Health Council of the Netherlands

β-Estradiol

Evaluation of the carcinogenicity and genotoxicity

2013/23

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Gezondheidsraad
Health Council of the Netherlands

Aan de minister van Sociale Zaken en Werkgelegenheid

Onderwerp : aanbieding advies *β-Estradiol*
Uw kenmerk : DGV/BMO-U-932542
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Geachte minister,

Graag bied ik u hierbij het advies aan over de gevolgen van beroepsmatige blootstelling aan β-oestradiol.

Dit advies maakt deel uit van een uitgebreide reeks waarin kankerverwekkende stoffen worden geclassificeerd volgens richtlijnen van de Europese Unie. Het gaat om stoffen waaraan mensen tijdens de beroepsmatige uitoefening kunnen worden blootgesteld.

Dit advies is opgesteld door een vaste subcommissie van de Commissie Gezondheid en beroepsmatige blootstelling aan stoffen (GBBS), de Subcommissie Classificatie van carcinogene stoffen. Het advies is getoetst door de Beraadsgroep Gezondheid en omgeving van de Gezondheidsraad.

Ik heb dit advies vandaag ter kennisname toegezonden aan de staatssecretaris van Infrastructuur en Milieu en aan de minister van Volksgezondheid, Welzijn en Sport.

Met vriendelijke groet,

prof. dr. W.A. van Gool,
voorzitter

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Evaluation of the carcinogenicity and genotoxicity

Subcommittee on the Classification of Carcinogenic Substances of the
Dutch Expert Committee on Occupational Safety,
a Committee of the Health Council of the Netherlands

to:

the Minister of Social Affairs and Employment

No. 2013/23, The Hague, October 18, 2013

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 and health (services) research..." (Section 22, Health Act).

The Health Council receives most requests for advice from the Ministers of Health, Welfare & Sport, Infrastructure & the Environment, Social Affairs & Employment, Economic Affairs, and Education, Culture & Science. 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.

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INAHTA

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Samenvatting

Op verzoek van de minister van Sociale Zaken en Werkgelegenheid evalueert en beoordeelt de Gezondheidsraad de kankerverwekkende eigenschappen van stoffen waaraan mensen tijdens de beroepsmatige uitoefening kunnen worden blootgesteld. In het voorliggende advies neemt de Subcommissie Classificatie van carcinogene stoffen van de Commissie Gezondheid en beroepsmatige blootstelling aan stoffen van de Raad, die deze evaluatie en beoordeling verricht, β-oestradiol onder de loep. β-Oestradiol is een natuurlijk vrouwelijk geslachthormoon en wordt onder andere gebruikt in anticonceptiemiddelen en hormonale therapie. Deze evaluatie betreft alleen externe blootstelling aan β-oestradiol.

Op basis van de beschikbare gegevens concludeert de commissie dat β-oestradiol kankerverwekkend is voor de mens en beveelt zij aan de stof te classificeren in categorie 1A*. De commissie concludeert dat β-oestradiol werkt via een niet-stochastisch genotoxisch mechanisme, er zijn echter ook aanwijzingen voor een stochastisch genotoxisch mechanisme.

* Volgens het classificatiesysteem van de Gezondheidsraad (zie bijlage G).

Executive summary

At request of the Minister of Social Affairs and Employment, the Health Council of the Netherlands evaluates and judges the carcinogenic properties of substances to which workers are occupationally exposed. The evaluation is performed by the Subcommittee on Classification of Carcinogenic Substances of the Dutch Expert Committee on Occupational Safety of the Health Council. In this report, the Committee evaluated β-estradiol. β-Estradiol is a natural female sex hormone that is used in among others contraceptives and hormonal therapy. This evaluation only concerns the external exposure to β-estradiol.

Based on the available information, the Committee concludes that β-estradiol is known to be carcinogenic to man, and recommends to classify the substance in category 1A*. The Committee concludes furthermore that β-estradiol acts by a non-stochastic genotoxic mechanism, however, there are also indications for a stochastic genotoxic mechanism.

* According to the classification system of the Health Council (see Annex G).

Chapter 1

Scope

1.1 Background

In the Netherlands a special policy is in force with respect to occupational use and exposure to carcinogenic substances. Regarding this policy, the Minister of Social Affairs and Employment has asked the Health Council of the Netherlands to evaluate the carcinogenic properties of substances and to propose a classification (see Annex A). In addition to classifying substances, the Health Council also assesses the genotoxic properties of the substance in question. The assessment and proposal for a classification are expressed in the form of standard sentences (see Annex G).

This report contains the evaluation of the carcinogenicity of β-estradiol.

1.2 Committee and procedures

The evaluation is performed by the Subcommittee on Classification of Carcinogenic Substances of the Dutch Expert Committee on Occupational Safety of the Health Council, hereafter called the Committee. The members of the Committee are listed in Annex B. The submission letter can be found in Annex C.

In 2013, the President of the Health Council released a draft of the report for public review. The individuals and organisations that commented on the draft are

listed in Annex D. The Committee has taken these comments into account in deciding on the final version of the report.

1.3 Data

The evaluation and recommendation of the Committee is standardly based on scientific data, which are publicly available. The starting points of the Committees' reports are, if possible, the monographs of the International Agency for Research on Cancer (IARC). This means that the original sources of the studies, which are mentioned in the IARC-monograph, are reviewed only by the Committee when these are considered most relevant in assessing the carcinogenicity and genotoxicity of the substance in question. In the case of β-estradiol, such monograph and several updates exist.[1-3] In the 1987 supplement, β-estradiol was reviewed as part of a group of steroidal estrogens.[3] In two subsequent monographs, the carcinogenicity of β-estradiol-containing contraceptives and hormonal therapies were evaluated.[4,5] The summaries and conclusions of the IARC monographs specifically addressing β-estradiol are inserted in Annex E.

As evaluations of 1999[4] and 2007[5] did not specifically address β-estradiol as such, the literature search for carcinogenic and genotoxic properties of β-estradiol was performed starting from 1987.

More recently published data were retrieved from the online databases Medline, Toxline, Chemical Abstracts, and RTECS. The last updated online search was in April 2013. The new relevant data were included in this report.

Chapter 2

General information

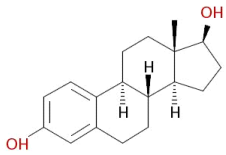
2.1 Identity and physico-chemical properties

β-Estradiol (CAS number 50-28-2) relates to the beta-isomeric form of estradiol, 17β-estradiol. The α-isomeric form is not addressed in this report. The data have been retrieved from the European Substance Information System (ESIS*), and the Hazardous Substances Data Bank (HSDB**).

The relevant physico-chemical properties of β-estradiol are presented below.

Chemical name	:	Estradiol
CAS registry number	:	50-28-2
EINECS number	:	200-023-8
Synonyms	:	Dihydrofollicular hormone; dihydrofolliculin; dihydromenformon; dihydrotheelin; dihydroxyestrin; 3,17β-dihydroxyestra-1,3,5(10)-triene; 3,17-epidihydroxyestratriene; β-estradiol; 17β-estradiol; 3,17β-estradiol; (D)-3,17β-estradiol; oestradiol-17β; 17β-oestradiol
Appearance	:	White or slightly yellow, small crystals or crystalline powder
Use	:	Medicine (estrogenic hormone)

* ESIS can be accessed via the ECB-site: http://esis.jrc.ec.europa.eu/.
** HSDB can be accessed via http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB.

Chemical formula	:	$C_{18}H_{24}O_2$
Structural formula		
Molecular weight	:	272.39 g/mol
Boiling point	:	-
Melting point	:	173 - 179 ˚C
Vapour pressure	:	-
Vapour density (air = 1)	:	-
Solubility	:	In water: 60 mg/L at 27 ˚C. Freely soluble in alcohol; soluble in acetone, dioxane, other organic solvents; solutions of fixed alkali hydroxides; sparingly soluble in vegetable oils.
Conversion factor	:	1 mg/m^3 = 0.09 × ppm
EU Classification (100% solution)	:	Not classified

2.2 IARC classification

In 1974, IARC concluded that estrogens for treatment of the menopausal syndrome and related conditions had not been shown to be associated with a risk of cancer.[1] IARC concluded in 1979 and 1987 that there was sufficient evidence for carcinogenicity of β-estradiol in experimental animals.[2,3] The summaries of these IARC evaluations are included in Annex E of this report.

β-Estradiol is primarily used as a component of oral contraceptives and post-menopausal therapies. In 1999, post-menopausal estrogen therapy was considered carcinogenic to humans (Group 1) and post-menopausal estrogen-progestogen therapy possibly carcinogenic to humans (Group 2B).[4] Combined estrogen-progestogen menopausal therapy was considered carcinogenic to humans (Group 1) in 2007.[5] However, for the 1999 and 2007 monographs, no specific data on β-estradiol were considered by IARC.

Chapter 3

Carcinogenicity studies

3.1 Observations in humans

No human studies on β-estradiol were evaluated by IARC.[2,3]

Cohort studies

A large number of cohort studies demonstrating a relationship between increased cancer risk and β-estradiol-containing pharmaceuticals (contraceptives, menopausal therapy etc.) was included in the IARC evaluations of 1999 and 2007.[4,5] However, in nearly all cases co-exposure to other estrogens and/or progestogens occurred, precluding the establishment of the relationship between exposure to β-estradiol only and cancer incidence in humans.

Thomas et al. performed a meta-analysis of 29 epidemiologic papers (covering the period of January 1966 to July 1996).[6] The criterion for considera-tion was that the study reported a point estimate for the measurement of β-estradiol in blood (or estrogen in urine) in postmenopausal breast cancer cases (retrospective or prospective) and controls. The ratio of the average estrogen concentration in the women with breast cancer to that in the women without breast cancer (and its 95% confidence interval (CI) was calculated for each study, and the results were summarised by calculating weighted averages of the log ratios. In six prospective studies on serum β-estradiol concentration, 329 women who subsequently

developed breast cancer had, overall, a 15% (CI = 6-24%, p = 0.0003) higher mean concentration of β-estradiol in their blood than the 1,105 women who remained free of cancer. The results of these prospective studies did not differ significantly from each other (χ^2 for heterogeneity = 8.7; degrees of freedom = 5; $p > 0.1$). Out of 16 case-control studies that compared the serum concentration of β-estradiol in breast cancer cases and controls, 7 reported a significantly higher mean concentration of β-estradiol than the controls. Over-all, the breast cancer cases had a 24% higher mean concentration of β-estradiol than the controls. There was, however, significant heterogeneity among the results of these studies ($\chi^2 = 57.2$, degrees of freedom = 15; $p < 0.00001$).

Beral et al.[7] reported on the results of the Million Women Study, a cohort study conducted between 1996 and 2001 that included 1,084,110 UK women aged 50-64 years. Half of the women in the study had used hormone-replacement therapy, who were compared to never users, after stratification by age, time since menopause, parity and age at first birth, family history of breast cancer, body-mass index, region, and deprivation index. All current users of hormone-replacement therapy at recruitment, but not past users, were more likely to develop, or die from invasive breast cancer. Results are summarised for users of preparations containing only oestrogen (including β-estradiol). The relative risk (RR) of breast cancer incidence was statistically significantly raised for all current oestrogen-only users versus all never users (RR = 1.30; 95% CI 1.22-1.38, $p < 0.0001$). This increased risk was dependent on treatment duration with the highest risk observed for women with a use of ≥ 10 years (RR = 1.37; 95% CI 1.22-1.54). Risks varied little between specific estrogens used (equine estrogen and ethinylestradiol), and doses applied. The relative risks were statistically significantly increased separately for oral, transdermal, and implanted oestrogen-only formulations (RR = 1.32; 95% CI 1.21-1.45, RR = 1.24; 95% CI 1.11-1.39, and RR = 1.65; 95% CI 1.26-2.16, respectively).

3.2 Carcinogenicity studies in animals

IARC reported on several animal carcinogenicity studies, e.g. with oral and subcutaneous administration, in various species.

Mammary, pituitary, uterine, cervical, vaginal, testicular, lymphoid and bone tumours were observed in mice. Also in rats, β-estradiol induced mammary and/or pituitary tumours. In hamsters malignant kidney tumours were noted and in guinea pigs, premalignant lesions were reported.[3] These studies are summarised in Annex F.

No inhalation studies are available.

Oral administration

Studies with mice

Several studies on the tumourigenic activity of β-estradiol have been conducted as part of the International Life Sciences Institute's (ILSI)/Health and Environmental Science Institute Alternatives to Carcinogenicity Testing (ACT) project).

McClain et al. reported the results of a neonatal mouse model in two oral gavage studies using CD-1 mice (following ILSI protocol).[8] The neonatal mouse model is known to be very sensitive for the detection of genotoxic carcinogens, and irresponsive to chemicals that act via epigenetic mechanisms. The two main target tissues for this model are considered to be the liver and the lungs. Animals are treated on days 8 and 15 of age, weaned around 22 days of age and then maintained until 1 year of age at which time they were sacrificed. Six litters containing 4 neonates/sex/litter were dosed for each group.

In one of the oral gavage studies, mice were treated with doses of 1, 2 and 4 mg/kg bw. No dose-dependent increase in adenoma or carcinoma of the liver or lung was reported. In the other study, after treatment with 2, 3 and 4 mg/kg bw, positive results were reported. A clear increase was observed in liver tumours in male mice, particularly carcinomas (5 in male mice, versus 0 in controls) and in lung tumours (6 lung adenomas in males and 4 in females, versus 0 in controls).

Van Kreijl et al. reported the results of β-estradiol tested in DNA repair deficient $Xpa^{-/-}$ and $Xpa^{-/-}$ / $p53^{+/-}$ knock-out mice in a C57BL/6 genetic background (further referred to as XPA and XPA/p53 model).[9] Groups of male and female mice (15 mice/sex/group) received β-estradiol by gavage at dose levels of 0 (control), 1, 2.5 and 5 mg/kg bw/day (XPA group) or 0 (control) and 5 mg/kg bw/day (XPA/p53 group), 7 days/week for 9 months (39 weeks), after which the animals were sacrificed and subjected to gross patho-logical and histological examinations. The observed spontaneous tumour formation in the XPA mice after 9 months was comparable to that of wild-type mice (total 6%); in the XPA/p53 mice it was somewhat higher (9% males; 13% females).

A positive tumour response was observed for β-estradiol in XPA/p53 group, but not in the XPA group. This conclusion was based on the increased incidence of osteosarcomas (3 in males receiving 5 mg/kg/bw, compared to none in the controls). The authors noted that although this increase was not statistically

significant, the response was considered positive in view of the rare nature of the tumour, and the presence of supporting fibro-osseous lesions.

Also within the ILSI framework, tumourigenicity of β-estradiol was evaluated in two 26-week short-term carcinogenicity studies in the $p53^{+/-}$ mice.[10] The protocol included administration by gavage of three dose levels of β-estradiol (0.1, 0.5 and 2.0 mg/kg bw/day and 0.5, 2.0 and 5.0 mg/kg bw/day, in two studies respectively), to groups of 15 mice/sex/dose for 26 weeks.

In the study using dose levels up to 2 mg/kg bw/day no treatment-related neoplastic effects were observed in any tissue. In the second study, testing up to a 2.5-fold higher dose (5.0 mg/kg bw/day) resulted in increased incidences of pituitary hyperplasias (in 12 out of 15 mice; 1 case of focal hyperplasia) and a single adenoma in the female high-dose mice. Although an incidence of 1/15 for adenoma was not statistically significant, if evaluated together with the evidence of a hyperplastic response, it was considered an equivocal response. High-dose wild-type mice in the same study showed a similar pattern, but at a lower incidence, of hyperplastic pituitary lesions (in 5 out of 15 mice).

β-Estradiol (0.1-5 mg/kg/day) has also been tested in the CB6F1-*ras*H2 mouse model, used in the ILSI project as a model that is responsive to genotoxic carcinogens.[11] Animals were dosed daily at levels of 0.1, 0.5, 2.0, and 5.0 mg/kg β-estradiol in an ethanol/methocel formulation for 6 months, followed by a 2-week recovery/respite from treatment. Control and high-dose groups with non-transgenic mice were included. The response to β-estradiol was reported to be negative, both in CB6F1-*ras*H2 animals and the non-transgenic control group.

Subcutaneous injection

Studies with mice

Martinez et al. studied the response of the inbred BALB/cCrgl newborn female mice to neonatal treatment with β-estradiol.[12] In mice, neonatal exposure to potent natural and synthetic estrogens results in the development of cervico-vaginal tumours, some of which resemble tumours in human females exposed to DES in utero. Beginning within 16 hours after birth, groups of 35 (control) and 43 (test group) mice were given 5 daily subcutaneous injections of either 5 µg β-estradiol or 20 µL sesame oil. Animals were weaned 21 days of age. All mice that survived 20 months of age were sacrificed and subjected to gross pathological and histopathological examinations. The only tumour seen in

control mice was 1 lymphoma. The incidence of malignant tumours was significantly greater in animals exposed to β-estradiol. The incidence of cervicovaginal tract carcinomas was 43%, and, for any tumour, 49% (some mice had more than one type of tumour detected). Also two cases of cholangiocarcinoma of the gallbladder, 1 case of ovarian granulosa cell tumour, 1 case of mammary gland carcinoma and 1 case of bronchoalveolar adenoma of the lung were detected.

The tumourigenicity in mice of β-estradiol was also studied by Newbold and Liehr.[13] The study was conducted to determine the potency of estrogens and estrogen metabolites to induce uterine adenocarcinoma in CD-1 mice, treated for the first 5 days of life. Outbred female CD-1 mice were treated with β-estradiol on days 1-5 of neonatal life (2 µg/pup/day) as subcutaneous injections in corn oil. Corn oil alone was used as a control. Animals were weaned at 21 days of age and sacrificed at 12 or 18 months of age.

One out of 15 mice treated with β-estradiol developed an uterine tumour. No tumours were observed in controls treated with corn oil.

Fujii reported the results of a newborn mouse tumourigenesis assay (NMTA) on 45 chemicals, including β-estradiol.[14] Forty male and female newborn ICR mice were injected subcutaneously within 24 hours of birth with 0.67 mg/kg bw β-estradiol. Control animals received 1% gelatin. The treated animals were weaned at 1 month, separated by sex and observed for 1 year. Animals were necropsied completely when moribund or dead.

All animals died within 30 months. In total 9 males and 2 females developed tumours (tumour incidence of 39% and 13%, respectively). A statistically significant increase in the number of liver tumours (4; 17% tumour incidence) was observed in male rats.

Subcutaneous implantation

Studies with rats

Mense et al. administered 3 mg of β-estradiol (only the total dose was reported) subcutaneously to female ACI rats in a form of cholesterol pellets (3 mg β-estradiol and 17 mg cholesterol).[15] All animals were divided into 4 subgroups, containing at least 10 rats each, which underwent their respective treatments for 7, 15, 120 or 240 days. At the end of each of these time periods, animals were

euthanised and subjected to gross pathological and histopatho-logical examination.

No mammary tumours were observed in 10 control rats, while the incidence of mammary tumours in rats treated with β-estradiol was 82% following 240 days (9 out of 11 rats). The first palpable breast tumour appeared after 128 days. The tumours were classified as adenocarcinoma and showed evidence of invasion. No significant morphological changes were noted in kidneys, uteri, lungs or brains. Treatment with the antioxidant vitamin C reduced both tumour incidence and latency, whereas the estrogen metabolic inhibitor α-naphthoflavone completely abrogated breast cancer development.

Singh et al.[16] investigated the effects of a phytoestrogen quercetin and its co-exposure with β-estradiol on the breast via subcutaneous implantation in female ACI rats. Feeding the rats (n = 6) with the quercetin-enriched diet (2.5 g/kg food) for 8 months did not induce breast tumours. However, when rats were implanted with β-estradiol pellets (3 mg β-estradiol, 17 mg cholesterol; only the total dose was reported) and fed the same diet (n = 10), 100% of the animals developed breast tumours within 8 months. A separate group of rats was implanted with β-estradiol in cholesterol pellets alone (n = 11) and not co-exposed to quercetin. Rats in the quercetin + β-estradiol group displayed an increased mammary tumour incidence relative to animals from the β-estradiol treatment group, where tumour incidence was equal to 82%. However, the increase in tumour incidence in the quercetin + β-estradiol group compared to the β-estradiol group was not statistically significant. Average tumour latency was significantly shorter for animals in the quercetin + estradiol group versus animals in the β-estradiol group, indicating that breast tumours appeared earlier in the quercetin + β-estradiol group compared to animals exposed to β-estradiol alone. No tumours were found in the control group (n = 10), which received the implantation of the vehicle alone.

Inoh et al. studied the carcinogenic effect of 5 mg β-estradiol (implanted subcutaneously as a pellet in female W/Fu rats), alone and in combination with nitrosobutylurea (NBU).[17] NBU alone (250 ppm in drinking water) for 14 days did not induce carcinogenic effects. Twelve out of 13 rats treated with β-estradiol alone developed mammary tumours ($p < 0.005$), while pituitary tumours were observed in 6 out of 13 animals ($p < 0.005$). No untreated control group was included.

Mills et al. implanted two groups of female August Copenhagen Irish (ACI) rats (26 and 20 animals, experiments I and II) subcutaneously under the back skin with a pellet containing 18 µmol of β-estradiol, corresponding to 4.9 mg of β-estradiol (only the total dose was reported in the paper).[18] Two separate groups of animals (24 for experiment 1, 10 for experiment 2) were used as controls and implanted with a vehicle alone (cholesterol).

All 46 rats developed large pituitary tumours within 7 months after the implantation and the experiment had to be terminated early. Histopathologically, major changes included the presence of extensive hyperplasia/adenoma, which were often coupled with multifocal hemorrhage, cystic change, and apoplectic necrosis. Histopathology suggested that they were essentially pituitary adenomas. In addition, 48% of all animals developed mammary tumours. In experiment I with 26 rats, the first palpable mammary tumour was observed at 126 days after the implantation. At the time of death, 9 animals (34.6%) were found to have one or multiple palpable mammary tumours and 4 other had early-stage mammary tumours (ductal carcinoma in situ), which brought the final mammary tumour incidence to 50%. A similar total mammary tumour incidence (45%) was observed in experiment II.

Turan et al. studied the potential induction of mammary tumours in female ACI rats (7-8 weeks of age) subcutaneously implanted with cholesterol pellets containing 1, 2 or 3 mg β-estradiol (only the total doses were reported).[19] Female rats were divided into 2 groups. The animals were implanted with a single 20 mg pellet containing cholesterol as an inert vehicle and β-estradiol (8 rats/dose). The rats were palpated for tumours twice weekly and terminated when a mammary tumour reached approximately 3 cm^2 in size, or at 36 weeks.

A 50% incidence of palpable mammary tumours was observed after 18 weeks for 3 mg β-estradiol, 19 weeks for 2 mg β-estradiol and 36 weeks for 1 mg β-estradiol in the first group. At termination after 36 weeks in the first group, the incidence of palpable mammary tumours was 100% for 3 mg β-estradiol, 73% for 2 mg β-estradiol and 50% for 1 mg β-estradiol. A 100% incidence of mammary tumours was observed after 24 weeks of treatment with 3 mg β-estradiol. No tumours were detected in controls.

Studies with hamsters

Li et al. studied the carcinogenic activity of β-estradiol, included in a panel of synthetic and natural estrogens, in the hamster kidney tumour model.[20] This model is one of the primary experimental systems to evaluate the carcinogenic

properties of estrogens.[21] Adult castrated-male Syrian (LAK:LVG outbred) hamsters exposed for approximately 9 months by subcutaneous implantation of 112 (± 15) mg β-estradiol pellets. Hormone pellets were renewed each 3 months. Renal tumour foci were distinguished microscopically. β-Estradiol induced bilateral and multiple renal tumours in 6/6 animals, with a combined number of tumour nodules in both kidneys of 18.0 ± 3.

In a study with a similar design, Li et al. studied the carcinogenic activity of β-estradiol as a 20 mg pellet.[22] The release rates of the pellets were adjusted so that mean daily absorptions were ca. 111 ± 11 µg (only the total dose was reported) After 9 months treatment, again a 100% kidney tumour incidence was observed in animals implanted with β-estradiol.

Liehr et al. confirmed the positive findings of β-estradiol in the kidney tumour model in Syrian hamsters, reporting incidences of 80-90%.[23,24]

Zhu and Liehr subsequently studied the influence of co-exposure to quercetin, an inhibitor of estradiol-2-hydroxylase, on the tumourigenicity of (25 mg) β-estradiol in male Syrian hamsters.[25] Whereas quercetin itself did not induce tumours, it increased the mean tumour size, the mean number of large tumour nodules and the incidence of abdominal metastases of kidney tumours as after treatment with β-estradiol.

Bhat et al. have investigated the role of oxidative stress in estrogen carcinogenesis using the hamster renal tumour model.[26] The authors implanted groups of 10 male Syrian hamsters subcutaneously with 25 mg pellets (of various estrogens, including β-estradiol). Hamsters were killed after 7 months and inspected macroscopically for tumour nodules. A 90% tumour incidence was found in the group treated with β-estradiol.

Other routes

Studies with mice

Mc Clain et al. reported the results of a neonatal mouse model with C56BL/6N mice receiving β-estradiol i.p. (following a protocol by National Center for Toxicological Research (NCTR)).[8] In this study, mice were administrated a total dose of 50 or 100 nmol (13.6 or 27.3 µg) β-estradiol. No increased incidence of neoplasms in liver or lung was reported.

3.3 Cell transformation assays

A number of cell transformation studies with β-estradiol have been published mainly using female cells, all reporting positive results. The cell transformation assays include the assessment of anchorage-independent growth of the immortalised but non-transformed human mammary epithelial cell line MCF-10A[27], phenotypical changes in immortalised human breast epithelial cells MCF-10F[28-31], microsatellite instability and neoplastic transformation of immortalised human endometrial (EM) glandular cells[32], anchorage independent growth in EM cells[32], and cellular transformation and genetic effects in Syrian hamster embryo (SHE) cells[33].

Transformation capability has also been observed in mammalian cells from male origin. β-Estradiol induced neoplastic transformation of rat prostatic epithelial NRP-152 cells, as was demonstrated by the expression of tumour markers and their capacity of forming colonies in soft agar and tumors in immunodeficient nude mice.[34] Transformation induction by β-estradiol, in the presence of testosterone, has also been described in a model for prostate carcinogenesis with human prostate stem-progenitor cells.[35]

3.4 Conclusion

The value of the available human data considers the Committee limited, as in virtually all cases, the use of oral contraceptives or hormone-replacement therapy involved other estrogens and/or progestogens besides β-estradiol present in the preparations. A positive correlation has been found between the mean serum concentrations of endogenous β-estradiol in women and the development of breast cancer. Furthermore, use of preparations containing estrogen only (including β-estradiol) is associated with an increased risk of breast cancer.

No animal inhalation carcinogenicity studies are available. From the animal data on other routes, the Committee concludes that β-estradiol is able to induce tumours in rats, mice and hamsters.

Chapter 4

Genotoxicity

4.1 Gene mutation assays

In vitro

Bacterial gene mutation assays have been consistently negative.[36,37]

In mammalian gene mutation assays, positive and negative results have been obtained. In several of these studies, V79 cells were applied. Drevon et al. co-cultured V79 cells with freshly isolated liver cells and β-estradiol (0, 25, 75 and 100 µM) for 48 h, before plating and assessment of azaguanine and ouabain resistance.[38] No increase in mutant colonies was observed.

Rajah and Pento also used the Chinese hamster lung fibroblast V79 cell line to study the mutagenicity of β-estradiol.[39] Cells were exposed for 4 days to 0.1, 1 and 10 nM concentrations of β-estradiol, in the absence of metabolic activation. At the higher doses (1 and 10 nM) β-estradiol was not mutagenic at the *hypoxanthine-guanine phosphoribosyltransferase* (*hprt*) locus of V79 cells, but at the lower dose (0.1 nM) it caused a 2-fold enhancement over the control. The survival of the cells did not vary between the dose levels.

Kong et al. found that β-estradiol significantly increased the mutant frequency of the *hprt* gene of Chinese hamster V79 cells at both physiological and pharmacological concentrations (2.57-, 3.45-, 2.63- and 8.78-fold, at concentrations of 0.01, 0.1 and 100, 1,000 nM, respectively), in the absence of metabolic activation.[40]

Cuendet et al. treated Chinese hamster V79 cells with concentrations of 10, 100 or 1,000 nM in the absence of a metabolic activation system.[27] No significant cytotoxicity was observed. At 10 and 100 nM, a concentration-dependent increase in the colony formation and a statistically significant difference in the colony formation in comparison to the solvent control was observed ($p < 0.0005$).

Tsutsui et al. allowed Syrian Hamster Embryo (SHE) cells to grow in the presence of β-estradiol for an expression time of 4 days, without metabolic activation, and plated and incubated cells for 7 days for colony formation.[41] Concentrations of 1, 3 and 10 µg/mL (3.7, 11.0 and 37 µM) β-estradiol did not induce the number of mutant colonies.

In a mouse lymphoma assay with L 5178Y $^{tk +/-}$ cells, equivocal responses were reported by Richold.[42] β-estradiol was tested at a concentration range of 25-255 µg/mL, in the presence or absence of S9-mix.

In vivo

In their genotoxicity evaluation of estradiol, Dhillon and Dhillon included an host-mediated assay (HMA) in mice.[43] Animals were treated with 0.1, 1 or 10 mg/kg bw estradiol (two i.p. injections with a 12-hour interval). DMSO (5 ml/kg bw) and 2-aminofluorene (20 mg/kg bw) served as negative and positive control, respectively. After 4 hours, 1.5 mL culture of *S. typhimurium* was injected into the tail vein. After an additional 2-hour incubation, mice were killed and livers removed, minced, washed and homogenised. The homogenates were transferred to agar plates and incubated for 48-hour to allow the growth of *His*$^+$ revertant colonies. No statistically significant increase in mutant colonies compared to concurrent controls was observed.

4.2 Cytogenicity assays

In vitro

Joosten et al. and Liehr reviewed the genotoxicity data of β-estradiol.[36,37] Several in vitro (structural) chromosomal aberration tests, both with positive[43-47] and negative results[33,41,48-51] have been reported in these reviews.

Positive results were reported in the in vitro micronucleus assay.[52-54] One in vitro sister chromatid exchange (SCE) assay was reported negative[50] and one positive[46]. In the review by Joosten et al., also six numerical chromosomal aberration tests were reported (both for polyploidy[47,48,55] and for

aneuploidy[33,41,50,51]), all showing positive results. In two Comet assays, positive results were reported.[54,56]

In addition, Eckert and Stopper[57] examined the ability of β-estradiol to induce micronucleus formation in the Chinese hamster V79 cell line, by exposing cells to concentrations up to and including 100 µM for 4 hours in the absence of a metabolic activation system. β-Estradiol induced an increase in cells with micronuclei in V79 cells in a concentration-dependent manner.

In vivo

In the majority of the in vivo micronucleus assays reviewed by Joosten et al.[36] (i.e. two performed in mouse bone marrow cells[58,59], one in mouse pheripheral lymphocytes[60], one in mouse spermatids[52], and two in rat bone marrow cells[58,59]), no induction of micronucleated cells was observed. In one bone marrow micronucleus assay in mice, positive results were reported.[43]

One in vivo chromosomal aberration test in Syrian hamster renal cells was reported to be positive.[45]

4.3 Miscellaneous

In vitro

Several unscheduled DNA synthesis (UDS) tests in rat hepatocytes showed negative results[50,61,62], whereas in an UDS test by Althaus et al., positive results were reported.[63]

Han and Liehr (cited in Joosten et al.), reported no increase in adducts in Syrian hamster DNA incubated with liver microsomes.[64]

Yagi et al.[65] studied the ability of β-estradiol and metabolites to induce DNA adducts in Syrian hamster embryo (SHE) cells using a ^{32}P-post-labeling assay. DNA adducts were detected in cells treated with 2- and 4-hydroxyestradiol. In contrast, DNA adducts were not detected in SHE cells treated with β-estradiol.

Van Aswegen et al. examined in vitro genetic toxicity of β-estradiol and its catechol metabolites, 2-hydroxy- and 4-hydroxyestradiol, using the Chromotest, in the absence of the liver homogenate S9.[66] The toxicity of estradiol and the catechol-estradiols was determined using the Toxi-Chromotest by measuring the activity of β-galactosidase in terms of absorbance. An increase in absorbance at low concentrations of estrogens was detected, reaching approximately a 2-fold

increase in absorption at 5 nM β-estradiol and 15 nM 4-hydroxyestradiol, while above these concentrations a decrease in enzyme production was observed, indicating cell damage due to toxicity.

Fernandez et al.[28,29] tested microsatellite instability and loss of heterozygosity of chromosomes 13 and 17 in MCF-10F cells after treatment with β-estradiol. MCF-10F cells treated with β-estradiol or 4-hydroxyestradiol alone or in combination with antiestrogen ICI182780 exhibited a loss of heterozygosity in the region 13q12.3 with the marker D13S893 in comparison to the parental cell line. Cells treated with β-estradiol or 4-hydroxyestradiol at doses of 0.007 and 70 nM and 2-hydroxyestradiol only at a higher dose (3.6 µM) showed a complete loss of 1 allele with D13S893. No further microsatellite changes were detected with any of the other 24 markers used for chromosome 13. For chromosome 17, differences were found using the marker TP53-Dint located in exon 4 of the tumour suppressor gene *p53*. Cells treated with β-estradiol or 4-hydroxyestradiol at doses of 0.007 nM and 70 nM and 2-hydroxyestradiol at a higher dose of 3.6 µM exhibited a 5 bp deletion in *p53* exon 4.

Continuing the work of Fernandez et al.[28,29], Russo and co-workers[67] demonstrated that the treatment of MCF-10F cells with 70 nM β-estradiol resulted in loss of genetic material. The first loss detected in β-estradiol treated cells was in chromosome 9p11-13. The same loss was maintained in the β-estradiol-treated transformed (invasive) cells, in the tumours formed by these cells in SCID mice, and in all cell lines derived from these tumours. β-Estradiol treated invasive cells also exhibited loss of chromosome 4p, which expanded to the loss of the complete chromosome in the tumours derived from these cells as well as in the cell lines derived from the tumours. Four additional losses appeared in all the tumours and in their derived cells, that included chromosomes 3p12.3-13, 8p11.1-21, 18q, and 9p21-pter, whereas the loss of chromosome 9p11-13 observed in previous cell lines was no longer evident. Gains in 1p and 5p15-qter were observed in the four tumours formed by β-estradiol treated invasive cells in SCID mice and the cell lines derived from them.

In vivo

In Joosten et al. several studies have been noted in which adduct formation was reported in Syrian hamster liver and kidney in vivo.[68-70]

Cavalieri et al.[71] treated female Sprague-Dawley rats at each of four mammary glands with the metabolites of β-estradiol, catechol estrogene-3,4-quinone and 4-hydroxyestradiol by intra-mammillary injection (200 nmol). The 4-hydroxy-

estradiol-1(α,β)-N7Glua depurinating adduct was detected at a level of 2.3 or 1.4 µmol/mol DNA-phosphate for treatment with the quinone and hydroxy derivative, respectively.

4.4 Conclusion

In several in vitro studies, an ability of β-estradiol to induce gene mutations and/or chromosome aberrations or aneuploidy in mammalian cell systems has been reported. From the available in vivo genotoxicity studies, there is evidence that β-estradiol can induce structural and numerical chromosome aberrations. The Committee concludes that β-estradiol is genotoxic.

Chapter 5

Classification

5.1 Evaluation of data on carcinogenesis and genotoxicity

No human data are available on the carcinogenicity of exclusive exposure to β-estradiol. Although a positive correlation has been observed between the mean serum concentrations of endogenous β-estradiol in women and the development of breast cancer, the Committee cannot establish a direct relationship between the exposure to β-estradiol and increased human cancer risk.

Only animal data on oral and subcutaneous routes of administration are available. These studies demonstrate that β-estradiol can induce the development of tumours, including mammary, pituitary, kidney and liver tumours in rats, mice and hamsters. Although these studies do not comply with the current guidelines, the Committee considers that there is sufficient evidence of carcinogenicity of β-estradiol in animals.

The Committee concludes that β-estradiol is genotoxic. In vitro, several studies are available that report the ability of β-estradiol to induce gene mutations, chromosome aberrations or aneuploidy. From the available in vivo genotoxicity studies, there is evidence that β-estradiol can induce structural and numerical chromosome aberrations. These observations are in line with a non-stochastic genotoxic mechanism. The weak mutagenic effects observed in vitro however, also indicate a stochastic genotoxic mechanism.

The Working Group of IARC classified steroidal estrogens, oral contraceptives (combined) and post-menopausal estrogen therapy as carcinogenic to

humans (Group 1) and post-menopausal oestrogen-progestogen therapy as possibly carcinogenic to humans (Group 2B), in one or more of its evaluations published in 1987[3], 1999[4] and 2007[5]. In all cases, β-estradiol was listed as one of the substances used in these preparations.

Based on the available data on carcinogenicity and mechanism of action of β-estradiol, and the available data on hormonal therapies including β-estradiol, the Committee considers β-estradiol to be carcinogenic in humans.

5.2 Recommendation for classification

Based on the available information, the Committee concludes that β-estradiol is known to be carcinogenic to man, and recommends to classify the substance in category 1A*. The Committee concludes furthermore that besides a non-stochastic genotoxic mechanism, there are also indications for a stochastic genotoxic mechanism.

5.3 Additional consideration

The Committee notes that β-estradiol is an essential endogeneous hormone, which should be taken into account when assessing the risk of external exposure to β-estradiol.

* According to the classification system of the Health Council (see Annex G).

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Annexes

Annex **A**

Request for advice

In a letter dated October 11, 1993, ref DGA/G/TOS/93/07732A, to, the State Secretary of Welfare, Health and Cultural Affairs, the Minister of Social Affairs and Employment wrote:

Some time ago a policy proposal has been formulated, as part of the simplification of the governmental advisory structure, to improve the integration of the development of recommendations for health based occupation standards and the development of comparable standards for the general population. A consequence of this policy proposal is the initiative to transfer the activities of the Dutch Expert Committee on Occupational Standards (DECOS) to the Health Council. DECOS has been established by ministerial decree of 2 June 1976. Its primary task is to recommend health based occupational exposure limits as the first step in the process of establishing Maximal Accepted Concentrations (MAC-values) for substances at the work place.

In an addendum, the Minister detailed his request to the Health Council as follows:

The Health Council should advice the Minister of Social Affairs and Employment on the hygienic aspects of his policy to protect workers against exposure to chemicals. Primarily, the Council should report on health based recommended exposure limits as a basis for (regulatory) exposure limits for air quality at the work place. This implies:

- A scientific evaluation of all relevant data on the health effects of exposure to substances using a criteria-document that will be made available to the Health Council as part of a specific request

for advice. If possible this evaluation should lead to a health based recommended exposure limit, or, in the case of genotoxic carcinogens, a 'exposure versus tumour incidence range' and a calculated concentration in air corresponding with reference tumour incidences of 10^{-4} and 10^{-6} per year.

- The evaluation of documents review the basis of occupational exposure limits that have been recently established in other countries.
- Recommending classifications for substances as part of the occupational hygiene policy of the government. In any case this regards the list of carcinogenic substances, for which the classification criteria of the Directive of the European Communities of 27 June 1967 (67/548/EEG) are used.
- Reporting on other subjects that will be specified at a later date.

In his letter of 14 December 1993, ref U 6102/WP/MK/459, to the Minister of Social Affairs and Employment the President of the Health Council agreed to establish DECOS as a Committee of the Health Council. The membership of the Committee is given in Annex B.

Annex **B**

The Committee

- R.A. Woutersen, *chairman*
  Toxicologic Pathologist, TNO Quality of Life, Zeist, and Professor of Translational Toxicology, Wageningen University and Research Centre, Wageningen
- J. van Benthem
  Genetic Toxicologist, National Institute for Public Health and the Environment, Bilthoven
- P.J. Boogaard
  Toxicologist, SHELL International BV, The Hague
- G.J. Mulder
  Emeritus Professor of Toxicology, Leiden University, Leiden
- Ms. M.J.M. Nivard
  Molecular Biologist and Genetic Toxicologist, Leiden University Medical Center, Leiden
- G.M.H. Swaen
  Epidemiologist, Dow Chemical NV, Terneuzen *(until April 1, 2013);*
  Exponent, Menlo Park, United States *(from August 15, 2013)*
- E.J.J. van Zoelen
  Professor of Cell Biology, Radboud University Nijmegen, Nijmegen
- S.R. Vink, *scientific secretary*
  Health Council of the Netherlands, The Hague

The Health Council and interests

Members of Health Council Committees are appointed in a personal capacity because of their special expertise in the matters to be addressed. Nonetheless, it is precisely because of this expertise that they may also have interests. This in itself does not necessarily present an obstacle for membership of a Health Council Committee. Transparency regarding possible conflicts of interest is nonetheless important, both for the chairperson and members of a Committee and for the President of the Health Council. On being invited to join a Committee, members are asked to submit a form detailing the functions they hold and any other material and immaterial interests which could be relevant for the Committee's work. It is the responsibility of the President of the Health Council to assess whether the interests indicated constitute grounds for non-appointment. An advisorship will then sometimes make it possible to exploit the expertise of the specialist involved. During the inaugural meeting the declarations issued are discussed, so that all members of the Committee are aware of each other's possible interests.

Annex **C**

The submission letter

Subject	: Submission of the advisory report *β-Estradiol*
Uw kenmerk	: DGV/BMO-U-932542
Ons kenmerk	: U-7912/SV/fs/246-A19
Enclosed	: 1
Date	: October 18, 2013

Dear Minister,

I hereby submit the advisory report on the effects of occupational exposure to β-estradiol.

This advisory report is part of an extensive series in which carcinogenic substances are classified in accordance with European Union guidelines. This involves substances to which people can be exposed while pursuing their occupation.

The advisory report was prepared by the Subcommittee on the Classification of Carcinogenic Substances, a permanent subcommittee of the Health Council's Dutch Expert Committee on Occupational Safety. The advisory report has been

assessed by the Health Council's Standing Committee on Health and the Environment.

I have today sent copies of this advisory report to the State Secretary of Infrastructure and the Environment and to the Minister of Health, Welfare and Sport, for their consideration.

Yours sincerely,

(signed)
Professor W.A. van Gool,
President

Annex **D**

Comments on the public review draft

A draft of the present report was released in 2013 for public review. The following organisations and persons have commented on the draft document:

- Mr. T.J. Lentz, National Institute for Occupational Safety and Health, USA.

Annex **E**

IARC evaluation and conclusion

OESTRADIOL-17b

VOL.: 6 (1974) (p. 99)

Summary of Data Reported and Evaluation

(N.B.: This section should be read in conjunction with the section 'General Conclusions on Hormones'.)

5.1 Animal carcinogenicity data

Oestradiol-17b was tested in mice, rats, hamsters and guinea-pigs by subcutaneous injection or implantation. Its administration resulted in an increased incidence of mammary, pituitary, uterine, cervical, vaginal and lymphoid tumours and interstitial-cell tumours of the testis in mice. In rats, there was an increased incidence of mammary and pituitary tumours. In hamsters, malignant kidney tumours occurred with a high incidence in intact or castrated males and in ovariectomized but not in intact females. In guinea-pigs, diffuse fibromyomatous abdominal lesions of uncertain histological interpretation were observed. Subcutaneous injections in neonatal mice resulted in precancerous and cancerous vaginal lesions in later life.

The studies in monkeys could not be assessed since they were limited in group size and duration.

Oestradiol-17b treatment increased the incidence of mammary and pituitary tumours in strains of mice having a spontaneous incidence of these tumours. The spontaneous occurrence can be related either to the presence of a virus or to a particular genetic susceptibility. No evidence of the possible role of a virus has been ascertained for rats.

The role of the hormonal balance in the development and persistence of these tumours and possible synergisms with other carcinogenic factors in increasing the incidence of lymphoid tumours has been discussed (see section, "General Remarks on the Sex Hormones", in this volume).

5.2 Human carcinogenicity data

No case reports or epidemiological studies were available to the Working Group. Epidemiological studies on steroid hormones used in oestrogen treatment have been summarized in the section, "Oestrogens and Progestins in Relation to Human Cancer" in this volume.

Subsequent evaluations: Vol. 21 (1979); Suppl. 7 (1987)

OESTRADIOL-17b, OESTRADIOL 3-BENZOATE, OESTRADIOL DIPROPIONATE, OESTRADIOL-17b-VALERATE AND POLYOESTRADIOL PHOSPHATE

VOL.: 21 (1979) (p. 279)

5. Summary of Data Reported and Evaluation

(N.B. - This section should be read in conjunction with the General Remarks on Sex Hormones and with the General Conclusions on Sex Hormones.)

5.1 Experimental data

Oestradiol-17b and its esters were tested in mice, rats, hamsters, guinea-pigs and monkeys by subcutaneous injection or implantation and in mice by oral administration. Its subcutaneous administration resulted in increased incidences of mammary, pituitary, uterine, cervical, vaginal and lymphoid tumours and interstitial-cell tumours of the testis in mice. In rats, there was an increased

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incidence of mammary and/or pituitary tumours. In hamsters, a high incidence of malignant kidney tumours occurred in intact and castrated males and in ovariectomized females, but not in intact females. In guinea-pigs, diffuse fibromyomatous uterine and abdominal lesions were observed. Oral administration of oestradiol-17b in mice led to an increased mammary tumour incidence. Subcutaneous injections in neonatal mice resulted in precancerous and cancerous cervical and vaginal lesions in later life and an increased incidence of mammary tumours.

Oestradiol-17b has teratogenic actions on the genital tract and possibly on other organs and impairs fertility.

5.2 Human data

No case reports or epidemiological studies on oestradiol-17b alone were available to the Working Group Epidemiological studies on steroid hormones used in oestrogen-progestin oral contraceptive preparations have been summarized in the section, 'Oestrogens and Progestins in Relation to Human Cancer'.

5.3 Evaluation

There is *sufficient evidence* for the carcinogenicity of oestradiol-17b in experimental animals. In the absence of adequate data in humans, it is reasonable, for practical purposes, to regard oestradiol-17b as if it presented a carcinogenic risk to humans. Studies in humans strongly suggest that the administration of oestrogens is causally related to an increased incidence of endometrial carcinoma; there is no evidence that oestradiol-17b is different from other oestrogens in this respect.

For definition of the italicized terms, see Preamble Evaluation.

Previous evaluation: Vol. 6 (1974)

Subsequent evaluation: Suppl. 7 (1987) (Steroidal oestrogens)

Excerpt from Volume: Supplement 7 (1987) (p. 280)

STEROIDAL OESTROGENS (Group 1*)

Oestradiol-17b and esters

A. Evidence for carcinogenicity to animals (*sufficient*)

Oestradiol-17b and its esters were tested in mice, rats, hamsters and guinea-pigs by oral and subcutaneous administration. Administration to mice increased the incidences of mammary, pituitary, uterine, cervical, vaginal, testicular, lymphoid and bone tumours [ref: 1-5]. In rats, there was an increased incidence of mammary and/or pituitary tumours [ref: 1,6]. Oestradiol-17b produced a nonstatistically significant increase in the incidence of foci of altered hepatocytes and hepatic nodules induced by partial hepatectomy and administration of *N*-nitrosodiethylamine in rats [ref: 7]. In hamsters, a high incidence of malignant kidney tumours occurred in intact and castrated males [ref: 1,8-10] and in ovariectomized females, but not in intact females [ref: 1]. In guinea-pigs, diffuse fibromyomatous uterine and abdominal lesions were observed [ref: 1].

B. Other relevant data

No data were available on the genetic and related effects of oestradiol-17b in humans.

Oestradiol-17b did not induce chromosomal aberrations in bone-marrow cells of mice treated in vivo. Unusual nucleotides were found in kidney DNA of treated hamsters. It induced micronuclei but not aneuploidy, chromosomal aberrations or sister chromatid exchanges in human cells in vitro. In rodent cells *in vitro*, it induced aneuploidy and unscheduled DNA synthesis but was not mutagenic and did not induce DNA strand breaks or sister chromatid exchanges. Oestradiol-17b was not mutagenic to bacteria [ref: 11].

* This evaluation applies to the group of chemicals as a whole and not necessarily to ail individual chemicals within the group.

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Annex **F**

Animal studies

Animal carcinogenicity data reported by IARC.[2]

Species, strain (sex)	Dose	Exposure duration/ frequency	Carcinogenic effects Other	References cited in IARC
Oral administration				
Mouse, C3H/HeJ (MTV+), (females) N=100	0.5 mg/L in drinking water	19 months	Mammary tumours (27/99 compared with 11/100 in controls)	Welsch et al. (1977)
Mouse, C3H/HeJ, (MTV+),(females) N=48	0, 100, 1,000 and 5,000 µg/kg diet	24 months	Mammary adenomas: 4/47 (controls) 0/35 (100 µg/kg) 6/36 (1,000 µg/kg) 8/48 (5,000 µg/kg) Other tumours: 1 adenocarcinoma of the cervix (100 µg/kg) 1 osteosarcoma of the cranium- (100 µg/kg) 2 adenomacarcinomas of the uterus (5,000 µg/kg) 3 adenocarcinomas of the cervix (5,000 µg/kg) 1 adenoacanthoma of the uterus (5,000 µg/kg) None other in controls	Highman et al. (1977)

Subcutaneous and/or intramuscular administration				
Mouse, Marsh-Buffalo, (MTV^+) (females) N=40	80 µg s.c. or i.m. (total dose 3.3-4.2 mg)	Twice weekly for 6 months	Mammary tumours: No increased incidence in intact and ovariectomized animals compared to controls. Lymphosarcomas: • 28% (intact animals) • 47% (ovariectomised animals) • 10% (controls)	Bischoff et al. (1942)
Mouse, Marsh-Buffalo, (males) N=36-43			Lymphoid tumours: • 8% (intact animals) • 34% (castrated animals) • 5% (controls)	
Mouse, 7 strains, N=822 (all strains)		Once a week s.c. for 10 weeks	β-estradiol resulted in a greater incidence of lymphoid tumours compared with other oestrogens tested (further not specified)	Gardner et al. (1944)
Subcutaneous implantation				
Mouse, (C3HxRIII)F1 (MTV^+) (castrated males)	0.5-1 mg in paraffin		Mammary tumours: • 15/16 (implant at 10 days) • 18/18 (implant at 70 days) • 7/41 (castrated controls)	Rudali et al. (1971)
Mouse, BALB/C (females) N=20	5 mg in cholesterol mixture	Every 3-4 months for 20 months	Precancerous lesions: • 2/17 Squamous cell carcinoma of the cervix/vagina: • 5/17 No cervical or vaginal lesions were observed in the 16 surviving controls	Munos (1973)
Mouse, (C3HxRIII)F1 (MTV^+) (both sexes)	0.64-0.85 mg in paraffin		Mammary tumours: • 15/16 females • 15/16 males (castrated) • 28/34 female controls • 10/61 male controls	Rudali et al. (1971)
Mouse, (C3HxRIII)F1 (castrated males)	0, 1, 2.5, 5, 10, and 100 µg in paraffin		Mammary tumours: • 11/33, 11/31, 23/27, 24/27, 27/2 and 23/24 (increasing doses)	

Rats, several species (females):	Two pellets of 5-6 mg	At 4 weeks and 1-3 months of age	Mammary tumours (adenocarcinomas, papillary carcinomas, aplastic carcinomas):	Gillman and Gilbert (1955)
Wistar albino			10/27	
Albino of the Royal Cancer Hospital strain			2/38	
Hooded rats originally derived from MRC			6/19 No equivalent numbers were observed in breeding or control rats	
Hamster (males and females)	20 mg	One or more pellets every 21 weeks	Renal tumours 15/15 (intact males) 12/12 (castrated males) 10/16 ovariectomised females) 0/6 (intact females) 0/145 (intact controls; either sex) 0/72 (castrated controls)	Kirkman (1959)
Guinea-pig (females)	20 or 50 mg		Fibromyomas in the uterine corpus, mesentery and other abdominal sites, 3 months after ovariectomy	Lipschütz and Vargas (1939)
Monkey, rhesus (females) N=5	575-825 mg	At intervals of 5-6 weeks, for 24-28 months	No tumours were found	Engle et al. (1943)
Monkey, Capuchin (females) N=5	250-700 µg/day (total amount of oestrogen; not specified for estradiol dipropionate/ β-estradiol)	29-145 weeks	A high degree of cystic and polypous glandular hyperplasia of the uterine mucosa developed in all animals	Iglesias and Lipschütz (1947)
Neonatal exposure				
Mouse, MTV$^+$	NS	NS	Increased tumourigenesis	Bern et al. (1975, 1976); Mori (1968a,b)
Mouse, C3H/MS (males)	NS	NS	Hyperplastic nodules or metaplastic lesions in various accessory sex organs	Mori (1967)

Mouse, A/Crgl;-BALB/cCrgl; C57BL/Crgl; RIII/Crgl; C3H/Crgl (females)	5 µg	5 consecutive days after birth	Hyperplastic and epidermoid vaginal lesions, mostly with vaginal concretions: • 16/23 (A/Crgl); • 6/14 (BALB/cCrgl); • 4/16 (C57BL/cCrgl); • 3/15 (RIII/Crgl) • 0/6 (C3H/Crgl) • 1/5 (C57BL/Crgl controls)	Takasugi and Bern (1964)
Mouse, BALB/cCrgl (females)	25, 5 and 0.1 µg	5 consecutive days after birth	Persistent vaginal cornification: 100% in 25, 5 µg group; 37/42 in 0.1 µg group; Vaginal epithelial downgrowths: • 16/16 (25 µg; intact) • 11/11 (5 µg; intact) • 16/19 (0.1 µg; intact) • 15/16 (25 µg; ovariectomised) • 5/10 (5 µg; ovariectomised) • 0/9 (0.1 µg; ovariectomised) • 5/10 (intact controls) Hyperplastic vaginal lesions: • 19/27 (25 or 5 µg; intact) • 8/26 (25 or 5 µg; ovariectomised) • 3/19 (0.1 µg; intact) • 0/9 (0.1 µg; ovariectomised) • 0/10 (intact control) Increased ovarian weight was observed in intact animals treated with β-estradiol	Kimura and Nandi (1967)
Mouse, BALB/cfC3H (MTV+), BALB/c (MTV-), C57BL (MTV-) (females)	5 and 20 µg	5 consecutive days	Mammary tumours: • 8/35 (5 µg) • 32/64 (20 µg) • 0/40 (controls)	Mori (1976)
Mouse, BALB/cfC3H	5 and 20 µg	5 consecutive days	Mammary adenocarcinoma: • 17/19 (5 µg) • 20/32 (5 µg + progesterone) • 4/11 (20 µg) • 33/44 (20 µg + progesterone) • 5/17 (controls)	Jones and Bern (1977)
Mouse, BALB/c (MTV-) (females)	40 µg	5 consecutive days after birth	More and different types of mammary dysplasias compared to controls given DMBA later in life.	Warner and Warner (1975)

Rat, Sprague-Dawley (females)	10-40 µg	30 consecutive days after birth	Treatment with β-estradiol completely inhibited DMBA-induced mammary tumourigenesis (none vs 15/27 in DMBA controls), but did lead to complete mammary gland regression	Nagasawa et al. (1974)
Rat, Sprague-Dawley	100 µg	Single dose	Treatment with β-estradiol did not influence the incidence of DMBA-induced ear-duct tumours	Yoshida and Fukunishi (1977)
Rat, Sprague-Dawley	100 µg	Single dose	DMBA-induced mammary dysplasia was significantly accelerated in rats given β-estradiol; mammary carcinoma incidence was significantly reduced	Yoshida and Fukunishi (1978)

Annex **G**

Carcinogenic classification of substances by the Committee

The Committee expresses its conclusions in the form of standard phrases:

Category	Judgement of the committee (GR_{GHS})	Comparable with EU Category	
		67/548/EEC (before 12/16/2008	EC No 1272/2008 (as from 12/16/2008
1A	The compound is known to be carcinogenic to humans. • It acts by a stochastic genotoxic mechanism. • It acts by a non-stochastic genotoxic mechanism. • It acts by a non-genotoxic mechanism. • Its potential genotoxicity has been insufficiently investigated. Therefore, it is unclear whether the compound is genotoxic.	1	1A
1B	The compound is presumed to be as carcinogenic to humans. • It acts by a stochastic genotoxic mechanism. • It acts by a non-stochastic genotoxic mechanism. • It acts by a non-genotoxic mechanism. • Its potential genotoxicity has been insufficiently investigated. Therefore, it is unclear whether the compound is genotoxic.	2	1B
2	The compound is suspected to be carcinogenic to man.	3	2
(3)	The available data are insufficient to evaluate the carcinogenic properties of the compound.	Not applicable	Not applicable
(4)	The compound is probably not carcinogenic to man.	Not applicable	Not applicable

Source: Health Council of the Netherlands. Guideline to the classification of carcinogenic compounds. The Hague: Health Council of the Netherlands, 2010; publication no. A10/07E.[72]

Health Council of the Netherlands

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