came to the conclusion that a number of important technical and scientific shortcomings are present in the study of M. Schlumpf et al.

Answer to the question ' *More generally, does the SCCNFP consider that organic UV filters used in cosmetic sunscreen products have any estrogenic effects which have the potential to affect human health?*' :

Based on the actual scientific knowledge, the SCCNFP is of the opinion that the organic UV-filters used in cosmetic sunscreen products, allowed in the EU market today, have no estrogenic effects that could potentially affect human health.

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Octyl methoxycinnamate: Two generation reproduction toxicity in Wistar rats by dietary administration

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Abstract

Wistar rats continuously received octyl methoxycinnamate (OMC) in the diet through two successive generations at nominal doses of 0, 150,450 or 1000 mg/kg bw/day. OMC had no adverse effects on estrous cycles, mating behavior, conception, parturition, lactation and weaning, spenn and follicle parameters, macropathology and histopathology of the sexual organs. 1000 mg/kg bw/day reduced parental food consumption and body weight (-14% to -16% in males, -4% to —5% females), increased liver weight, produced hepatic cytoplasmic eosinophilia and erosion/ulceration of glandular stomach mucosa. and led to a slightly decreased implantation rate in the top dose PO and FI dams. The high dose FI and F2 pups had reduced lactation weight gain and organ weights and delayed sexual maturation landmarks. There was no evidence of a selective influence of the test compound on pups' sexual landmarks. The NOAEL (no observed adverse effect level) is 450 mg/kg bw/day for fertility and reproductive performance, for systemic parental and developmental toxicity.
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Keywords: CAS 5466-77-3; Octyl methoxycinnamate; OMC; Two-generation toxicity study; Rat; Fertility; Development; Reproduction

1. Introduction

Octyl methoxycinnamate (=2-ethylhexyl-4-methoxy-cinnamate = p-methoxycinnamic acid 2-ethylhexyl ester = octinoxate = OMC; $C_{18}H_{26}O$" 290 glmol; CAS# 5466-77-3, EINECS/ELINCS# 226-775-7) is an ultraviolet absorber marketed under the trade name Uvinul" MC 80 N. In addition to its use as a light stabilizer, OMC is an FDA Category I sunscreen, approved worldwide at concentrations up to 10%, and is the most **frequently used sunscreening agent. At room tempera**ture it is a colorless or slightly yellow liquid, freely soluble in organic solvents and oils, but insoluble in water (estimated log *Pow* = 5.80).

In animal studies, OMC is acutely non-toxic by oral administration (rat oral LD50 > 5 glkg), does not **irritate the skin and mucous membranes, has no sensi**tizing effect (SCC, 1996) and is not mntagenic in the Ames test (Zeiger et aI., 1985; BASF AG, unpublished data).

OMC has been reported to display no androgenic or **antiandrogenic activity at androgen receptors in the** human breast carcinoma cell line, MDA-kb2 at any tested concentration (I nM-IO µM) (Ma et aI., 2003). It has been reported, however, by Schlumpf et aI., 2001, to stimulate in vitro proliferation of MCF-7 cells

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(human estrogen-sensitive breast cancer cells) with an EC50 of 2.37 J.lM (although secretion of the estrogen-regulated protein pS2 in these cells was not significantly increased), and to have estrogenic activity in the utero-trophic assay in immature Long-Evans rats, with an ED50 of 935 mg/kg bodyweight (bw)/day and a NOEL of 522 mg/kg bw/day. However, no estrogenic activity of OMC has been found in the uterotrophic assay using immature Wistar rats at doses up to 1000 mg/kg bw/day (SCCNFP, 2001; BASF AG, unpublished data). In addition, OMC does not bind in vitro to estrogen recep-tors from the porcine uterus or to recombinant ERα and ERβ proteins and there are no indications of an antian-drogenic activity in the Hershberger assay in castrated male rats (BASF AG, unpublished data). These some-what conflicting data on the potential estrogenic activity of OMC in screening systems suggest that at the most the compound has a very weak estrogenic potential at high dosages only and that the toxicological relevance for the reproductive cycle is therefore questionable.

There are no data on the potential effects of OMC on fertility and reproductive performance after continuous multigeneration exposure throughout development from gametogenesis to weaning and sexual maturity. This study was conducted to address this need, i.e. to deter-mine the potential hazard of OMC concerning fertility and reproduction.

2. Materials and methods

2.1. Study design

This two-generation reproduction toxicity study was designed in accordance with OECD test guideline 416 (OECD, 2001), and was conducted in accordance with Good Laboratory Practice guidelines and applicable animal welfare legislation (OECD, 1981; German Chemicals Act, 1994).

2.2. Test animals

Male and female Wistar rats (CrlGlxBrlHan:WI), 27-29 days old on arrival, were supplied by Charles River, Germany. Males and females were from different litters, to exclude the possibility of sibling matings. All animals were free from clinical signs of disease, and the females were nulliparous and non-pregnant. These animals were the FO generation parental animals. All other animals on study (FI and F2 pups, FI parental rats and F2 young adults) were bred from these animals.

Parental adults were uniquely identified with an ear tattoo identification number, and were housed individu-ally (except during mating) in stainless steel wire-mesh cages (DK III, Becker & Co., Castrop-Rauxel, Ger-many) or, during cohabitation for mating and in females

from day 18 of pregnancy until day 14 of lactation, in Makrolon type M III cages containing nesting material (certified cellulose wadding, supplied by Ssniff Spezial-diaeten GmbH, Soest, Germany). All live pups were identified by skin tattoo on day I post partum and with picric acid marking of the fur between day 10 and 15 post partum. The animal quarters were air-conditioned (20-24 °C, 30-70% relative humidity) with a 12 h light/12 h dark cycle; walls and floors were washed weekly. Certified feed (ground Kliba maintenance diet rat/mouse meal, Provimi Kliba SA, Kaiseraugst, Switzerland) was available *ad libitum* throughout the study, as was tap water (human drinking quality) in drinking bottles.

2.3. Treatment

After acclimatization to the housing facility for one week, groups of 25 male and 25 female rats were allo-cated randomly, stratified by weight, to one of four treatment groups, and received OMC (Uvinul MC 80 N, batch UV2-01.019, purity 99.9% by HPLC-UV) ad-mixed to dry ground diet to produce nominal target doses of 0 (control), 150, 450 or 1000 mg/kg bw/day. Doses were based on a preceding I-generation range finding study in rats dosed at 0, 1500, 4500 or 10,000 ppm in the diet (fixed concentrations) resulting in test substance uptakes of 180, 525 and 1190 mg/kg bw/day. In that study, 10,000 ppm reduced final male bodyweight by 10% (relative to controls), decreased female plasma urea, creatinine, total protein and albu-increased cholesterol, and reduced ovary weight by 20%; pup weaning body weights were reduced 37% at 10,000 ppm and 10% at 4500 ppm; treatment had no effects on clinical observations, food consumption, fertility, reproductive performance, offspring viability and lactation indices, or parental and offspring gross necropsy findings (BASF, unpublished data). In the present study, dietary test substance concentrations were adjusted weekly in both sexes during the premating per-iod, based on actual food consumption and body weight, and also weekly in males during the gestation, lactation and post-weaning periods. During mating in both sexes and during gestation and post-weaning peri-ods in females, dietary test substance concentrations were kept the same as in the last week of the premating period. During lactation, dietary test substance concen-trations in females were set to 50% of those used during the last week of the premating period, to compensate for the increased maternal food intake during this period. Post-weaning FI and F2 pups were dosed on the basis of historical body weight and food consumption data for rats of similar ages. To achieve the final doses of 0, 150, 450 and 1000 mg/kg bw/day, the nominal die-tary concentrations used ranged between 674 and 16,090 ppm; actual concentrations were confirmed by UV-HPLC to be 94.0-105.6% of nominal.

FO parental animals were exposed to test substance throughout a premating period of at least 73 days (Le. **for more than one complete cycle of spermatogenesis in males, for several complete cycles of oogenesis in** females), through cohabitation for mating (maximum 21 days), and then throughout gestation (about 21 days), parturition and lactation up to weaning of the FI offspring (about 21 days), ending about 16 h before necropsy. From the FI pups, 25 males and 25 females per dose group were selected as FI parental animals, and dietary dosing continued for at least 74 days prior to cohabitation for mating, and then **during mating, gestation and lactation up to** weaning of the F2 offspring, ending about 16 h before necropsy. Dosing continued in F2 offspring until necropsy.

Parental animals were cohabitated overnight for mating (one male and one non-sibling same-dose female per cage) daily until positive mating (sperm in vaginal **smear;::: day 0 post-coitum) or for a maximum of** 2 weeks.

2.4. Observations

Parental animal health status and clinical signs were checked daily. Food and water consumption and body weights were measured weekly throughout the study, and in mated females during gestation on days 0, 7, 14 and 20 post-coitum, and in those with litters during lactation on days I, 4, 7, 14 and (body weights only) 21 **post partum. Food consumption was not measured** from day 14 to 21 post partum since by this time pups **also consume considerable amounts of solid food, and so maternal food consumption alone cannot be quanti**fied during this time. Estrous cycle length and cytologi**cal normality were evaluated daily from vaginal smears** for all FO and FI female parental rats for a minimum of 3 weeks prior to mating and throughout the mating period until positive mating.

All offspring (Fl and F2) were examined as soon as possible on the day of birth (day 0 post partum) to determine the number of liveborn and stillborn per litter; they were sexed based on anus to genital tubercle distance, and subsequently by anogenital distance and mammary line appearance, and at necropsy. Pup health status and clinical signs were checked at least once daily throughout lactation.

Litters (both FI and F2) were randomly culled on day 4 post partum to 4 male and 4 female pups where possible. Pups were weighed on the day after birth (day I post partum) and on days 4, 7, 14 and 21 post partum. To quantify sexual maturation, female vaginal opening was assessed daily from day 27 post partum and male preputial separation daily from day 40 post partum in FI offspring selected as FI parental animals and in all surviving F2 offspring.

2.5. Pathology

FO and FI parental animals, as well as offspring not selected for mating, were necropsied at or after litter weaning on day 21 post partum. Parental animals were killed by decapitation under CO_2 anesthesia. Whole body, liver, kidneys, epididymides and testes weights, **and major organ macropathology were recorded in all animals. Implantation sites were counted after uterine staining for about 5 min in 10% ammonium sulfide** solution (Salewski, 1964). Histopathology of vagina, **cervix uteri, uterus, ovaries, oviducts, left testis and epididymis (caput, corpus and cauda), seminal vesicles,** coagulating gland, prostate gland, adrenal glands, brain, pituitary gland, spleen and kidneys was recorded in all control and high dose (1000 mg/kg bw/day) Fa and FI parental animals, and in all low and mid-dose animals with suspected impaired fertility. Liver and spleen (males only) were examined in all groups. For **gross lesions detected macroscopically and liver, histo**pathology was recorded in all animals from all groups. **Testes, epididymides and ovaries were fixed in Bouin's** solution, the other organs in formaldehyde solution. **Standard stain was hematoxylin and eosin; to investigate observed brown pigmentation in male spleens,** these were treated with Perls stain (for iron). Differen**tial ovarian follicle counts were performed in control** and high dose (1000 mg/kg bw/day) FI females, folli**cles were quantified in serial sections of both ovaries** as primordial (types I, 2, 3a and b) or growing (types 4, Sa and b) according to Plowchalk et aJ. (1993). Sperm motility was microscopically quantified immediately after necropsy and organ weight determination in the right testis and cauda epididymis from all males (in randomized order) according to Slott et aJ. (1991). **Mean number of homogenization-resistant testicular** spermatids and caudal epididymal sperm, and percentages of morphologically abnormal sperm were quantified based on Feuston et aJ. (1989) in all control and high dose group males.

Pups culled on day 4 post partum and superfluous FI and F2 pups at weaning were killed by CO_2 inhalation. Post-weaning F2 pups were sacrificed by cervical dislocation. All these pups, including stillborn and sub**sequent mortalities, were examined externally, eviscer**ated and their organs assessed macroscopically. At scheduled necropsy in FI and F2 pups, brain, spleen and thymus were weighed in I pup/sex and litter; relative organ weights were calculated relative to in-life body weight on day 21 post partum.

2.6. Reproductive parameters

Reproductive performance of Fa and Fl parental **animals was summarized by the following indices: male** mating index (%): (number of males with confirmed

mating = vaginal sperm or pregnancy in cohabited female x 100)/uumber of males placed with females; male fertility index (%): (number of males proving their **fertility = parturition or presence of embryos or fetuses** in utero in cohabited female x 100)/number of males placed with females; female mating index (%): (number of females mated = vaginal sperm or pregnancy x 100)/ number of females placed with males); female fertility index (%): (number of females pregnant = embryos or fetuses in utero or giving birth x 100)/number of females placed with males); gestation index (%): (number of females with liveborn pups x 100)/number of females pregnant; livebirth index (%): (number of liveborn pups x 100)/number of liveborn + stillborn pups; post-implantation loss (%): (number of implantations-number of pups delivered) x 100/number of implantations; viability index (%): (number of pups alive on day 4 post partum (preculling) x 100)/number of liveborn pups; lactation index (%): (number of pups alive on day 21 post partum x 100)/number of pups alive on day 4 post partum (postculling).

2.7. Statistical analysis

The experimental unit of analysis was the parental **animal or litter, except for livebirth, viability and** tation indices, which were analysed by dose group. Dunnett's test (Dunnett, 1955; Dunnett, 1964) was used for simultaneous comparison of all dose groups with the control group in food and water consumption (g/ parental animal), body weights and body weight change (parental animals and litters), estrus cycle length, number of mating days to successful mating, duration of gestation, number of pups delivered per litter, and time **to sexual maturation (days to vaginal opening or pre-**putial separation). Kruskal-Wallis (2-sided) tests followed if significant by pairwise Wilcoxon tests (Hettmansperger, 1984; Nijenhuis and Wilf, 1978; Siegel, 1956). Wilcoxon tests were also used to assess group differences in terminal parental body weights and parental and pup absolute and relative organ weights, proportion of pups per litter with necropsy observations, differential follicle counts, total spermatids/g testis or cauda epididymides, and % sperm motility, for which variable Bonferoni-Holm adjustment (Holm, 1979) was applied. Fisher's Exact test of equal proportions (Siegel, 1956), one-sided, was used for pairwise comparison of each dose group with the control for male and female mating and fertility indices, gestation index, females with liveborn, stillborn and with all stillborn pups, livebirth index, pups stillborn, pups **dead, pups cannibalized, pups sacrificed moribund, via-**bility and lactation indices, number of litters containing pups with necropsy findings, sexual maturation data (vaginal opening or preputial separation), and males with >4% abnormal sperm.

3. Results

3.1. FO parental observations

Calculated test substance intake in the 150, 450 and 1000 mg/kg bw/day groups for the premating phase was 153, 460 and 1015 mg/kg bw/day body weight/day in males (mean of weeks 0-17) and 156, 468 and 1039 mg/kg bw/day for females (mean of weeks 0-10). For females test substance intake was 152, 451 and 1025 mg/kg bw/day during gestation (mean of days 0-20), and 137, 413 and 867 mg/kg bw/day during lactation (mean of days 1-14).

Except for two 1000 mg/kg bw/day males with urine-smeared fur (week 7-17), there were no treatment-related clinical signs. One non-pregnant 150 mg/kg bw/ day female was found dead in week 19 (the last week of treatment) with severe chronic progressive glomerulo-nephropathy; this death was not considered treatment-related.

Food consumption was reduced by up to 10% compared to controls in 1000 mg/kg bw/day males, starting **in premating week 5. Treatment had less consistent effects on female food consumption, which was reduced** at 1000 mg/kg bw/day during lactation days 4-14, and at 450 mg/kg bw/day during premating weeks 2-9 and gestation days 0-14).

Body weight was reduced at 1000 mg/kg bw/day in **males, starting in premating week 3-4; mean terminal** body weight was significantly lower than controls (-16%). Effects in females were not so marked or consistent; at 1000 mg/kg bw/day, body weights were reduced during gestation days 7-20 and throughout lactation; lactation (days 0-20), but mean terminal body weight was not significantly lower than controls (-5%).

At necropsy, significant effects on organ weights (defined as statistical significance in both absolute and **relative weights, and consistent with a dose-response** relationship) were seen only in females at 1000 mgt kg bw/day, with increased liver weight (117% of control mean absolute weight and 123% of control mean relative weight) and reduced ovary weight (83% of control mean absolute weight and 86% of control mean relative weight). Histopathology revealed minimal or slight hepatic cytoplasmic eosinophilia in most 1000 mgt kg bw/day animals (males *0/010/22,* females 0/0/0/23, in the 0, 150, 450 and 1000 mg/kg bw/day groups, respectively). There was no histopathological correlate for the reduced ovary weights. As summarized in Table 3, erosion/ulceration of the glandular stomach mucosa was noted in a single 1000 mg/kg bw/day male and few dosed females (011/3/4 in the 0, 150, 450 and 1000 mgt kg bw/day groups, respectively). Treatment had no ad-**verse effects On spermatid and spenn number, morphol-**ogy or motility (Table 7).

3.2. FO reproductioll/Fl pup data

As shown in Table I, treatment had no effect on **estrus cycle length, time to positive mating, duration** of gestation, post-implantation losses, offspring sex and survival at and after birth, and the associated mating, fertility, livebirth, viability and lactation indices. At 1000 mg/kg bw/day, there was a significantly reduced **mean number of implantation sites per dam and corre-**spondingly reduced litter size, reduced pup weight gain from day 4 to 21 post partum, and delayed vaginal opening and preputial separation. Vaginal opening was also delayed at 450 mg/kg bw/day. From these findings only reduced pup body weight gain was considered to be related to the test substance. The average number of implants per dam and the litter size of the high dose group (10.0 and 9.2) were just marginally below the his-**torical control range, whereas the number of implants** per dam in the control group (12.0) was unusually high and above the historical range of the test facility (10.2-11.5). The statistically significantly lower litter size is a **direct consequence of this phenomenon and is therefore** not considered to be an independent toxicologically relevant adverse effect of the test compound. Furthermore, in the preceding I-generation range finding study in rats, even at 10,000 ppm in the diet (about 1190 mg/kg bw/day) no test substance-related effects on implantation and litter size were recorded (BASF AG, unpublished data).

Vaginal patency was within the historical control range (30.8-33.8 days) in all groups, the control value (31.3 days) being close to the lower limit and the high dose value (33.8 days) being close to the upper limit of the historical range. Thus, the statistically significant difference between control and high dose group is likely to be the caused by an unusually low control value rather than indicating a particular effect of the test compound **on sexual maturation. Therefore, this finding is consid-**ered to be an incidental event. Preputial separation (45.4 days) in high dose males slightly exceeded the historical range of the test facility (42.5-45.0 days). However, the average body weights of these males were significantly below the concurrent control, which presumably contributed to this slight delay of sexual maturation. Thus, this apparent slight delay is probably the result of a general retardation of the development of the male FI pups rather than a specific effect of the test compound.

As shown in Table 2, the litter incidence of pups at weaning with dilated renal pelvis was statistically significantly increased at 450 and 1000 mg/kg bw/day (2.4% and 2.9%, respectively). Dilated renal pelvis is a physiological stage of development of this organ and is frequently observed (historical control range for affected pups per litter 0-3.5%). Thus, an association of the slightly increased incidence of this finding to the test substance is not assumed. All other pup necropsy observations were not related to dose. Changes of high dose

Table I
FO reproductive *data/FI* progeny

Dose group (mglkg bw/day)	O	150	450	1000
FO mean ± SD estrus cycle length (days)	4.1 ± 0.7	**4.3 ± 1.4**	4.7± 3.1	4.2 ± 1.3
FO male mating index (%)	100	100	100	100
FO male fertility index (%)	96	88	92	100
FQ female mating index (%)	100	100	100	100
FO female fertility index (%)	96	88	92	100
Mean ± SD days to positive mating	**2.6 ± 1.2**	**2.5 ± 0.9**	2.4 ± 1.0	**2.5 ± 0.9**
Mean ± SD duration of gestation (days)	**22.1 ± 0.6**	21.7 ± 0.6	**22.0 ± 0.7**	**22.0 ± 0.5**
FO gestation index (%)	100	100	96	100
Number of FI litters	24	22	22	25
Mean implantation sites per dam	**12.0 ± 2.3**	12.3 ± 1.0	**11.3±2.4**	10.0 ± 2.0"
Mean ± SD % post-implantation loss	**8.1 ± 11.2**	**3.6 ± 4.7**	11.8 ± 22.6	**7.6 ± 8.8**
Mean ± SD FI pups delivered per litter	**11.0 ± 2.5**	11.9±0.9	10.9 ± 2.1	**9.2 ± 2.0"**
FI livebirth index (%)	99	99	99	99
Sex ratio of live Fl newborns (% male)	46	49	53	44
Number of Fl pups stillborn	3	3	2	3
Number of FI pups died preweaning	3	2	5	6
Number of Fl pups cannibalized	1	O	O	5
FI viability index (survival day 0–4 post partum) (%)	98	100	98	96
FI lactation index (survival day 4--21 post partum) (%)	100	99	99	99
FI pup weight, mean ± SD per litter, day 1 post partum (g)	6.3 ±0.6	6.1 ± 0.5	6.4 ± 0.7	**6.2 ± 0.7**
FI pup weight gain, mean ± SD per litter, day 1-4 post partum, precull (g)	**3.1 ± 0.7**	2.9 ±0.6	3.0 ± 0.6	2.7 ± 0.8
FI pup weight gain, mean ± SD per litter, day 4--21 post partum, postcuII (g)	37.1 ± 2.7	**36.7 ± 2.6**	36.8 ± 2.8	**31.6 ± 4.2ᵒᵒ**
FI mean ± SD age at vaginal opening (days)	31.3 ± 1.6	32.5 ± 1.3	**33.6 ±**	33.8 ± 2.2"
FI mean ± SD weight at vaginal opening (g)	90.8 ± 10.6	95.0 ± 10.1	98.7 ± 10.1'	91.9 ± 8.2
F2 mean ± SD age at preputial separation (days)	**43.6 ± 1.2**	**43.7 ± 1.4**	43.4 ± 1.3	45.4 ± 1.5"
FI mean ± SD weight at preputial separation (g)	**172.5 ± 10.3**	175.7 ± 15.3	173.9 ± 10.4	168.7 ± 15.8

* $p \leqslant 0.05$; ** $ll \leqslant 0.01$ versus controL

Table 2
FI pup necropsy observations

Number of affected pups!litters

Dose group (mg/kg bw/day)	O	150	450	1000
Number of pups/litters evaluated	213/24	207/22	189/22	171/25
Cardiomegaly (globular shaped heart) (%)	0.0	0.0	0.8	0.0
Dilated renal pelvis (%)	0.0	0.5	2.4*	2.9"
Hemorrhagic thymus (%)	0.0	0.0	2.1'	1.0
Malpositioncd carotid branch (abnomlal course of carotids) (%)	0.6	0.0	0.0	0.0
Misshapen spleen (%)	0.0	0.5	0.0	0.0
Small testis (%)	0.0	0.0	0.5	0.0
Partly cannibalized (%)	0.0	0.0	0.6	0.0
Incisors sloped (%)	1.0	0.5	0.0	0.0
Post-mortem autolysis (%)	1.0	0.0	0.6	2.3
Number with any finding (%)	2.7	1.5	6.9'	6.2

Terminology according to Wise   al. (1997).

* $p \leqslant 0.05$ versus control.

Table 3
FO parental pathology

Sex	Male				Female			
Number of animals finding (most relevant observations; 1I-examined = 25 per dose alld sex)								
Dose group (mg/kg bw/day)	0	ISO	450	1000	0	150	450	1000
Glandular stomach-erosion/ulcer	0	0	0	I	0	1	3	4
Liver-minimal or slight cytoplasmic eosinophilia	0	0	0	22	0	0	0	23
Pituitary-cyst, pars intennedia	0	0	0	3	1	0	0	0
Spleen-marked hemosiderin (perls stain)	7	7	5	18	0	0	0	0

pup brain, spleen andlor thymus weights were secondary to the observed body weight decrements.

3.3. Fl parental observations

Calculated test substance intake in the 150, 450 and 1000 mg/kg bwlday groups was 154, 461 and 1028 mg/kg bwlday in males (mean of weeks 0-15). In females, it was 158,474 and 1057 mglkg bwlday during the premating period (mean of weeks 0-10); 149, 443 and 976 mg/kg bw/day during gestation (mean of days 0-20), and 133, 396 and 873 mg/kg bw/day during lactation (mean of days 1-14).

Except for four males and one female at 1000 mg/kg bw/day with urine-smeared fur starting in week 7-**15, there were no treatment-related clinical signs. One** 450 mg/kg bw/day female was sacrificed moribund on the first day of the premating period (i.e. just after selection as a parental animal), with severe dilation of the cecum and moderate dilation of the jejunum (both with liquid contents), but no other notable pathology; this animal was not replaced. One 450 mg/kg bw/day female was found dead, unable to deliver on day 23 of gestation, with no notable pathology findings. Neither of these deaths was considered treatment-related.

Food consumption was reduced at 1000 mg/kg *bwl* day, in males during the first 7 weeks of the premating

period, and in females during gestation and lactation, by about 10% compared to controls.

Mean body weight at 1000 mg/kg bw/day was reduced by approximately 10% in both males and females throughout the premating, gestation and lacatation periods. At necropsy, mean terminal body weights **were significantly lower than controls in both males** (-14%) and females (-4%). Significant effects on organ weights, defined as statistical significance in both abso**lute and relative weights, and consistent with a dose-response relationship, were seen only in females: at** 450 and 1000 mg/kg bw/day, absolute liver weight was significantly increased compared to controls (Il1% and 116%, respectively) as well as relative organ weight (110% and 121%, respectively). Histopathology revealed minimal or slight hepatic cytoplasmic eosinophilia at 1000 mg/kg bw/day in all males and half of the females (males *010/0/25*, females *0/010112,* in the 0, 150, 450 and 1000 mg/kg bwlday groups, respectively). Absolute and relative ovary weight was reduced only at 1000 mg/kg bw/day (82% and 86% of control, respectively). There was no histopathological correlate for the reduced ovary weights.

As summarized in Table 6, erosion/ulceration of the glandular stomach mucosa was noted in 1000 mg/kg bw/day males (group incidences *0101014* in males and *3/312/4* in females). One 1000 mg/kg bw/day male had unilateral reduced testis size and diffuse tubular

Table 4
FI reproductive data/F2 progeny

Dose group (mg/kg bw/day)	0	150	450	1000
FI mean ± SD estrus cycle length (days)	4.1 ± 0.5	**4.2 ± 0.5**	3.9 ± 0.3	4.2 ± 0.6
FI male mating index (%)	100	100	100	100
FI male fertility index (%)	96	96	96	100
FI female mating index (%)	100	100	100	100
FI female fertility index (%)	96	96	96	100
Mean ± SD days to positive mating, FI	3.0 ± 1.4	**3.0 ± 1.2**	3.1 ± 1.4	**2.6 ± 0.7**
Mean ± SO duration of gestation, FI (days)	22.0 ± 0.4	22.3 ± 0.5	**22.3 ± 0.5**	**22.3 ± 0.5**
FI gestation index (%)	100	100	96	100
Number of F2 litters	24	24	22	25
Mean ± SO implantation sites per dam	12.4 ± 2.3	**11.5 ± 2.4**	10.7 ± 2.8'	10.3 ± 1.8"
Mean ± SD % post-implantation loss	8.8 ± 9.4	13.5 ± 19.2	8.3 ± 12.5	**5.7 ± 8.4**
Mean ± SO F2 pups delivered per litter	11.4 ± 2.4	**10.2 ± 3.2**	9.8 ± 2.9	9.7 ± 1.8
F2 livebirth index (%)	99	97	96	97
Sex ratio of live F2 newborns (% male)	47	54	46	48
Number of F2 pups stillborn	2	7	9'	8'
Number of F2 pups died preweaning	4	2	4	6
Number of F2 pups cannibalized	5	6	0	12*
F2 viability index (survival day 0–4 post partum) (%)	97	97	98	92
F2 lactation index (survival day 4-21 post partum) (%)	99	100	100	100
F2 pup weight, mean ± SD per litter, day I post partum (g)	5.9 ± 0.6	**6.3 ± 0.8**	**6.6 ± 0.9****	**5.9 ± 0.7**
F2 pup weight gain, mean ± SO per litter, day 1-4 post partum, precuU (g)	2.8 ± 0.5	3.2 ± 0.7	3.5 ± 0.8"	2.8 ± 0.6
F2 pup weight gain, mean ± SD per litter, day 4-21 post partum, postcull (g)	36.5 ± 3.1	36.5 ± 4.1	38.0 ± 4.7	**29.3 ± 4.0****
F2 mean ± SO age at vaginal opening (days)	32.9 ± 2.7	33.5 ± 3.4	33.8 ± 3.7	35.5 ± 3.5"
F2 mean ± SO weight at vaginal opening (g)	93.5 ± 10.9	96.8 ± 13.4	**100.2 ± 12.6**	95.3 ± 13.4
F2 mean ± SO age at preputial separation (days)	42.8 ± 1.5	**42.8 ± 1.3**	42.9 ± 1.3	45.1 ± 1.9"
F2 mean ± SO weight at preputial separation (g)	165.5 ± 8.9	165.8 ± 13.5	170.0 ± 9.3	**153.9 ± 12.8"**

• $p \leqslant 0.05$; ** $p \leqslant 0.01$ versus control.

Table 5
F2 pup gross pathology (day 0-21 p.p.)

Number of affected pups/litters

Dose group (mg/kg bw/day)	0	150	450	1000
Number of pups/litters evaluated	*215/24*	*190/23*	165/22	181/25
Conjoined twins (%)	**0.0**	**0.4**	0.0	0.0
Dilated renal pelvis (%)	**0.8**	**0.0**	0.0	1.7
Empty stomach (%)	**0.0**	**0.0**	0.6	0.0
Hemorrhagic thymus (%)	**0.0**	**0.0**	0.6	1.8
Hydronephrosis (%)	**0.0**	**0.0**	0.0	0.5
Hydroureter (%)	**0.0**	**0.0**	0.0	0.5
Incisors sloped ('Yo)	**0.0**	**0.0**	0.9	0.0
Post-mortem autolysis ('Yo)	**4.5**	**3.9**	4.1	5.8
Situs inversus (%)	**0.5**	**0.0**	0.0	0.0
Small testis ('Yo)	**0.4**	**0.4**	0.0	0.8
Number with any finding ('Yo)	**6.3**	**4.7**	6.2	10.1'

Terminology according to Wise et al. (1997).
• $p \leqslant 0.05$ versus control.

degeneration (seminiferous tubuli contained only Sertoli cells), and corresponding left epididymal reduced size and aspermia, but the contralateral (right) testis and epididymis were grossly normal with normal sperm counts, and the animal mated successfully. This isolated finding **has been observed in historical controls; it was considered spontaneous and unrelated to treatment. Minimal** or slight focal testicular tubular degeneration was observed in one control and two high dose males, but **sperm parameters were normal and all three animals** mated successfully; this finding was also considered **unrelated to treatment.**

Treatment had no adverse effects on spermatid and sperm number, morphology or motility, or on differential follicle counts (Table 7). (The significant difference in epididymal spermatids between 1000 mglkg bw/day and control groups is attributable to anomalously high control values, as noted in Table 7.)

3.4. Fl reproduction/F2 pup data

Calculated test substance intake of the F2 offspring in the 150, 450 and 1000 mglkg bw/day groups was 175, 523 and 1In mglkg bw/day in males (mean of weeks 0–3 post-weaning) and 173, 519 and 1077 mglkg bw/day in females (mean of weeks 0–2 post-weaning).

As shown in Table 4, treatment had no significant **effect on estrus cycle length, time to positive mating, duration of gestation, post-implantation losses, off**spring sex and survival after birth, and the associated mating, fertility, livebirth, viability and lactation indices.

The mean number of implantation sites was statistically significantly reduced at 450 mglkg bw/day and 1000 mglkg bw/day (10.7 and 10.3, respectively) compared to controls (12.4). However, the average number of implants per dam in the control group was unusually high and considerably above the historical range of the test facility (10.2-11.5), whereas the number of implants

Table 6
FI parental pathology

Sex	Male				Female			
Number of animals \Vii" finding (most relevant observations; ll-examined = 25 per dose and sex)								
Dose group (mg/kg bw/day)	0	150	450	1000	0	150	450	1000
Glandular stomach-erosion/ulcer	0	0	0	4	3	3	2	4
Liver-minimal or slight cytoplasmic eosinophilia	0	0	0	25	0	0	0	12
Pituitary-cyst, pars intermedia	2	0	0	5	0	0	0	3
Spleen-marked hemosiderin (Perls stain)	3	3	4	6	0	0	0	0

Table 7
Parental spenn analyses and follicle counts (mean ± SD)

Dose group (mg/kg bw/day)	O	1000
Number evaluated per sex	25	25
FO spenn analysis		
Total spermatids/g testis	**125 ± 16.2**	**125 ± 27.7**
Total spermatids/g cauda epididymis	677 ± 133.7	641 ± 141.2
% abnormal sperm	1.4 ± 0.9	2.0 ± 1.4
% motility (mean ± SD)	88±11.0	86±11.2
Fl spenn analysis		
Total spermatids/g testis	119±11.5	128 ± 14.0
Total spennatids/g cauda epididymis	763 ± 108.9	700 ± 135.7'
% abnormal sperm	1.3±1.1	1.5 ± 1.5
% motility (mean ± SD)	89 ± 7.7	87 ± 10.5
FI differential follicle counts		
Primordial follicles (mean)	**224**	243
Growing follicles (mean)	42	43

• $p \leqslant 0.05$ **versus control, due to anomalously high control value,** which exceeded historical control range (in 19 control groups from 10 previous studies of this type, total spermatids/g cauda epididymis means ranged from 517 to 727; mean of historical means = 625).

per dam in the high dose group was well within the historical range. These findings were therefore considered **to be incidental and not associated to the test substance.**

The incidence of stillborn pups was statistically significantly higher than controls in the 450 and 1000 mg/kg bw/day groups (4.2% and 3.3%, respectively). **ever, this increase was not related to dose and the rates** of stillborn pups were well within the historical range of the test facility (0-4.3%), and was therefore considered not test substance-related.

The viability index as indicator for pup mortality (pups died and cannibalized) between days 0 and 4 p.p. was statistically significantly reduced in the 1000 mg/kg bw/day group. Loss of one single complete litter was mainly responsible for the reduced viability index in this dose group as all of the delivered pups died or were cannibalized between day I and 4 p.p. This is **considered to be an incidental event and not due to** the test compound.

At 1000 mg/kg bw/day, pup weight gain was reduced from day 4 to 21 post partum and in the selected off-**spring also during entire rearing; subsequently vaginal** opening and preputial separation were slightly delayed (Table 4). F2 gross pathology did not reveal any other treatment related findings (Table 5). Changes of high

dose pup brain, spleen and/or thymus weights were secondary to the observed body weight decrements.

4. Discussion

In the present two generation reproduction toxicity study, OMC had no adverse effect on estrus cycle, sperm number, morphology and motility, differential follicle counts, mating, fertility, gestation and parturition. Parental food consumption and body weight were reduced at 1000 mg/kg bw/day (necropsy bodyweight -14% to -16% in males, -4% to -5% females). General systemic effects were evident at 1000 *mg/kg* bw/day in tenns of increased liver weight and hepatic cytoplasmic **eosinophilia due to hepatic enzyme induction and are** considered to be an adaptive rather than a frank toxicological effect. **An apparent increase in the incidence of erosion/ulceration of the glandular stomach mucosa** was noted in FO females *(0/1/3/4)* as well as in F1 males *(0101014)* and females *(31312/4).* From the F1 females it can be seen that this finding is also observed in control **animals. However, an incidence of 4 affected animals was not observed in this study and also not in our his-**torical control data. Therefore, it is concluded that the **increased OCCurrence of erosion/ulceration of the glan-**dular stomach mucosa at 1000 mg/kg bw/day may have been related to treatment. An effect of OMC on liver **was previously reported in a 13-week repeat-dose toxic-**ity study, in which females at the high dose (1000 mg/kg bw/day) had increased glutamate dehydrogenase **activity which reversed during the recovery period** (SCC, 1996), but there are no previous reports of an OMC effect on glandular stomach mucosa.

There was a statistically significantly reduction of the number of implantation sites at 1000 mg/kg bw/day in both parental generations, and also at 450 mg/kg bwl day in F1 parents, compared to controls. It should be noted that the number of implantation sites in the FO and FI female control animals was particularly high, **in fact exceeded the historical range recorded to date** in the test facility. The number of implantation sites in the FO females at 1000 mg/kg bw/day (10.0 ± 2.0) was in fact very close to the historical control range (10.2-11.5). In the F1 generation females the number of

implantation sites at 450 mglkg bw/day (10.7 ± 2.8) and 1000 mglkg bw/day (10.3 ± 1.8) was fully within the historical range. The fact that subsequent follicle counts were normal in all FI parents indicates that if the **reduction in implantation rate was truly related to treatment,** it was not related to egg maturation.

Moreover, in both generations, post-implantation loss was normal for all groups, again indicating the absence of a treatment related effect on this parameter. Since a marginally lower implantation rate was noted in both parental generations, it is possible that this is a secondary, albeit a small effect, at the high dose. **Reductions in the implantation rate are not unusual in animals** which are showing toxicity, as is the case in the high dose females here, which had reduced food consumption and body weight, liver effects and stomach erosion. A small pre-implantation loss in a multigeneration study such as this, at a dose where the parents show signs of **toxicity, is not unusual and is most likely secondary to** the maternal toxicity. Therefore, we conclude that the slight reduction in the number of implantation sites in the high dose FO and FI females was unlikely to be directly related to treatment.

In both generations, pup weight gain was reduced at 1000 *mg/kg* bw/day from day 4 to 21 oflactation, resulting in pup body weights that were 13-16% below controls at weaning. At 1000 mglkg bw/day, significantly lower body weights were also noted post-weaning in the reared offspring of both generations. In weeks 2 and 3 after weaning (the time when sexual maturation was recorded), the average body weights ranged 6-**12% below concurrent controls. Sexual maturation (vag-**inal opening and preputial separation) was slightly delayed, I.e. occurred a few days later than in control animals, in male and female offspring of both Fl and F2 generations at 1000 mglkg bw/day. The conclusion that this is a test substance-specific effect could be made **if time to sexual maturation were the only relevant parameter. However, if body weight rather than** dar age is the essential component driving sexual ration, then a different conclusion would need to be drawn. In this study the body weights of the 1000 mgl kg bw/day offspring (both sexes) on the day of sexual **maturity were quite comparable to the concurrent** trol values at the time when they reached sexual maturation (i.e. 2-3 days earlier). It is not unlikely that delayed body weight development would result in a delay in sexual maturation. To better determine the relationship between body weight development and the time to reach sexual maturity, we recommend that a daily determination of body weight during recording of vaginal patency (from day 27 p.p. onwards) and preputial separation (from day 40 p.p. onwards) should be performed. Such data may help to assess more correctly if a primary (selective) or secondary test substance-related effect is implicated.

As noted in the Introduction, OMC has no andro-**genic or antiandrogenic activity, and no or, at high dos-**ages only, very weak estrogenic potential. The results of the present study indicate that OMC has no estrogenic potential in vivo in parental animals and their offspring **when continuously treated over two generations during premating, gestation, lactation and sexual maturation.**

In conclusion, the no observed adverse effect level (NOAEL) of OMC by continuous dietary administration in this study is 450 mglkg bw/day for fertility and reproduction parameters, for systemic parental and developmental toxicity. This is based on reduced body weights, increased liver weight and hepatic cytoplasmic **eosinophilia in the parents, a secondary reduction in** implantation rate, and reduced body weights and delayed sexual maturation of the pups at 1000 mgl kgbw/day.

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