Ethyl methanesulphonate

Evaluation of the effects on reproduction, recommendation for classification

Gezondheidsraad **Voorzitter**
Health Council of the Netherlands

Aan de Staatssecretaris Sociale Zaken en Werkgelegenheid

Onderwerp : Aanbieding advies 'Ethyl methanesulphonate'
Uw kenmerk : DGV/MBO/U-932542
Ons kenmerk : U 1049/AvdB/543-B8
Bijlagen : 1
Datum : 30 september 2004

Mijnheer de staatssecretaris,

Bij brief van 3 december 1993, nr DGV/MBO/U-932542, verzocht de Staatssecretaris van Welzijn, Volksgezondheid en Cultuur namens de Minister van Sociale Zaken en Werkgelegenheid om naast het afleiden van gezondheidskundige advieswaarden ook te adviseren over andere onderwerpen ten behoeve van de bescherming van beroepsmatig aan stoffen blootgestelde personen. In 1995 heeft de Staatssecretaris van Sociale Zaken en Werkgelegenheid besloten tot het opstellen van een zogenaamde niet-limitatieve lijst van voor de voortplanting vergiftige stoffen. Op deze lijst komen stoffen die volgens de richtlijnen van de Europese Unie ingedeeld moeten worden in categorie 1, 2 en 3 wat betreft effecten op de voortplanting en stoffen die schadelijk kunnen zijn voor het nageslacht via de borstvoeding. De Gezondheidsraad is verzocht om voor stoffen een classificatie volgens de EU-criteria voor te stellen.

In dit kader bied ik u hierbij een advies aan over ethyl methaansulfonaat. Dit advies is opgesteld door de Commissie Reproductietoxische stoffen van de Gezondheidsraad en beoordeeld door de Beraadsgroep Gezondheid en Omgeving.

Ik heb deze publicatie heden ter kennisname aan de Minister van Volksgezondheid, Welzijn en Sport en aan de Staatssecretaris van de Volkshuisvesting, Ruimtelijke Ordening en Milieu gestuurd.

Hoogachtend,

Prof. dr JA Knottnerus

Bezoekadres
Parnassusplein 5
2511 VX Den Haag
Telefoon (070) 340 7520
E-mail: A.vd.burght@gr.nl

Postadres
Postbus 16052
2500 BB Den Haag
Telefax (070) 340 75 23
www.gr.nl

Ethyl methanesulphonate

Evaluation of the effects on reproduction, recommendation for classification

Committee for Compounds Toxic to Reproduction,
a committee of the Health Council of the Netherlands

to:

the Minister and State Secretary of Social Affairs and Employment

No. 2004/08OSH, The Hague, September 9, 2004

The Health Council of the Netherlands, established in 1902, is an independent scientific advisory body. Its remit is "to advise the government and Parliament on the current level of knowledge with respect to public health issues..." (Section 21, Health Act).

The Health Council receives most requests for advice from the Ministers of Health, Welfare & Sport, Housing, Spatial Planning & the Environment, Social Affairs & Employment, and Agriculture, Nature & Food Quality. The Council can publish advisory reports on its own initiative. It usually does this in order to ask attention for developments or trends that are thought to be relevant to government policy.

Most Health Council reports are prepared by multidisciplinary committees of Dutch or, sometimes, foreign experts, appointed in a personal capacity. The reports are available to the public.

This report can be downloaded from www.healthcouncil.nl.

Preferred citation:
Health Council of the Netherlands. Committee for Compounds Toxic to Reproduction. Ethyl methanesulphonate; Evaluation of the effects on reproduction, recommendation for classification. The Hague: Health Council of the Netherlands, 2004; publication no. 2004/08OSH.

ISBN: 90-5549-540-9

Contents

Samenvatting

In het voorliggende advies heeft de Gezondheidsraad ethyl methaansulfonaat (EMS) onder de loep genomen. EMS is een direct alkylerende verbinding, die met name gebruikt wordt als modelstof in biomedisch onderzoek. Dit advies past in een reeks adviezen waarin de Gezondheidsraad op verzoek van de minister van Sociale Zaken en Werkgelegenheid de effecten van stoffen op de voortplanting beoordeelt. Het gaat vooral om stoffen waaraan mensen tijdens de beroepsuitoefening kunnen worden blootgesteld. De Commissie Reproductietoxische stoffen, een commissie van de raad, kijkt naar effecten op de vruchtbaarheid van mannen en vrouwen zowel als op de ontwikkeling van het nageslacht. Bovendien worden effecten van blootstelling van de zuigeling via de moedermelk beoordeeld.

Op basis van Richtlijn 93/21/EEC van de Europese Unie doet de commissie een voorstel voor classificatie. Voor EMS komt de commissie tot de volgende aanbevelingen:

- Voor effecten op de fertiliteit adviseert de commissie omEMS te classificeren in categorie 2 (*Stoffen die dienen te worden beschouwd alsof zij bij de mens de vruchtbaarheid schaden*) en te kenmerken met T;R60.
- Voor effecten op de ontwikkeling van het nageslacht adviseert de commissie om EMS te classificeren in categorie 3 (*Stoffen die in verband met hun mogelijke voor de ontwikkeling van de mens schadelijke effecten reden geven tot bezorgdheid)* en te kenmerken met Xn;R63.
- Voor effecten tijdens lactatie, adviseert de commissie om EMS niet te kenmerken wegens onvoldoende geschikte gegevens.

Op grond van wat bekend is uit onderzoek naar ontwikkelingsstoornissen als gevolg van blootstelling aan EMS, classificeert de commissie EMS in categorie 3 (er is reden voor bezorgdheid). Op basis van het toxicologisch werkingsmechanisme van direct alkylerende stoffen kan echter gesteld worden dat EMS waarschijnlijk schadelijke effecten heeft op de ontwikkeling.

Executive summary

In the present report the Health Council of the Netherlands reviewed ethyl methanesulphonate (EMS). EMS is a direct alkylating substance, which is among other things used as a model compound in biomedical research. This report is part of a series, in which the Health Council evaluates the effects of substances on reproduction, at request of the Minister of Social Affairs and Employment. It mainly concerns substances to which man can be occupationally exposed. The Committee for Compounds toxic to reproduction, a committee of the Health Council, evaluates the effects on male and female fertility and on the development of the progeny. Moreover, the effects of exposure on lactation are considered.

According to the Directive 93/21/EEC of the European Union, the committee recommends a classification. The committee's recommendations for EMS are:

- For effects on fertility, the committee recommends classifying EMS in category 2 (*substances which should be regarded as if they impair fertility in humans*) and labeling EMS with T;R60.
- For developmental toxicity, the committee recommends classifying EMS in category 3 (*Substances which cause concern for humans owing to possible developmental toxic effects)* and labeling EMS with Xn;R63.
- For effects during lactation, the committee is of the opinion that EMS cannot be labelled due to a lack of appropriate data.

Based on the available studies concerning the effects of exposure to EMS on development, the committee recommends classifying EMS in category 3 (cause concern for humans). However, general information about the mechanism of toxicity of direct alkylating compounds shows that adverse effects on development are to be expected after exposure to EMS.

Chapter 1

Scope

1.1 Background

As a result of the Dutch regulation on registration of compounds toxic to reproduction that came into force on 1 April 1995, the Minister of Social Affairs and Employment requested the Health Council of the Netherlands to classify compounds toxic to reproduction. The classification is performed by the Health Council's Committee for Compounds toxic to reproduction according to the guidelines of the European Union (Directive 93/21/EEC). The committee's advice on the classification will be applied by the Ministry of Social Affairs and Employment to extend the existing list of compounds classified as toxic to reproduction (category 1, 2 or 3) or labelled as may cause harm to breastfed babies (R64).

1.2 Committee and procedure

The present document contains the classification of ethyl methanesulphonate by the Health Council's Committee for Compounds toxic to reproduction. The members of the committee are listed in Annex A. The first draft of this report was prepared by Dr. W. Mennes, Centre for Substances and Integrated Risk Asssessment, RIVM, Bilthoven, The Netherlands, by contract with the Ministry of Social Affairs and Employment. The classification is based on the evaluation of published human and animal studies concerning adverse effects with respect to fertility and development and lactation of the above mentioned compound.

Classification and labelling was performed according to the guidelines of the European Union listed in Annex C.

Classification for fertility and development:	
Category 1	Substances known to impair fertility in humans (R60)
	Substances known to cause developmental toxicity in humans (R61)
Category 2	Substances which should be regarded as if they impair fertility in humans (R60)
	Substances which should be regarded as if they cause developmental toxicity in humans (R61)
Category 3	Substances which cause concern for human fertility (R62)
	Substances which cause concern for humans owing to possible developmental toxic effects (R63)
No classification for effects on fertility or development	
Labelling for lactation:	
	May cause harm to breastfed babies (R64)
	No labelling for lactation

In 2003, the President of the Health Council released a draft of the report for public review. The individuals and organisations that commented on the draft report are listed in Annex B. The committee has taken these comments into account in deciding on the final version of the report.

1.3 Additional considerations

The classification of compounds toxic to reproduction on the basis of the Directive 93/21/EEC is ultimately dependent on an integrated assessment of the nature of all parental and developmental effects observed, their specificity and adversity, and the dosages at which the various effects occur. The directive necessarily leaves room for interpretation, dependent on the specific data set under consideration. In the process of using the directive, the committee has agreed upon a number of additional considerations.

- If there is sufficient evidence to establish a causal relationship between human exposure to the substance and impaired fertility or subsequent developmental toxic effects in the progeny, the compound will be classified in category 1, irrespective the general toxic effects (see Annex C, 4.2.3.1 category 1).
- Adverse effects in a reproductive or developmental study, in the absence of data on parental toxicity, occurring at dose levels which cause severe toxicity in other studies, need not necessarily lead to a category 2 classification.
- If, after prenatal exposure, small reversible changes in foetal growth and in skeletal development (e.g. wavy ribs, short rib XIII, incomplete ossification) in offspring occur at a higher incidence than in the control group in the absence of maternal

effects, the substance will be classified in category 3 for developmental toxicity. If these effects occur in the presence of maternal toxicity, they will be considered as a consequence of this and therefore the substance will not be classified for developmental toxicity (see Annex C, 4.2.3.3 developmental toxicity final paragraph).

- Clear adverse reproductive effects will not be disregarded on the basis of reversibility per se.
- Effects on sex organs in a general toxicity study (e.g. in a subchronic or chronic toxicity study) may warrant classification for fertility.
- The committee not only uses guideline studies (studies performed according to OECD standard protocols* for the classification of compounds), but non-guideline studies are taken into consideration as well.

1.4 Labelling for lactation

The recommendation for labelling substances for effects during lactation is also based on Directive 93/21/EEC. The Directive defines that substances which are absorbed by women and may interfere with lactation or which may be present (including metabolites) in breast milk in amounts sufficient to cause concern for the health of a breastfed child, should be labelled with R64. Unlike the classification of substances for fertility and developmental effects, which is based on a hazard identification only (largely independent of dosage), the labelling for effects during lactation is based on a risk characterisation and therefore also includes consideration of the level of exposure of the breastfed child.

Consequently, a substance should be labelled for effects during lactation when it is likely that the substance would be present in breast milk in potentially toxic levels. The committee considers a concentration of a compound as potentially toxic to the breastfed child when this concentration leads to exceedence of the exposure limit for the general population, eg the acceptable daily intake (ADI).

1.5 Data

Literature searches were conducted in the on-line databases Toxline+, Toxcenter and Medline, starting from 1965 upto 2003. Literature was selected primarily on the basis of the text of the abstracts. Publications cited in the selected articles, but not selected during the primary search, were reviewed if considered appropriate. In addition, handbooks and a collection of most recent reviews were consulted. References are divided in literature cited and literature consulted but not cited.

* Organisation for Economic Cooperation and Development

The committee chose to describe both the human and animal studies in the text. The animal data are described in more detail in Annex D as well. Of each study the quality of the study design (performed according to internationally acknowledged guidelines) and the quality of documentation are considered.

1.6 Presentation of conclusions

The classification is given with key effects, species and references specified. In case a substance is not classified as toxic to reproduction, one of two reasons is given:

- Lack of appropriate data preclude assessment of the compound for reproductive toxicity.
- Sufficient data show that no classification for toxic to reproduction is indicated.

1.7 Final remark

The classification of compounds is based on hazard evaluation* only, which is one of a series of elements guiding the risk evaluation process. The committee emphasises that for derivation of health based occupational exposure limits these classifications should be placed in a wider context. For a comprehensive risk evaluation, hazard evaluation should be combined with dose-response assessment, human risk characterization, human exposure assessment and recommendations of other organisations.

* for definitions see Tox95

Chapter 2

Ethyl methanesulphonate (EMS)

2.1 Introduction

Name	:	Ethyl methanesulphonate
CAS-no	:	62-50-0
Synonyms	:	Methanesulphonic acid ethyl ester; EMS; half-myleran;
Use	:	Biomedical research / experimental mutagen
Mol weight	:	124.15
Chem formula	:	CH_3-CH_2-O-SO_2-CH_3
Conversion factor	:	1 ppm = 5.1 mg/m^3 (101kPa, 25^o C); 1 mg = 0.20 ppm 1% = 10000 ppm = 50746 mg/m^3

Ethyl methanesulphonate (EMS) is possibly carcinogenic to humans (IARC74, IARC87), based on sufficient evidence in experimental animals. The Dutch Expert Committee on Occupational Substances considered EMS as a genotoxic carcinogen.

EMS is an alkylating substance which has been and still is extensively used for the study of toxic events, resulting from the ethylation of tissue macromolecules, notably DNA (DEC89). Depending on the ability of the compound to reach the molecular target, direct alkylating compounds may also react with the genetic material of germ cells and somatic cells of the foetus. As mutations can be the basis of abnormal development, the Committee Compounds toxic to reproduction is of the opinion that due to these properties, effects on reproduction are to be expected after exposure to EMS.

2.2 Human studies

Fertility

No publications were found concerning effects of EMS on human fertility.

Development

No publications were found concerning developmental effects of EMS in man.

Lactation

No publications were found concerning the excretion of EMS in human breast milk.

2.3 Animal studies

Tables 1 and 2 (Annex D) summarize the fertility and developmental toxicity studies with EMS in experimental animals.

General introduction

Studies have been performed to acquire insight into the heritable effects of EMS, either via a direct action on the fertilised egg, embryo and foetus or on the effects of these substances on gametogenesis and gametes in males. OECD has incorporated EMS as a positive reference substance in a number of testing guidelines for genotoxicity, among which guideline 478 for the assessment of dominant lethality in rodents (OEC96).

However, standard fertility or developmental toxicity studies are not available for EMS. Most of the studies are limited to parenteral (in particular intraperitoneal) and usually single dosing. Information on general or parental toxicity is commonly not provided, but in some studies, parental lethality has been observed. In other studies, not necessarily at lower dose levels, no effects on parental survival or even sublethal effects have been mentioned.

In the subsequent sections, a number of studies have been reviewed in more detail. These are chosen because they provide a sufficient level of experimental detail, or because they are illustrative for other aspects, e.g. effects on sperm quality, effects on

glutathione or covalent binding to biomacromolecules. By no means are these studies performed according to regulatory guidelines for reproductive toxicity studies.

Fertility studies

Fertility studies (only in males) with oral exposure have been summarized in table 1.1, Annex D. Parenteral fertility studies have been summarized in tables 1.2 and 1.3 for females and males, respectively, while in table 1.4 studies on effects on sperm cells and covalent binding studies have been described.

Most of the studies on fertility are dominant lethal tests for heritable genotoxicity, and have been carried out in males (oral or i.p.) or in females (i.p. only). In addition, sperm morphology studies have been incorporated. Most of the sperm morphology studies have been carried out in animals treated via i.p. injection.

One oral study into sperm quality is available (Tak00). The alkylating activity of EMS in the developing sperm cell has also been studied with radioactive EMS (Seg78). Although the latter study is no dominant lethal or sperm quality study *per se,* it provides a mechanistic background for the observations in these studies and is therefore incorporated in this section. In addition, the role of the male reproductive tract GSH status on the reproductive toxicity of EMS in male animals has been studied (Gan92, Tea85, Tea87).

The most relevant studies are summarized below.

Oral exposure

The effects of EMS on male fertility were studied in a combined dominant lethal / sperm morphology / sperm motility study in rats (Tak00). Male animals (Crj:CD(sd)IGS strain; n = 9 per group for dominant lethal study and n = 2 x 8 for sperm analysis study) were treated with 0 or 100 mg/kg bw/d during 5 consecutive days by oral gavage. Males were allowed to mate with untreated females 24 h after the last treatment or after a 28-day withdrawal period. Pregnant females were sacrificed at day 20 of pregnancy and offspring were examined for postimplantation losses and foetal abnormalities. Sperm analysis was carried out in the epididymides at the same points in time. No clinical signs were observed during treatment, apart from a decrease in body weight in the males. At the end of the withdrawal period, the body weight had not fully recovered.

No effects on libido were observed. Immediately after treatment, an insignificant decrease in the number of implantations per female was observed, but after the withdrawal period this decrease became significant. Immediately after treatment, a decreased number of live foetuses was found. This effect was less pronounced, after the

28 day withdrawal period. Increased foetal body weight was seen in foetuses sired by treated males probably due to smaller live litter size that was observed. EMS did not affect offspring sex ratio and no external anomalies were observed. In treated males, decreased absolute testis and epididymis weights were found only at the end of the withdrawal period. No treatment-related deviant sperm motility and morphology parameters were seen in males immediately after treatment. After the withdrawal period, decreases in percentage motile sperm cells and sperm cell velocity, lower sperm counts and increased percentages of tailless and anomalous sperm cells were seen (Tak00).

In a dominant lethal study with male mice of the C3D2F1 and DBA/2J strains (n = 15 per treatment group), animals were treated with a single oral or i.p. dose with 0 or 200 mg/kg bw. At 48 hours after exposure, males were mated for three periods of 7-days with untreated females (Soa76). Offspring were studied at gestation day 17. Results were compared with a pooled control group (including those for the anaesthetic) and with results obtained from a second group of males treated with 200 mg/kg via intraperitoneal injection. General toxicity effects were not reported.

After oral dosing, no effects on the percentage of fertile matings were observed for both strains. For C3D2F1 males at week 1 and 2 but not at week 3, a slight increase in pre-implantation loss was observed after both oral and i.p. administration but statistical significance was only reached at week 2 after i.p. dosing. For DBA/2J males, significantly increased pre-implantation loss was observed for all mating intervals and for both routes. In both strains and after both oral and i.p. administration, increased post implantation loss was observed in litters sired during week 1 or 2 post treatment, but not in litters conceived in week 3. The extent of the post-implantation loss was independent of strain or route of administration. Pre-implantation loss was only based on reduction in the number of implants per female in comparison with controls. No account was taken for the number of corpora lutea, but females were not treated (Soa76).

In a fertility study, male rats (n = unspecified) were mated to untreated females (one to one) for 13 consecutive weeks post treatment with EMS either via a single i.p. dose of 300 mg/kg bw or via 15 consecutive oral daily doses of 20 mg/kg/d (Jac61). After the single i.p. dose, loss of body weight was observed, but not after the repeated exposure. Fertility was monitored as live foetuses per litter.

Complete loss of fertility was observed during week 1-3 following the single i.p. dose. This recovered during week 4 and 5 to normal during the rest of the study. The repeated oral dose caused a transient loss of fertility during weeks 3-5 post treatment (Jac61).

Intraperitoneal exposure

Male mice of the (SECxC57BL)F1, (101xC3H)F1 or T-stock strains (10 per treatment group; control group not specified) were submitted to a dominant lethal assay (Gen69). The animals were dosed with 0, 300 or 400 mg/kg bw, via a single i.p. injection. Immediately after treatment, males were mated up to day 19.5. The females that had mated were removed at 12 h post treatment and every other day thereafter. Females were examined at day 13-17 of gestation and scored for corpora lutea, living embryos and dead implants.

No lethality was observed at 300 mg/kg bw. At 400 mg/kg bw (not tested for dominant lethality) mortality was 28%, 8% or 5% in (SECxC57BL)F1, (101xC3H)F1 or T-stock strains respectively. Sub-lethal effects were not mentioned.
Dominant lethal events were observed in all three strains of mice. Males from the T-stock strain were slightly more resistant to EMS than males of the two hybrid strains. The most sensitive period was from post treatment days 2.5 to 13.5 i.e. the spermatozoa and late spermatid stages. After day 13.5, no difference in dominant lethal frequency between treated and control animals was observed (Gen69).

Female mice of the (SECxC57BL)F1, (101xC3H)F1 or T-stock strains (size of treatment groups: n=34-70; 32-56 or 19-43, respectively) were submitted to a dominant lethal study for detection of differences in strain sensitivity (Gen69). EMS was administered as a single i.p. injection at dose levels of 0 and 300 or 400 mg/kg bw.

The females were mated to males at 24 h post treatment. Ovaries and uteri of mated females were examined at day 13 -17 of pregnancy. The females were subdivided in groups that mated between day 1.5-4.5 or day 4.9 - 9.5 post tretament. At 300 mg/kg bw mortality rates were 6%, 10% or 3%, and at 400 mg/kg bw (not tested for dominant lethality) 78%, 88% and 38% in (SECxC57BL)F1, (101xC3H)F1 or T-stock strains, respectively. For the females of the (101xC3H)F1 the occurrence of a "post-treatment sick phase" was mentioned, which might have resulted in a small reduction of the frequency of pregnant matings during the first mating interval.
T-stock females were most sensitive to EMS. In this strain the percentage of fertile matings was reduced to 40 or 59%, depending on the moment of copulation. In controls 93 % of the matings were fertile. In fertile females, dominant lethal frequency was approximately 68% and when the non-fertile matings were also included in the calculations (presumed 100% pre-implantation loss) the frequency of dominant lethals rose to ca. 83%. In the (101xC3H)F1 strain, EMS reduced the percentage of fertile matings from 96% to 83% in the first mating interval and from 95% to 89% in the second. 9.7% and 5.4% dominant lethals (fertile matings only) were observed in first and second mating interval, respectively. In the (SECxC57BL)F1 strain the percentage of fertile matings

was not affected, and dominant lethal frequency was 5 or 2% (first and second mating interval, respectively) (Gen69).

At 24 h post treatment, in a total reproductive capacity test, female (SECxC57BL)F1 mice were permanently cohabited with a young untreated male until the end of the female's reproductive lifespan (Gen71). EMS was administered at 0, 200 and 325 mg/kg bw via a single i.p. injection to groups of 71 controls and 25 and 26 per group at 200 and 325 mg/kg bw , respectively. Per group, some females were sacrificed at 3 days, 6 and 10 months after treatment, for examination of the ovaries. At the highest dose level, 3 out of 26 females died within 30 days of exposure; other effects were not reported.

Both low and high dose EMS caused a decrease in the mean number of young per female. The high dose also caused a decrease in the mean number of litters per female. Decreases in fertility (both litter size and number of litters produced) became more pronounced later in reproductive life. EMS did not affect the number of oocytes in the ovaria at any time point during the study, indicating that the reduced fertility was not the result of a direct oocyte killing effect, but the precise mechanism has not been elucidated (Gen71).

In a sperm alkylation assay, male (C3Hx101)F1 mice were treated with ethyl-tritiated EMS. Ethyl-tritiated EMS was administered at dose levels of 0 and 200 mg/kg bw tritium-EMS via single i.p. injection. Sperm heads were collected during 1-15 days post treatment (Sea78). Alkylation of sperm head DNA and protamine was monitored in sperm cells collected from vas deferens and cauda epididymis. Signs of general toxicity were not reported.

The number of alkylations per sperm head closely followed the dominant -lethal frequency curve (as determined in other studies) with a peak during day 7-14. Sperm head DNA alkylation was greatest at 4 h post treatment and declined steadily during the rest of the study, without an additional peak. The peak in total sperm head alkylation was associated with a peak in protamine alkylation rather than in DNA alkylation (Seg78).

Male F344 rats, submitted to a dominant lethal test (n = 9 group) were administered at 0, 50 and 100 mg/kg bw via single i.p. injection. Each male was caged with a virgin female at 24 h before treatment and with a new female during the 5 days following treatment (Tea85). At day 6-7 the female was removed and a new female was introduced at day 8. This procedure was repeated for 6 consecutive weeks. Uteri were examined at day 18 of gestation.

GSH levels in testes, epididymis and vas deferens were determined at 1.5 h post treatment at levels of 0, 125 and 250 mg kg bw via single i.p. injection in 4 animals per group. No overt toxicity, measured as male body weight changes was observed.

Treatment caused an increase in dominant lethals and in particular in pre-implantation loss in post-meiotic stages of rat spermatogenesis (during weeks 2 and 3 post treatment), already at 50 mg/kg bw but especially at 100 mg/kg bw. No effect on libido was observed. EMS caused a decrease in GSH levels in testes (only the high dose), and more prominently in epididymis and vas deferens tissue (Tea85).

In a dominant lethal study, male F344 rats (9 per group) were pre-treated with buthionine sulphoximine (BSO) to deplete sex organ GSH levels (Tea87). Animals were allowed to mate during 6 mating periods of 5 days per week following treatment with EMS. The following dose levels and regimens were used: EMS: 0 or 50 mg/kg bw via single i.p. injection. BSO: 0 or 3 mmol/kg BSO in 3 s.c. injections on 12 hrs intervals. In combination studies the EMS injection took place at 12 hrs following the last BSO injection. Reproductive effects were correlated with tissue GSH levels. In addition a sperm head alkylation study was performed using the same dosing regimens for BSO and ethyl-tritiated EMS.

Neither BSO, nor EMS induced general toxicity. No effect or a small effect on dominant lethal index was observed after treatment with BSO alone. With EMS alone a slight increase in dominant lethality was observed in week 3 litters. A much stronger increase in dominant lethality was observed when EMS was administered after pre-treatment with BSO, especially in litters sired at week 2 and 3 post EMS treatment. Treatment with EMS or BSO+EMS did not result in increased pre-implantation loss.

The BSO treatment caused a 22% decrease in testicular GSH concentration and a 50% decrease in both epididymal and vas deferens GSH concentration.
When animals were treated with tritium-EMS or BSO+tritium-EMS, little alkylation of sperm heads was observed at day 1 post treatment. A clear increase in sperm head alkylation was seen at day 8 and at day 15, while sperm head alkylation was much lower again at day 22. Only at day 15 BSO+EMS shows a higher alkylation rate than EMS alone (Tea87).

In a dominant lethal study, female (C3HxC57BL)F1 mice were treated with EMS via single i.p. injection either before mating or within 25 h thereafter (Gen88). Animal numbers were 50 to 65 and 30 to 50 in treatment and control groups, respectively. Dose levels 25 hours before mating were 0 or 250 mg/kg bw but in two additionally post mating studies, dose levels were 0 or 250 mg/kg bw at 1, 6, 9 or 25 h post mating or 0, 150 and 250 mg/kg bw at 6 h post mating. At day 17 of gestation uteri were investigated to score fertility, post-implantation loss and abnormal foetuses. Data on general toxicity were not provided.
No effects on offspring were observed when animals were treated either before mating or at 25 h thereafter. When females were treated at 1 or 6 h post-mating, with 250 mg/kg

bw about 60% of the conceptuses were lost, half of them as early resorptions, half of them as mid- and late gestational death. When animals were treated with the same dose at 9 h post-mating the effects were less pronounced, as was the case with the lower dose level at 6 h.

With 250 mg/kg bw at 6 h post mating, about 30% of all living foetuses showed morphological abnormalities (not further specified), in comparison with 1% in controls. According to the authors, embryolethality of EMS was specifically elicited during the moment of sperm entry and during the early pronuclear stage of the developing zygote (Gen88).

In a dominant lethal test, male CBA mice (numbers unspecified) were treated with 0 or 200 mg/kg bw EMS via single i.p. injection and after treatment mated for 5 consecutive weekly intervals with 2-3 virgin females of the C57Bl/6J strain (Pas90). At day 16 - 19 of pregnancy uterine contents was investigated for dominant lethals. Using polyacrylamide gel-elctrophoresis, livers of viable foetuses and both parents were screened to detect biochemical variants of several enzymes.

General toxicity effects were not reported. Effects on male fertility were not observed. Increased frequencies of post-implantation lethality were seen in litters sired during week 1 and 2 post treatment but not in later litters. In pups from the week 1 and 2 litters, increased incidences of liver enzyme variants could be detected, which according to the authors were indicative for mutational events (Pas90).

Subcuteaneous

Male Slc-ICR mice (4 per group) were dosed with 0, 175 or 350 mg EMS/kg bw via single s.c. injection (Has87). In a pre-test, these dose levels showed toxic effects in the bone marrow. Other effects were not reported. In the first experiment effects on bone marrow cells, spermatogonia en spermacytes were studied in treated mice, sacrificed 1-6 weeks after exposure.

In a second experiment, treated males were mated with untreated females at 1 to 6 weeks after EMS administration (0, 175 or 350 mg EMS/kg bw). 16 hours after ovulation females were killed and the development of fertilized eggs (a chromosomal aberration test in first cleavage metaphase fertilised eggs) was studied.

Bone marrow chromosomal breaks were found at 1 but not at 2 or 3 weeks post dosing (both concentrations). With EMS in spermatogonia at week 1 but not at week 2 or 3, a slight increase in numerical aberrations but not in chromatid or chromosomal breaks was observed. No chromosomal changes were observed in spermatocytes. A slight increase in percentage of hook-less sperm heads was seen at 2 and 3 weeks post-dosing. However, in females mated 1 to 3 weeks post-treatment to exposed males, increased

numbers of fertilised eggs with chromosomal aberrations were observed. At week 4-6 no increased frequency of chromosomal aberration in fertilised eggs was seen (Has87).

Developmental toxicity

In Annex D, table 2.1, tests are included which have specifically paid attention to morphological changes in the offspring. Embryo- and foetal lethality are covered by the dominant lethal studies in Annex D (see table 1.1 to 1.3) and treated above in the section on fertility, but it should be noted that in a number of *in vivo* developmental studies embryo- or foetotoxicity resulting in increased pre- or post-implantation loss was also observed.

With respect to the *in vivo* studies, no data are available from oral developmental toxicity studies. All available data come from studies with i.p. administration, either in males or in females. The studies with females comprise both pre-mating and post-mating exposure. Only occasionally, developmental effects have been studied on a *per litter* basis.

The developmental toxicity of EMS has been studied in female NMRI mice. The study was intended to provide information on the phase of foetal development, which was most sensitive to EMS (phase specificity study; n=8 to 29 females per group) and to reveal the dose-relationship for EMS-induced developmental tocixity at the most susceptible developmental stage (dose dependency study; n= 6 to 53 females per group).

Pregnant females were dosed at day 9, 10, 11 or 12 with 0 or 215 mg/kg bw for the phase specificity study, or at day 11 of pregnancy with dose levels of 0, 50, 100, 150, 175, 195, 215 or 250 mg/kg bw the dose dependency study. In both studies, EMS was administered to the animals via a single i.p. injection. Foetuses were examined at day 18 of gestation. General maternal toxicity effects were not reported.
Treatment of dams with 215 mg/kg bw did not result in embryolethality but a decreased foetal body weight was observed, especially when treated at day 10 or 11. Treatment-related malformations included cleft palate, limb and paw malformations, asymmetry or fusion of pelvic bones, fusion of ventral and lateral ossification centres of lumbar vertebrae. Day 10 and 11 were the most sensitive to the induction of teratogenic effects.

In the dose dependency study, again no embryolethality was observed despite growth retardation and teratogenicity at all dose levels > 100 mg/kg bw. At the highest dose level, no foetuses without malformations were found at all. The most frequently observed malformations were cleft palate, fusion of ventral and lateral ossification centres of lumbar vertebrae, hindpaw adactyly and missing or fused cranial bones (Pla82).

Lactation

No studies were available

2.4 Conclusion

No human data were available to evaluate the reproductive toxicity of EMS. With respect to animal studies on fertility, all available data were variations of the dominant lethal test. Such tests have been carried out in female and more extensively in male mice and rats. Most studies used intraperitoneal exposure, few were done with oral exposure. Most studies were single dose studies and the rare multidose studies available had few dose groups. In addition, sperm quality studies (morphology, motility and counts), chromosome aberration studies in fertilised eggs, and sperm alkylation assays have been performed.

Invariably, the dominant lethal assays have shown that in males treated with EMS, sensitive stages of spem cells, in particular spermatozoa and late spermatids are alkylated by EMS resulting in increased pre- and post implantation loss. This phenomenon was observed after i.p as well as after oral administration (Soa76, Tak00, Pas90, Tea85). In female mice, mated to exposed males 1 to 3 weeks post treatment, increased numbers of fertilised eggs with chromosomal aberrations were observed and it has been speculated that these chromosomal breaks are causally related to the dominant lethal effects (Has87).

The available sperm alkylation studies show that protamine alkylation is most extensive at the stages of spermatogenesis which are also most sensitive with respect to the induction of dominant lethals. No clear correlation between DNA alkylation and induction of dominant lethals was observed. It has also been demonstrated that low GSH levels in the male reproductive tract may render the sperm cells more vulnerable to EMS (Tea85, Tea87). In addition liver GSH is also relevant, possibly because low liver GSH levels may enhance systemic circulation of EMS.

Also in females EMS is able to induce dominant lethal events, in oocytes or fertilised egg cells, but females have been studied less extensively than males. In one study female reproduction appeared particularly affected at the moment of sperm entry and during the early pronuclear stage of the developing zygote (Gen88). It has also been demonstrated that a single i.p. injection with EMS is capable of reducing the life span reproductive capacity of female mice (Gen71).

Based on the results from the dominant lethal studies with oral administration, supported by the outcome of the studies with intraperitoneal administration and the sperm morphology and sperm alkylation studies, the committee recommends classifying EMS

in category 2 for fertility (substances which should be regarded as if they impair fertility in humans) and labelling the compound with R60 (May impair fertility).

Foetal malformations have been observed in studies in which EMS has been administered to females via intraperitoneal injection. No oral teratogenicity studies are available. In one *in vivo* study in the mouse, with a detailed description of the malformations, the most frequently observed malformations were cleft palate, fusion of ventral and lateral ossification centres of lumbar vertebrae, hindpaw adactyly and missing or fused cranial bones.

In the few *in vivo* studies available, all single dose i.p. studies, the administered amounts of EMS were close to those which induced parental lethality in several dominant lethal studies. No information is available on developmental or maternal toxicity at lower dose levels.

It has been clearly demonstrated that EMS may elicit foetal morphological abnormalities after intraperitoneal injection. The intraperitoneal route is considered an inappropriate route for classification for developmental toxicity. Data from the oral fertility studies indicate that EMS may become systemically available. This indicates that EMS may elicit developmental toxicity (as is observed after ip administration) after oral administration. Therefore, the committee recommends classifying EMS in category 3 ('substances which cause concern for humans owing to possible developmental toxic effects') and labeling with R63 ('may cause harm to the unborn child').

However, general information about the mechanism of toxicity of direct alkylating compounds shows that adverse effects on reproduction are to be expected after exposure to EMS.

As no studies on EMS in breast milk were available, the committee recommends not labelling EMS for effects during lactation because of a lack of appropriate data.

Proposed classification for fertility

Category 2, T;R60

Proposed classification for developmental toxicity

Category 3, Xn;R63

Proposed labelling for effect during lactation

Lack of appropriate data precludes the assessment of EMS for labelling for effects during lactation.

References

And77	Anderson D, McGregor DB, Purchase IF, Hodge MC, Cuthbert JA. Dominant-lethal test results with known mutagens in two laboratories. Mutat. Res. 1977; 43: 231-46.
And81	Anderson D, McGregor DB, Weight TM. The relationship between early deaths and implants in control and mutagen-treated CD-1 mice in dominant lethal assays. Mutat. Res. 1981; 81: 187-96.
Arn76	Arnold DW, Kennedy GL Jr, keplinger ML, Calandra JC. Dominant lethal studies with alkylating agents: dose-response relationships. Toxicol. Appl. Pharmacol. 1976; 38: 79-84.
DEC89	Dutch Expert Committee on Occupational Substances, Health based Recommended occupational exposure limits for Ethylmethanesulphonate and Methylmethanesulphonate RA4/89, 1989
Ehl68	Ehling UH, Cumming RB , Malling HV. Induction of dominant lethal mutations by alkylating agents in male mice. Mutat. Res. 1968; 5: 417-428.
Fav78	Favor J, Soares ER, Crenshaw JW Jr. Chemical and radiation induced late dominant lethal effects in mice. Mutat. Res. 1978; 54: 333-342.
Fic88	Ficsor G, Goldner L, Panda BB. Comparison of behaviors for detection of heritable mutations. Teratog. Carcinog. Mutagen. 1988; 8: 153-160.
Gan92	Gandy J, Bates HK, Conder LA, Harbison RD. Effects of reproductive tract glutathione enhancement and depletion on ethyl methanesulphonate-induced dominant lethal mutations in Sprague-Dawley rats. Teratog. Carcinog. Mutagen. 1992; 12: 61-70.
Gen69	Generoso WM, Rutledge WL. Strain and sex variations in the sensitivity of mice to dominant-lethal induction with ethyl methanesulphonate. Mutat. res. 1969, 589-598.
Gen71	Generoso WM, Stout S, Huff, SW. Effects of alkylating chemicals on reproductive capacity of adult female mice. Mutat. Res. 1971, 13, 171-184.

Gen88 Generoso WM, Rutledge JC, Cain KT, Hughes LA, Downing DJ. Mutagen-induced fetal anomalies and death following treatment of females within hours after mating. Mutat. Res. 1988; 199: 175-181.

Has87 Hashimoto T, Takeuchi K, Ohno S, Komatsu S, Ohno Y. Cytogenic analysis of dominant lethal effects of mitomycin C and ethyl methanesulphonate. J. Toxicol. Sci. 1987; 12: 33-45.

Hem68 Hemsworth BN. Embryopathies in the rat due to alkane sulphonates. J. Reprod. Fertil. 1968; 17: 325-334 .

Iar74 International Agency for Research on Cancer. IARC Monograph on the evaluation of the carcinogenic risk of chemicals to man. Some Anti-thyroid and related substances, nitrofurans and industrial chemicals. 1974, 7:326

Iar87 International Agency for Research on Cancer. IARC Monograph on the evaluation of the carcinogenic risk of chemicals to humans. Overall evaluations of carcinogenicity. 1974, suppl. 7:440

Jac61 Jackson H, Fox BW, Craig AW. Antifertility substances and their assessment in the male rodent. J. Reprod. Fertil. 1961; 2: 447-465 .

Kat89 Katoh M, Cacheiro NLA, Cornett CV, Cain KT, Rutledge JC, Generoso WM. Fetal anomalies produced subsequent to treatment of zygotes with ethylene oxide or ethyl methanesulphonate are not likely due to the usual genetic causes. Mutat. Res. 1989; 210: 337-344.

Kau97 Kaur R, Parshad VR. Ethyl methanesulphonate induced changes in the differentiation, structure and functions of spermatozoa of the house rat, Rattus rattus L. J. Biosci. (Bangalore, India) 1997; 22: 357-365.

Kra78 Kratochvilova J. Evaluation of pre-implantation loss in dominant-lethal assay in the mouse. Mutat. Res. 1978; 54: 47-54.

Lyo86 Lyon MF, Renshaw R. Induction of congenital malformations in the offspring of mutagen treated mice. In: Ramel CB, Lambert Magnusson J (Ed.) Progress in Clinical and Biological Research, Vol. 209B. Genetic toxicology of environmental chemicals, Part B: Genetic effects and applied mutagenesis 1986; 449-458, Alan R. Liss, Inc. New York, N.Y., USA.

Moh90 Mohr KL, Working PK. An in vitro technique to detect dominant lethal mutations induced in mouse oocytes by ethyl methanesulphonate exposure in vivo. Toxic. in Vitro 1990; 4: 115-122.

Mou75 Moutschen J, Colizzi A. Absence of acrosome: an efficient tool in mammalian mutation research. Mutat. Res. 1975; 30: 267-272.

OEC96 OECD guidelines for testing of chemicals. Organisation for Economic Co-operation and Development, 1996 Paris.

Par63 Partington M, Jackson H. The induction of dominant lethal mutations in rats by alkyl sulphonic esters. Genetical Res. Cambridge 1963; 4: 333-345 .

Pas90 Paschin YuV. Presumed gene mutations detected in F1 progeny of mouse males treated with ethyl methanesulphonate. Mutat. Res. 1990; 229: 185-7.

Pla82 Platzek T, Bochert G, Schneider W, Neubert D. Embryotoxicity induced by alkylating agents: I ethylmethanesulphonate as a teratogen in mice. Arch. Toxicol. 1982; 51: 1-25 .

Seg78 Sega GA, Owens JG. Ethylation of DNA and protamine by ethyl methanesulphonate in the germ cells of male mice and the relevancy of these molecular targets to the induction of dominant lethals. Mutat. Res. 1978; 52: 187-106.

Sin81 Singh H, Tate F. Antispermatogenic effects of ethyl methanesulphonate and benzo[a]pyrene in PD4 Lakeview hamsters. J.-Toxicol. Environ. Health. 1981; 8: 929-937.

Soa76 Soares ER. Studies of factors influencing the mutagenicity of EMS in mice. Mutat. Res. 1976; 37: 245-252.

Sut76 Suter KE, Generoso WM. Chemical induction of presumed dominant-lethal mutations in postcopulation germ cells and zygotes of mice. II. Sensitivity of different postcopulation-precleavage stages to three alkylating chemicals. Mutat. Res. 1976; 34: 259-270.

Tak00 Takagi H, Satoh A, Shirane R, Hashimoto T, Inoue T, Kimura M. Effects of an agent inducing dominant lethals on rat sperm - Examination with ethyl methanesulphonate. J. Toxicol. Sci. 2000; 25: 25-31.

Tea85 Teaf CM, Harbison RD, Bischop JB. Germ-cell mutagenesis and GSH depletion in reproductive tissue of the F-344 rat induced by ethyl methanesulphonate. Mutat. Res, 1985; 144: 93-98.

Tea87 Teaf CM, Bishop JB, Harbison RD. Depression of glutathione in male reproductive tissues and potentiation of EMS-induced germ cell mutagenesis by L-buthionine sulfoximine. Teratog. Carcinog. Mutagen. 1987; 7: 497-513.

Tox95 Niesink RJM, De vries J, Hollinger MA, eds, Toxicology, Principles and Applications, Boca Raton: CRC Press, 1995: 385.

Wyr75 Wyrobeck AJ, Bruce WR. Chemical induction of sperm head abnormalities in mice. Proc. Ntl. Acad. Sci USA, 1975; 72: 4425-4429.

Literature consulted but not cited

Boc87 Bochert G, Platzek T, Neubert D. DNA modification in murine embryos: a primary cause of embryotoxic effects. In: Nau H, Williams JS Jr, Pharmacokinetics in Teratogenesis 1987; Vol II:73-82, CRC Press, Boca Raton, USA.

Das95 Daston GP, Manson JM. Critical periods of exposure and developmental outcome. Inhal. Toxicol. 1995; 7:863-871.

Ehl80 Ehling UH. Comparison of the muytagenic effect of chemicals and ionizing radiation in germ cells of the mouse. Alacevic (eds) Environmental Mutagenesis. 1980, p47-58, Elsevier, Amsterdam, NL.

Ehl86 Ehling UH. Dozovye zavisimosti mutatsii polovykh kletok u myshei. [Dose dependencies of mutations of germ cells in mice]. Tsitol. Genet. 1986; 20: 180-190.

Ehl89 Ehling UH, Neuhäuser-Klaus A. Induction of specific-locus mutations in male mice by ethyl methanesulphonate (EMS). Mutat. Res. 1989; 227: 91-95.

Eve93 Evenson DP, Jost LK, Gandy J. Glutathione depletion potentiates ethyl methanesulphonate-induced damage to sperm chromatin structure. Reprod. Toxicol. 1993; 7: 297-304.

Fah75 Fahrig R. A mammalian spot test: induction of genetic alterations in pigment cells of mouse embryos with x-rays and chemical mutagens. Mol. Gen. Genet. 1975; 138: 309-314.

Fau89 Faustman EM, Kirby Z, Gage D, Varnum M. In vitro developmental toxicity of five direct-acting alkylating agents in rodent embryos: structure-activity patterns. Teratology. 1989; 40: 199-210.

Gen71 Generoso WM, Huff SW, Stout SK. Chemically induced dominant-lethal mutations and cell killing in mouse oocytes in the advanced stages of follicular development. Mutat. Res. 1971; 11: 411-420.

Gen79 Generoso WM, Huff SW, Cain KT. Relative rates at which dominant-lethal mutations and heritable translocations are induced by alkylating chemicals in postmeiotic male germ cells of mice. Genetics 1979; 93: 163-171.

Gen81 Generoso WM, Cain KT, Krishna M, Cunningham EB, Hellwig CS. Evidence that chromosome rearrangements occur after fertilization following postmeiotic treatment of male-mice germ cells with EMS. Mutat. Res. 1981; 91: 137-140.

Jac64 Jackson H. The effects of alkylating agents on fertility. B. Med. Bull. 1964; 20: 107-114.

Jel85 Jelinek R, Peterka M, Rychter Z. Chick embryotoxicity screening test - 130 substances tested. Indian J. Exp. Biol. 1985; 23: 588-595.

Ker87 Kerr JB, Knell CM. The regenerative capacity of testicular interstitial tissue: A recurrent pattern of experimental destruction and regeneration of rat Leydig cells. Wilhelm Roux's Arch. Dev. Biol. 1987; 196: 467-471.

Mai94 Mailhes JB, Marchetti F. Chemically-induced aneuploidy in mammalian oocytes. Mutat. Res. 1994; 320: 87-111.

Moh87 Mohr KL, Working PK. In vitro dominant lethal assessment of ethyl methanesulphonate (EMS)-induced oocyte damage in the mouse. Environ.Mutagen. 1987; 9 (suppl. 8): 74-75. .

Nag98 Nagao T, Fujikawa K. Modified susceptibility to teratogenesis in the offspring of male mice exposed to mutagens. Congen. Anom. 1998; 38: 1-8.

Nit91 Nito S, Ariyuki F, Nakayama Y. A new in vitro screening method for teratogens using human embryonic palatal mesenchymal cells. Congen. Anom. 1991; 31: 329-336.

Pie97 Piegorsch WW, Lockhart AC, Carr GJ, Margolin BH, Brooks T, Douglas GR, Liegibel UM, Suzuki T, ThybaudV, Van Delft JHM, Gorelick NJ. Sources of variability in data from a positive selection lacZ transgenic mouse mutation assay: An interlaboratory study. Mutat. Res. 1997; 388: 249-289.

Pla00 Platzek T, Bochert G. DNA alkylation studies of combined treatment with methylnitrosourea and ethylmethanesulphonate in mice. Teratog. Carcinog. Mutagen. 2000; 20: 27-34.

Pla82 Platzek T, Bochert G, Rahm U, Schneider W. Alkylating agents as teratogens. A model for dose response relationship. Teratology 1982; 25: 19A.

Pla90 Platzek T, Bochert G. DNA adduct formation induced by ethyl methanesulphonate in mouse embryos. Naunyn-Schmiedeberg's Arch. Pharmacol. 1990, 341 (SUPPL.); R26.

Pla91 Platzek T, Bochert G, Rahm U, Pauli B. Dosimetry as a tool in prenatal toxic risk estimation of low doses of alkylating agents. Teratology 1991; 44: 22A.

Pla95 Platzek T, Bochert G, Meister R. Embryotoxicity induced by alkylating agents: 9. Low dose prenatal-toxic risk estimation of ethylmethanesulphonate based on no-observed-adverse-effect-level risk factor approach, dose-response relationships, and molecular dosimetry. Teratog. Carcinog. Mutagen. 1995; 15: 81-92.

Pla95 Platzek T, Bochert G. Embryotoxicity induced by alkylating agents: 10. Analysis of the combined teratogenic effects of methylnitrosourea and ethylmethanesulphonate in mice. Teratog. Carcinog. Mutagen. 1995; 15: 201-214.

Rom85 Rommerts FFG, Grootenhuis AJ, Hoogerbrugge JW, Van der Molen HJ. Ethane dimethanesulphonate (EDS) specifically inhibits LH stimulated steroidogenesis in Leydig cells isolated from mature rats but not in cells from immature rats. Mol. Cell. Endocrinol. 1985; 42: 105-111.

Rut98 Rutledge JC, Generoso WM. Malformations in pregastrulation developmental toxicology. Reproduct. Dev. Toxicol. 1998; :73-86.

Sch78 Schneider J, Warzok R, Schreiber D, Heiderstadt R. Zur postnatalen und transplazentaren kanzerogenen Wirkung von Methylmethansulfonat (MMS) und Athylmethansulfonat (AMS) bei der Haubenratte. [Postnatal and transplacental carcinogenic effects of methyl methanesulphonate (MMS) and ethyl methanesulphonate (EMS) in hooded rats (author's transl)]. Exp. Pathol. (Jena). 1978; 16: 157-167.

See91 Seeley MS, Silbernagel SM, Faustman EM. Effects of nitrosoureas on in vitro rat embryo limb cell differentiation: comparisons with whole embryo culture effects. Toxicologist 1991; 11: 293.

See95 Seeley MR, Faustman EM. Toxicity of four alkylating agents on in vitro rat embryo differentiation and development. Fundam. Appl. Toxicol. 1995; 26:136-142.

Ski83 Skinner MJ, DiValerio M, Yu RL, Nichols WW, Miller RC. The detection of viable heritable translocations by chromosome banding procedures. Environ. Mutagen. 1983; 5: 153-159.

Van97 Van Delft JHM, Bergmans A, Baan RA. Germ-cell mutagenesis in lambda lacZ transgenic mice treated with ethylating and methylating agents: Comparison with specific-locus test. Mutat. Res. 1997; 388: 165-173.

Annexes

Annex **A**

The committee

- BJ Blaauboer, *chairman*
  Toxicologist, Institute for Risk Assessment Sciences, Utrecht
- AM Bongers, *advisor*
  Ministry of Social Affairs and Employment, Den Haag
- JHJ Copius Peereboom-Stegeman
  Toxicologist, University Medical Centre St Radboud, Nijmegen
- HFP Joosten
  Toxicologist, NV Organon, Department of Toxicology and Drug Disposition, Oss
- D Lindhout
  professor of Medical Genetics, paediatrician, University Medical Centre, Utrecht
- AH Piersma
  Reproductive toxicologist, National Institute for Public Health and the Environment, Bilthoven
- N Roeleveld
  Epidemiologist, University Medical Centre St Radboud, Nijmegen
- DH Waalkens-Berendsen
  Reproductive toxicologist, TNO Nutrition and Food Research, Zeist
- PJJM Weterings
  Toxicologist, Weterings Consultancy BV, Rosmalen
- ASAM van der Burght, *scientific secretary*
  Health Council of the Netherlands, Den Haag

The first draft of the present document was prepared by Ing. JJA Muller from the RIVM in Bilthoven, by contract with the Ministry of Social Affairs and Employment.

Secretarial assistance:
Lay-out: J van Kan.

Annex **B**

Comments on the public draft

A draft of the present report was released in 2003 for public review. The following persons and organisations have commented on the draft review:

- RD Zumwalde,
  Centers for Disease Control and Prevention, NIOSH, USA

Annex **C**

Directive (93/21/EEC) of the European Community

4.2.3 Substances toxic to reproduction

4.2.3.1 For the purposes of classification and labelling and having regard to the present state of knowledge, such substances are divided into 3 categories:

Category 1:

Substances known to impair fertility in humans

There is sufficient evidence to establish a causal relationship between human exposure to the substance and impaired fertility.

Substances known to cause developmental toxicity in humans

There is sufficient evidence to establish a causal relationship between human exposure to the substance and subsequent developmental toxic effects in the progeny.

Category 2:

Substances which should be regarded as if they impair fertility in humans:

There is sufficient evidence to provide a strong presumption that human exposure to the substance may result in impaired fertility on the basis of:

- Clear evidence in animal studies of impaired fertility in the absence of toxic effects, or, evidence of impaired fertility occurring at around the same dose levels as other toxic effects but which is not a secondary non-specific consequence of the other toxic effects.
- Other relevant information.

Substances which should be regarded as if they cause developmental toxicity to humans:

There is sufficient evidence to provide a strong presumption that human exposure to the substance may result in developmental toxicity, generally on the basis of:

- Clear results in appropriate animal studies where effects have been observed in the absence of signs of marked maternal toxicity, or at around the same dose levels as other toxic effects but which are not a secondary non-specific consequence of the other toxic effects.
- Other relevant information.

Category 3:

Substances which cause concern for human fertility:

Generally on the basis of:

- Results in appropriate animal studies which provide sufficient evidence to cause a strong suspicion of impaired fertility in the absence of toxic effects, or evidence of impaired fertility occurring at around the same dose levels as other toxic effects, but which is not a secondary non-specific consequence of the other toxic effects, but where the evidence is insufficient to place the substance in Category 2.
- Other relevant information.

Substances which cause concern for humans owing to possible developmental toxic effects:

Generally on the basis of:

- Results in appropriate animal studies which provide sufficient evidence to cause a strong suspicion of developmental toxicity in the absence of signs of marked maternal toxicity, or at around the same dose levels as other toxic effects but which are not a secondary non-specific consequence of the other toxic effects, but where the evidence is insufficient to place the substance in Category 2.
- Other relevant information.

4.2.3.2 The following symbols and specific risk phrases apply:

Category 1:

For substances that impair fertility in humans:

T; R60: May impair fertility

For substances that cause developmental toxicity:

T; R61: May cause harm to the unborn child

Category 2:

For substances that should be regarded as if they impair fertility in humans:

T; R60: May impair fertility

For substances that should be regarded as if they cause developmental toxicity in humans:

T; R61: May cause harm to the unborn child.

Category 3:

For substances which cause concern for human fertility:

Xn; R62: Possible risk of impaired fertility

For substances which cause concern for humans owing to possible developmental toxic effects:

Xn; R63: Possible risk of harm to the unborn child.

4.2.3.3 Comments regarding the categorisation of substances toxic to reproduction

Reproductive toxicity includes impairment of male and female reproductive functions or capacity and the induction of non-inheritable harmful effects on the progeny. This may be classified under two main headings of 1) Effects on male or female fertility, 2) Developmental toxicity.

1 *Effects on male or female fertility*, includes adverse effects on libido, sexual behaviour, any aspect of spermatogenesis or oogenesis, or on hormonal activity or physiological response which would interfere

with the capacity to fertilise, fertilisation itself or the development of the fertilised ovum up to and including implantation.

2 *Developmental toxicity*, is taken in its widest sense to include any effect interfering with normal development, both before and after birth. It includes effects induced or manifested prenatally as well as those manifested postnatally. This includes embrytoxic/fetotoxic effects such as reduced body weight, growth and developmental retardation, organ toxicity, death, abortion, structural defects (teratogenic effects), functional defects, peripostnatal defects, and impaired postnatalmental or physical development up to and including normal pubertal development.

Classification of chemicals as toxic to reproduction is intended to be used for chemicals which have an intrinsic or specific property to produce such toxic effects. Chemicals should not be classified as toxic to reproduction where such effects are solely produced as a non-specific secondary consequence of other toxic effects. Chemicals of most concern are those which are toxic to reproduction at exposure levels which do not produce other signs of toxicity.

The placing of a compound in Category 1 for effects on Fertility and/or Developmental Toxicity is done on the basis of epidemiological data. Placing into Categories 2 or 3 is done primarily on the basis of animal data. Data from *in vitro* studies, or studies on avian eggs, are regarded as 'supportive evidence' and would only exceptionally lead to classification in the absence of *in vivo* data.

In common with most other types of toxic effect, substances demonstrating reproductive toxicity will be expected to have a threshold below which adverse effects would not be demonstrated. Even when clear effects have been demonstrated in animal studies the relevance for humans may be doubtful because of the doses administrated, for example, where effects have been demonstrated only at high doses, or where marked toxicokinetic differences exist, or the route of administration is inappropriate. For these or similar reasons it may be that classification in Category 3, or even no classification, will be warranted.

Annex V of the Directive specifies a limit test in the case of substances of low toxicity. If a dose level of at least 1000 mg/kg orally produces no evidence of effects toxic to reproduction, studies at other dose levels may not be considered necessary. If data are available from studies carried out with doses higher than the above limit dose, this data must be evaluated together with other relevant data. Under normal circumstances it is considered that effects seen only at doses in excess of the limit dose would not necessarily lead to classification as Toxic to Reproduction.

Effects on fertility

For the classification of a substance into Category 2 for impaired fertility, there should normally be clear evidence in one animal species, with supporting evidence on mechanism of action or site of action, or chemical relationship to other known antifertility agents or other information from humans which would lead to the conclusion that effects would be likely to be seen in humans. Where there are studies in only one species without other relevant supporting evidence then classification in Category 3 may be appropriate.

Since impaired fertility may occur as a non-specific accompaniment to severe generalised toxicity or where there is severe inanition, classification into Category 2 should only be made where there is evidence that there is some degree of specificity of toxicity for the reproductive system. If it was demonstrated that impaired fertility in animal studies was due to failure to mate, then for classification into Category 2, it would normally be necessary to have evidence on the mechanism of action in order to interpret whether any adverse effect such as alteration in pattern of hormonal release would be likely to occur in humans.

Developmental toxicity

For classification into Category 2 there should be clear evidence of adverse effects in well conducted studies in one or more species. Since adverse effects in pregnancy or postnatally may result as a secondary consequence of maternal toxicity, reduced food or water intake, maternal stress, lack of maternal care, specific dietary deficiencies, poor animal husbandry, intercurrent infections, and so on, it is important that the effects observed should occur in well conducted studies and at dose levels which are not associated with marked maternal toxicity. The route of exposue is also important. In particular, the injection of irritant material intraperitoneally may result in local damage to the uterus and its contents, and the results of such studies must be interpreted with caution and on their own would not normally lead to classification.

Classification into Category 3 is based on similar criteria as for Category 2 but may be used where the experimental design has deficiencies which make the conclusions less convincing, or where the possibility that the effects may have been due to non-specific influences such as generalised toxicity cannot be excluded.

In general, classification in category 3 or no category would be assigned on an ad hoc basis where the only effects recorded are small changes in the incidences of spontaneous defects, small changes in the proportions of common variants such as are observed in skeletal examinations, or small differences in postnatal developmental assessments.

Effects during Lactation

Substances which are classified as toxic to reproduction and which also cause concern due to their effects on lactation should in addition be labelled with R64 (see criteria in section 3.2.8).

For the purpose of classification, toxic effects on offspring resulting *only* from exposure via the breast milk, or toxic effects resulting from *direct* exposure of children will not be regarded as 'Toxic to Reproduction', unless such effects result in impaired development of the offspring.

Substances which are not classified as toxic to reproduction but which cause concern due to toxicity when transferred to the baby during the period of lactation should be labelled with R64 (see criteria in section 3.2.8). This R-phrase may also be appropriate for substances which affect the quantity or quality of the milk.

R64 would normally be assigned on the basis of:

a toxicokinetic studies that would indicate the likelihood that the substance would be present in potentially toxic levels in breast milk, and/or

b on the basis of results of one or two generation studies in animals which in- dicate the presence of adverse effects on the offspring due to transfer in the milk, and/or

c on the basis of evidence in humans indicating a risk to babies during the lactational period. Substances which are known to accumulate in the body and which subsequently may be released into milk during lactation may be labelled with R33 and R64.

Annex **D**

Fertility and developmental toxicity studies

Table 1.1 Oral fertility studies in males.

Authors	species	Experimental period/design	Dose and route	General toxicity	Effects on reproductive organs and reproduction
And77	Male CD-1 mice (n=~30 in control and ~15 in treatment groups)	Dominant lethal test; exposure for 5 days prior to mating.	0, 100, 150, 200 mg/kg bw/d; oral by gavage	Not reported	Reduced fertility which recovered during the 3rd week post-dosing; reduced numbers of implantations and increased numbers of early death per pregnancy, which recovered after 3 weeks post dosing.
And81	Male CD-1 mice (n= not stated)	Dominant lethal test; exposure for 5 days prior to mating.	0, 100, 150 mg/kg bw/d; oral by gavage	Not reported	Increased numbers of early death per pregnancy.
Fav78	Male DBA/1J mice (n=50)	Dominant lethal test; single exposure 48 h before mating.	0, 175 mg/kg bw, oral	Not reported	Increased ratio late death / alive foetuses; but stat. non-sign.
Jac61	Male rat; (n=n.s.)	Fertility study; animals were mated for 13 consecutive weeks post treatment	Single i.p. dose of 300 mg/kg bw; or 15 doses oral of 20 mg/kg/d	weight loss after single i.p. dose; not after repeated exposure.	Complete but reversible loss of fertility was observed following the single i.p. dose. The repeated oral dose also caused a transient loss of fertility.

Soa76	Male mice, C3D2F1 and DBA/2J strains, (n=15)	Dominant lethal study. single exposure at 48 h before mating.	0, 200 mg/kg bw via single oral or single i.p. dose; 0, 200 mg/kg testis weight in each testis (=21 µg/testes; ~2 mg/kg bw) via single intratesticular injection.	not reported	Oral dosing: No effects on percentage fertile matings in both strains. For C3D2F1 and DBA/2J males a slight increase and a significant increase in pre-implantation losses observed, respectively, were without a clear route effect. Increased post implantation loss in week 1 or 2 litters, but not in week 3 litters without route or strain effect. Testicular injection did not affect the percentage of fertile matings. Similarly, no effect of intratesticular injection was seen on pre- or post-implantation loss
Tak00	Male rats Crj:CD(sd)IGS (n=9 for copulation; n= 2 x 8 for sperm analysis)	Sperm motility / morphology + dominant lethal test; exposure for 5 days. Mating immediately after treatment and at day 28.	0, 100 mg/kg bw/d oral by gavage	Reduced body weight gain during treatment period	Decreased absolute testis and epididymis weight at day 28. No effects on libido. No significantly decreased number of implantations immediately post treatement but significant at day 28. Immediately after treatment decreased percentages of live foetuses; less pronounced at day 28. No deviant sperm motility and morphology parameters immediately after treatment. At day 28, decreased percentage motile sperm cells, sperm cell velocity and sperm counts. Increased percentage tailless and anomalous sperm cells were observed.

Table 1.2 Parental fertility studies in females.

Authors	species	Experimental period/design	Dose and route	General toxicity	Effects on reproductive organs and reproduction
Gen69	Mice of (SECxC57BL)F1, (101xC3H)F1 or T-stock strains Size ot treatment groups: n=34-70; 32-56 or 19-43, respectively)	Dominant lethal study for detection of differences in strain sensitivity. Mating at 24 h post treatment. Ovaries and uteri of mated females were examined at day 13-17 of pregnancy. The females were subdivided in groups that mated between day 1.5-4.5 or day 4.9 - 9.5 post treatment.	0, 300 or 400 mg/kg via single i.p. injection.	At 400 mg/kg bw (not tested for dominant lethality) 78%, 88% and 38%; same order.At 300 mg/kg also lethality but less extensive. "post-treatment sick phase" was mentioned for (101xC3H)F1 females.	In T-stock females fertile matings was reduced from 93 to 40 to 59%. Dominant lethal frequency in fertile females was approximately 68%. In (101xC3H)F1 strain, redution of fertile matings from 96% to 83% or 89% depending on mating interval. 9.7% and 5.4% dominant lethals (fertile matings only). In (SECxC57BL)F1 strain percentage of fertile matings not affected. Dominant lethal frequency 5 or 2%.

Gen71	(SECxC57BL)F1 mice (n=71, 25 or 26)	Total reproductive capacity test. Treated females permanently cohabited with untreated males. Ovaria analysed at 3, 180 and 300 days post reatment.	0, 200 and 325 mg/kg bw; single i.p. administration,	At the highest dose level 3/26 death, no other effects reported	EMS caused a decrease in the mean number of young per female. At the high dose decreased mean number of litters per female, mainly later in life. No effect on number of oocytes.
Gen88	(C3HxC57BL)F1 mice (n=50-65 (treated) or 30-50 control)	Dominant lethal test. Females injected pre- or post mating. Fertility, postimplantation loss foetal abnormalities scored at gestation day 17.	Single i.p. injection: 0, 250 mg/kg bw pre-mating or 0, 250 mg/kg bw at 1, 6, 9 or 25 h post-mating. 0, 150 and 250 mg/kg bw at 6 h post mating.	not reported	When treated at 1 or 6 h post mating, at 250 mg/kg bw ca. 60% loss of conceptuses, When treated at 9 h post mating with 250 mg/kg or with lower doses at 6h less pronounced effects. No effects after treatment at 25 h pre or post-mating
Moh90	B6C3F1 mice (n=26-30)	Dominant lethal test in ex vivo grown fertilised oocytes, treated in vivo, ~20 hrs before ovulation.	0, 125 or 250 mg/kg bw via single i.p. injection	Not reported, but ex vivo study	No effect on ovulation rate, 15% decrease in fertilisation rate ; increased percentage of embryos that failed to enter morula / blastula stadium after both treatments. Disturbance of trophectoderm outgrowth and Inner Cell Mass formation.
Sut76	(C3Hx101)F1 mice (= JH); (Gs/Yx(SECxCs7BL)F1 mice (= SBGS).	Dominant-lethal test. Females treated 12-13 h before cohabitation (dictyate oocyte stage). Alternatively, females were cohabited and treated after detection of a vaginal plug (postcopulation-precleavage and zygote stages). Uterine examination at gestation day 12- 14.	Dictyate oocyte treatment: 0, 300 mg/kg bw via single i.p. dose postcopulation-precleavage and zygote stages: 0, 250 and 300 mg/kg bw via single i.p. dose.	not reported	*Dictyate oocyte stage treatment:* Considerable pre- and post implantation loss was observed. resulting in dose related increase in dominant lethals. *Postcopulation-pre cleavage and zygote stage treatment:* Egg cell / zygote sensitivity highest for second meiotic division and for sperm cells. Similar pattern in JH or SBGS females. No dominant lethal effects in oocytes treated at dictyate phase.

Table 1.3 Parenteral fertility studies in males.

Authors	species	Experimental period/ design	Dose and route	General toxicity	Effects on reproductive organs and reproduction
Arn76	albino mice; (n=12, in duplicate)	Dominant-lethal test. Substance administrations was followed by 3 consecutive mating sessions of one week each.	0, 50, 100, 200, 300, 400 mg/kg bw. single i.p. injection	Lethality, hypothermia, hypoactivity diuresis and reduced mating ability were observed at 400 mg/kg.	At ≥ 200 mg/kg bw increased early resorptions increased. At 300 and 400 mg/kg bw, reduced male fertility. Decreased numbers of implantation and live embryos and increased numbers of early death per litter at 300 and 400 mg/kg bw Effects were most pronounced at week 2 post treatment.
Ehl68	male (101xC3H)F1 mice (n=n.s.)	Dominant-lethal test. Substance administrations was followed by 3 consecutive mating sessions of one week each	0, 100, 200, 250, 400, 450 and 500 mg/kg bw, as single i.p. injection	Loss of fertility and mortality at dose levels of 400 mg/kg bw and above.	Reduced male fertility, litter sizes and percentage of live implants were observed at 200 and 250 mg/kg bw at the same dose levels during mating week 1; more strongly during week 2.
Gan92	Male Sprague-Dawley rats; (n=10)	Dominant lethal test. Mating during week 2 and three post-treatment. Study of the influence of liver and reproductive tract GSH on EMS-induced dominant lethal mutations.	0, and 100 (after NAC) or 50 (after phorone) mg/kg bw single i.p. injection with EMS	not reported	Epididymal and testicular GSH levels increased by treatment with NAC. Phorone gave reduced liver, epididymal and testicular GSH. With EMS only, extensive dominant lethality at week 2 and 3. Mutation frequency reduced when EMS followed NAC. No effect of GSH delpetion on dominant lethal frequency in females mated during week 2 but increase in dominant lethals was observed in week 3 matings.
Gen69	Mice of (SECxC57BL) F1, (101xC3H)F1 or T-stock strains. (n=10 per treatment group; control group size n.s.)	Dominant lethal assay. Mating immediately after treatment. Females were examined at day 13-17 of gestation.	0, 300 mg/kg bw, via single i.p. injection.	Mortality at 400 mg/kg bw (not tested for dominant lethality). No lethality at 300 mg/kg bw. Sub-lethal effects were not mentioned.	Dominant lethal events in all three strains. T-stock males slightly more resistant to EMS than hybrid strain males. Most sensitive period was during spermatozoa and late spermatid stages. No increased dominant lethal frequency ater day 13.5.
Kra78	male (101XC3H)F1 mice (n=40),	Dominant lethal study. Mating at day 1-2 post treatment and there after. Egg morphology studied at 24 or 30-40 h post coitus. Dominant lethals examined at day 14-17 post coitus.	0 or 450 mg/kg bw, single i.p. injection.	450 mg/kg bw is MTD.	No effect observed on percentage fertilised ova. In females mated at day 1-22 post treatment, increased preimplantation loss (up to 98% during day 7-14) and increased post-implantation loss (up to 27% during day 15-18) were seen.

Par62	Male rats strain unspecified,	Dominant lethal test, spermatid counts. Mating at various time intervals up to week 10 post treatment. Testicular spermatid cells studied up to 42 days post treatment	0, 100, 200 mg/kg bw via single i.p. injection	not reported	At 100 and 200 mg/kg bw, reversibly increased percentages of pre- and post-implantation loss. No changes in spermatid counts were observed.
Pas90	Male CBA mice mated with C57Bl/6J females (n=2-3).	Dominant lethal test. Treated males were mated weekly for 5 weeks. Offspring were investigated at day 16 - 19 of pregnancy Livers of living foetuses screened for biochemical variants.	0, 200 mg/kg bw, via single i.p. injection.	not reported	No effects on male fertility. Increased frequencies of post-implantation lethality in week 1 and 2 litters but not later. Increased incidences of liver enzyme variants in week 1 and 2 litter pups.
Tea85	Male rats, F344 strain (dominant lethal n=9; GSH study: n=4))	Dominant lethal test, Mating at 24 h before treatment and for 7 consecutive weekly periods post treatment. Uterine examination at day 18 of gestation. GSH levels in testes, epididymis and vas deferens.	Dominant lethal test: 0, 50 and 100 mg/kg bw via single i.p. injection. GSH assay: 0, 125 and 250 mg kg bw via single i.p. injection.	No effects on body weight.	Increased dominant lethal frequencies and pre-implantation loss in post-meiotic stages of rat spermatogenesis at 50 and 100 mg/kg bw. No effect on libido. EMS related decrease in testes (only the high dose) and in epididymis and vas deferens tissue GSH levels.

Table 1.4 Sperm cell morphology and sperm cell alkylation studies.

Authors	species	Experimental period/ design	Dose and route	General toxicity	Effects on reproductive organs and reproduction

Has87	Male Slc-ICR mice; (n=4)	Germ cell cytogenicity assay, studiy Sperm head abnormality test, Cromosomal aberration test in fetilised eggs.	0, 175, 350 mg/kg; single s.c. injection,	Bone marrow toxicity in pretest. No other effects reported	EMS caused slight increase in numerical aberrations (both doses) but not in chromatid or chromosomal breaks in spermatogonia at week 1 but not at week 2 or 3. No chromosomal changes in spermatocytes. Slight increase in abnormal sperm heads. Increased numbers of fertilised eggs with chromosomal aberrations untill week 3 post dosing.
Kau97	wild rats; (n=18)	sperm cell abnormality test at day 7, 14 or 28 post treatment of 5 days.	0, 500 or 625 mg/kg bw, divided over 5 single daily i.p. injections	lethality at 500 and 625 mg/kg bw within 9 days of first injection. During treatment, loss of body weight	Testes weight unaffected, epididymal weights reduced. Reduction of sperm concentration, sperm motility and viability. Increased percentages of abnormal sperm cells. All these effects had partly recovered at day 28 post treatment. At 625 mg/kg bw reduction of seminiferous tubule diameter and epithelium height. Decreased numbers of preleptotene and pachytene spermatocytes and of round spermatids.
Mou75	Q-strain, Tailless, C57BL and C57BR mice; (n= n.s.)	Sperm head abnormality test in ductus deferens sperm cells at 31 days post treatment	0, 25 mg/kg bw by single i.p. injection	not reported	No differences in frequency of acrosomeless sperm cells.
Seg78	Male mice, (C3Hx101)F1 ,	Sperm alkylation assay. Sperm heads from vas deferens and cauda epididymis were collected during 1-15 days post treatment.	0, 200 mg/kg bw tritium-EMS via single i.p. injection.	not reported	Number of alkylations per sperm head closely followed dominant -lethal frequency curve with peak during day 7-14. Sperm head DNA alkylation greatest at 4 h post treatment declining during the rest of study. Peak in total sperm head alkylation was associated with peak in protamine alkylation.
Sin81	Male PD4 lakeview hamsters; (n=6-9)	Sperm head abnormalities, assayed in caudal epididymis at 1, 4 or 10 weeks post treatment.	0, 5, 25 125 mg/kg bw in five daily i.p. injections	sub-lethal dose levels; body weight and testes / epididymal weights not affected	Week 1: slight increase in sperm head abnormalities at 1235 mg/kg. Week 4: abnormal sperm cells in all dose groups, Week 10: abnormal sperm cells at 5 and aspermia at 25 and 125 mg/kg. Week 1: 70% reduced sperm numbers (all doses). Week 4: normal sperm counts 5 and reduced to 88% and 32% at 25 and 125 mg/kg bw. week 10: 98% reduced (low dose) or 100% (mid and high dose). At week 1 and 4 reduced testes weights at high dose and in all dose groups at week 10.

Tak00	Male rats Crj:CD(sd)IGS (n=9 for copulation; n= 2 x 8 for sperm analysis)	Sperm motility and morphology and dominant lethal test; exposure for 5 days. Males were allowed to mate 24 h after the last treatment or after a 28-day withdrawal period. Sperm analysis was performed at the same time points	0, 100 mg/kg bw/d oral by gavage	body weight loss during treatment period, not fully recovered after 28 days; no other clinical signs of toxicity	Decreased testis and epididymis weights at end of withdrawal period; not immediately post treatment. No effects on libido. Decreased number of implantations per female after withdrawal period, but non-significant immediately after treatment. Decreased live litter size; less pronounced after withdrawal period. No effects on sperm motility and morphology immediately post treatment. After withdrawal period, decreased sperm motility and sperm velocity, lower sperm counts and more tailless and anomalous sperm cells.
Tea87	Male rats, F344 strain (n= 9; ominant lethal; 4-8 for GSH studies)	Dominant lethal / covalent binding study. Pe-treated with 3 BSO doses. Mating during 6 periods of 5 days per week following EMS treatment. Reproductive effects correlated with GSH levels sperm head alkylation rate.	0, 50 mg/kg bw via single i.p. injection. EMS injection at 12 hrs following the third BSO (mmol/kg) injection.	No toxicity observed with BSO, no toxicity for EMS	With EMS slight increase in dominant lethality in the week 3 litter and more pronounced after after pre-treatment with BSO. No increased pre-implantation loss. BSO treatment caused 22%, 50%, 50% decrease in testicular epididymal and vas deferens GSH levels respectively. Little sperm heads alkylation observed at day 1 with EMS or BSO + EMS). Increase in sperm head alkylation at days 8 and 15, which diminished towards day 22. Only at day 15 BSO+EMS showed higher alkylation rate than EMS.
Wyr75	Male (C57BLxC3H/Anf)F1 or (C57BL/6xC3H/He)f1 mice; (n=~4)	Sperm head abnormality test; dosing for 5 consecutive days, sperm analysis at 1, 4 and 10 weeks post treatment	0 and 4 dose levels up to 200 mg/kg bw/d; via single s.c. injection	lethality at 200 mg/kg bw/d	Increased percentages of abnormal sperm at ≥ ~ 100 mg/kg bw after 4 weeks. Less pronounced effects at week 1 and week 10.

Table 2.1 Developmental studies in animals.

Authors	species	Experimental period/ design	Dose and route	Parental toxicity	Effects on reproductive organs and reproduction
Fic88	male HA(ICR) mice; (n=~5)	Heritable behavioural mutations. Mating with untreated females at two and five weeks post treatment. Pups studied in behavioural test battery.	0, 60, 150, 300, 600 mg/kg bw, single i.p. injection	Death in 3 of 5 parent males at 600 mg/kg bw	No litters produced from males at 600 mg/kg bw after 2 weeks At 5 weeks, these males still had reduced fertility. Litter size was not affected. EMS caused heritable behavioural mutations in pre- and post-meiotic germ cells.
Gen88	Female (C3HxC57BL)F1 mice; (n=50-65 (treated) or 30-50 control)	Females were injected pre- or post mating. Fertility, post-implantation loss and abnormal foetuses were scored at day 17 of gestation	Dosing via single i.p. injection: 0, 150 and 250 mg/kg bw at 6 h post mating.	not reported	With 250 mg/kg bw at 6 h post mating, morphological abnormalities in 30% of all living foetuses
Hem68	Female Wistar rat; (n=5)	Teratogenicity test, test for testicular abnormalities in day-old, 15-day old and 6 months old progeny. Pups kept for a subsequent cohabitation study	single i.p. dose per dam At gestation day: 13 or 16: 0 or 200 mg/kg bw. At gestation day 5, 9, 16 or 20: 250 mg/kg bw At day 14, 15 or 16: 200 mg/kg bw	200 mg/kg near-lethal dose. No effect on offspring body weight, 15% reduction in testicular weight, at 200 mg/kg bw on day 15 of gestation.	After 200 mg/kg bw on day 13, cleft palate, retarded lower jaw development, limb defects but not in control dams. At 250 mg/kg at day 5, 9 or 20 complete death of progeny and at day 16 strongly reduced average litter size. No effects on seminiferous epithelium or supporting cell numbers in pups from dams treated with 200 mg/kg bw at day 16 of gestation. In 15-day old pups reduced number of first generation spermatocytes. Treatment with 200 mg/kg at day 14, 15 or 16 did not affect fertility of pups.
Kat89	Female (C3HxC57BL)F1 mice; (n=6 -12 transplantation studies), 19-111 embryos (developmental delay study), 59-141 zygotes (embryo chromosomal aberrations); 10-26 foetuses (foetal chromosomal aberrations)	Animals treated 6h after mating. Zygotes from treated females transplanted into non-treated females. Zygotes studied for early development inhibition and for chromosomal aberrations. Metaphase chromosomes studied in 14-day old foetuses.	0 or 250 mg/kg bw via i.p. injection	Not reported.	Treatment of donor females resulted in mid-gestational or late death, or in structural defects (limb, tail and abdominal wall defects, hydropia, exencephalia and anaemia). No such effects were seen when recipient females were treated. EMS caused delay in embryonal development. At morula stage EMS-induced embryo degeneration. No numerical or structural chromosomal aberrations in early embryos and 14 day old foetuses.

Lyo85	Male mice strain not specified, numbers not specified	Teratogenicity test. Males were treated and mated up to day 12 post h treatment.	0, 200 mg/kg bw via single i.p. injection	not reported	Slight increase in foetuses with malformations in all litters. No specification of type of malformations. No reporting on post-implantation loss.
Pla82	Female NMRI mice (*phase specificity study:* n=8-29; *dose dependency study:* n=6-53)	Teratogenicity test Pregnant females were dosed at day 9-12. (*phase specificity study*) or at day 11 with varying doses (*dose dependency study*). Foetuses were examined at day 18 of gestation	*phase specificity study:* 0, 215 mg/kg bw; single i.p. injection *dependency study:* 0, 50, 100, 150, 175, 195, 215 or 250 mg/kg bw: single i.p. injection	not reported	*Phase specificity study:* No increased embryolethality, decreased foetal body weight. Treatment-related malformations (mainly day 10-11): cleft palate, limb and paw malformations, asymmetry or fusion of pelvic bones, fusion of ventral and lateral ossification centres of lumbar vertebrae. *Dose dependency study:* No embryolethality but growth retardation and teratogenicity at all dose levels > 100 mg/kg bw.

Annex **E**

Abbreviations

Abbreviations used:

bw	body weight
d	day
EMS	ethyl methane sulponate
F	female(s)
i.p.	intraperitoneal
i.v.	intravenous
M	male(s)
n	number
n.s.	not stated
NOAEL	no adverse effect level
OECD	Organisation for Economic Cooperation and Development
p.o.	per os
s.c.	subcutaneous
PN	postnatal