European Medicines Agency
Veterinary Medicines and Inspections

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COMMITTEE FOR VETERINARY MEDICINAL PRODUCTS

Report of the CVMP on the Safety Evaluation of Steroidal Sex Hormones in particular for 17β-Oestradiol, Progesterone, Altrenogest, Flugestone acetate and Norgestomet in the Light of New Data/Information made available by the European Commission

1. General Introduction

Article 4 of Council Directive 81/851/EEC requires that a Member State shall not authorise the placing on the market of a veterinary medicinal product intended for administration to food producing animals [...] unless the active substance [...] contained in the product:

a) were authorised for use in other veterinary medicinal products in the Member State concerned on the day of entry into force of Council Regulation (EEC) No 2377/90;

b) is or are mentioned in Annex I, II, or III of the aforementioned Regulation.

With regard to substances with hormonal activity, their use in veterinary medicine is restriced by Council Directive 96/22/EC concerning the prohibition of certain substances having hormonal or thyreostatic action and of beta-agonists, and repealing Directives 81/602/EEC, 88/146/EEC and 88/299/EEC, to therapeutic and zootechnical uses. Application as growth promoting agents is not allowed according to the aforementioned legislation.

In the past few years, CVMP has evaluated MRL applications for 17β-oestradiol, progesterone, altrenogest (allyltrenbolone), cronolone (further referred to as "flugestone acetate" in the text of this report) and norgestomet.

Upon evaluation, CVMP recommended the inclusion in Annex II of Council Regulation (EEC) No 2377/90 for 17β-oestradiol and progesterone for bovines and equidae for therapeutic and zootechnical uses only, and for norgestomet for zootechnical purposes in bovines only. With respect to altrenogest, CVMP recommended its inclusion in Annex III of Council Regulation (EEC) No 2377/90 for porcine species and equidae for zootechnical purposes only, establishing provisional MRLs of 3 µg/kg for the target tissues fat liver and kidney. For flugestone acetate, the CVMP set a provisional MRL of 1 µg/kg for ovine and caprine milk (Annex III) but considered it not necessary to set MRLs for ovine and caprine tissues, thus recommending inclu sion in Annex II.

Only in the case of 17β-oestradiol the CVMP recommendation was adopted by the Commission, with a slight modification in 'Animal species'. This decision was published in the Official Journal of the European Communities as Commission Regulation (EEC) No 3059/94 of 15 December 1994:

Pharmacologically active substance(s)	Animal species	Other provisions
17β-Oestradiol	All food producing mammals	For therapeutic and zootechnical uses only

In 1997, in application of Article 9 of Council Regulation (EEC) No 2377/90, the Commission brought new data/information on 17β-oestradiol and progesterone, indicating safety concerns with regard to the genotoxic potential of these substances, to the attention of the CVMP and requested the CVMP to review the previous assessment on 17β-oestradiol. The CVMP was also asked to re-consider

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progesterone and altrenogest, and to indicate if the previous recommendations needed to be amended in the light of the new data/information that became recently available to the Commission.

During evaluation the Commision asked to also take into account any new relevant data that was available to the Scientific Committee on Veterinary Measures Relating to Public Health (SCVPH) and reported in their assessment report (SCVPH, 1999).

As in the meantime also the CVMP recommendations for flugestone acetate and norgestomet were not adopted by the Commission, the additional assessment should also cover the impact of the new data on the evaluation of these substances.

In chapter 2 of this assessment report, the previous CVMP evaluations of the genotoxic and carcinogenic hazards and risks arising from exogenous exposure of consumers to 17β-oestradiol, progesterone, altrenogest, flugestone acetate and norgestomet are shortly summarized. In chapters 3 and 4 the new data on genotoxic and carcinogenic potential of hormones and new metabolic and mechanistic studies are evaluated, followed by an updated hazard and risk assessment for these five hormones in chapter 5, and the conclusions in chapter 6.

In the report of the SCVPH, attention was also given to possible effects on the immune system and on human development and reproduction. As in general for those effects either threshold levels of exposure can be assumed, or they are related to mutagenic effects, the CVMP felt that for the substances with hormonal effects the evaluation of the genotoxicity was of major importance.

2. PREVIOUS HAZARD/RISK ASSESSMENTS FOR 17β-OESTRADIOL, PROGESTERONE, ALTRENOGEST, FLUGESTONE ACETATE, NORGESTOMET

2.1 17β-OESTRADIOL

Upon evaluation of the MRL applications for oestradiol benzoate and oestradiol hexahydrobenzoate, it was concluded that after administration these synthetic oestradiol esters are hydrolysed to the active, naturally occurring estrogen 17β-oestradiol. In the assessment and summary report (CVMP 1994) the following was concluded with respect to the genotoxicity and carcinogenicity of oestradiol, and the exposure of consumers to oestradiol residues:

- 17β-oestradiol does not induce gene mutations *in vitro*, but conflicting results are found in chromosomal aberration assays. 17β-Oestradiol can induce unscheduled DNA synthesis and DNA-adducts by indirect interaction rather than by a direct electrophilic interaction;
- following long term exposure to 17β-oestradiol at levels considerably higher than those required for a physiological response, the incidence of tumours in tissues with a high level of hormone receptors is increased. It is concluded that the carcinogenic effects occur as an extension of the physiological effects of 17β-oestradiol;
- the bioavailability of 17β-oestradiol esters after oral administration is low (3% as unchanged 17β-oestradiol), but might be higher if estron, an estrogenic active metabolite, is included;
- after therapeutic and zootechnical treatment with 17β-oestradiol esters, milk and plasma levels are at or within physiological limits. Although tissue levels might be somewhat higher than the physiological limits immediately after treatment, it was concluded that compared to the lowest human daily production rates of 17β-oestradiol in prepubertal boys, and compared to the amount of 17β-oestradiol in other food stuffs that are part of the human diet, the amount of exogenous 17β-oestradiol that humans will be exposed to through ingestion of tissue from treated animals is biologically insignificant, and will be incapable of exerting an hormonal effect in human beings.

Hence, it was concluded that for the therapeutic and zootechnical use of 17β-oestradiol no ADI or MRLs need to be established. This resulted in an Annex II recommendation for 17β-oestradiol, with the restriction that in veterinary medicine 17β-oestradiol is only allowed for therapeutic and zootechnical uses.

2.2 PROGESTERONE

Based on the data provided in the MRL applications for progesterone the following was concluded on the genotoxicity, carcinogenicity and exposure of consumers to the naturally occurring hormone progesterone (EMEA, 1996):

- progesterone does not exhibit mutagenic activity in most *in vitro* and *in vivo* tests performed;
- progesterone is known to increase the tumour incidence in endocrine target tissues after continuous (parenteral) doses clearly above the physiological levels. Progesterone is not carcinogenic per se, but acts via an epigenetic mechanism associated with its endocrine activity, i.e. its ability to cause a hyperproliferative effect at cellular levels mediated by the steroid-hormone receptor interaction. Hence, tumours will not result from ingestion of progesterone at levels that do not produce any hormonal effects;
- the human oral bioavailability of progesterone is less than 10%;
- after therapeutic and zootechnical treatment with progesterone, milk, tissue and plasma levels are at or within physiological limits.

Hence, it was concluded that for the therapeutic and zootechnical use of progesterone no ADI or MRLs need to be established. This resulted in an Annex II recommendation for progesterone, with the restriction that in veterinary medicine progesterone is only allowed for therapeutic and zootechnical uses.

2.3 ALTRENOGEST

Based on the data provided with the MRL application for the synthetic hormone altrenogest (or allyltrenbolone) it was concluded that in an adequate set of mutagenicity tests (*in vitro*: Ames test, forward mutation tests, chromosome aberration test, DNA repair tests; *in vivo*: chromosome aberration test in rats) altrenogest did not show a genotoxic potential, and that therefore chronic toxicity/carcinogenicity data were not deemed necessary (EMEA, 1997). Besides, it was argued that a potential oncogenic effect of altrenogest, being a steroidal hormone, is supposedly correlated to the hormonal activity of altrenogest. Hence, for altrenogest an ADI was set on the basis of a threshold approach, i.e. applying a safety factor to a (pharmacological) NOEL. As the residue profile after zootechnical use of this synthetic hormone indicated the need for MRLs, provisional MRLs were set for altrenogest, resulting in an Annex III recommendation, with the restriction that in veterinary medicine altrenogest is only allowed for zootechnical use.

2.4 FLUGESTONE ACETATE

Based on the data provided with the MRL application for the synthetic progestagen flugestone acetate, the substance tested negative in *in vitro* tests for gene mutations in bacteria and mouse lymphoma cells, and for chromosomal aberrations in human lymphocytes (EMEA, 1998a). It was concluded by the CVMP that flugestone acetate, like other progestagens, can be considered a non-genotoxic compound. Carcinogenicity studies were considered not necessary as flugestone acetate belongs to a class of non-genotoxic compounds and tested negative in *in vitro* mutagenicity tests. It was argued that the possible tumourigenic effects of progestagens are related to epigenetic mechanisms that are secundary to the progestational effects of these compounds. Hence, also for flugestone acetate an ADI was set on the basis of a threshold approach, i.e. applying a safety factor to the overall NOEL. As the residue profile after zootechnical use of this synthetic hormone indicated need for MRLs for milk but not for tissues, flugestone acetate was included in Annex II for tissues and in Annex III for milk, with the restrictions that in veterinary medicine flugestone is only allowed for intravaginal use for zootechnical purposes.

2.5 NORGESTOMET

For norgestomet, a synthetic derivative of progesterone, it was concluded on the basis of the data provided with the MRL application that in an adequate set of mutagenicity tests (*in vitro*: Ames test, gene mutations in mouse lymphoma cells, chromosomal aberrations in human lymphocytes; *in vivo*: micronucleus test in rats) norgestomet did not show a genotoxic potential (EMEA, 1998b). The CVMP considered that norgestomet was a non-genotoxic compound and it was therefore concluded that carcinogenicity studies were not necessary. Hence, also for norgestomet an ADI was set on the basis of a threshold approach, i.e. applying a safety factor to the pharmacological NOEL. As the residue profile

after zootechnical use of this synthetic hormone indicated no need for MRLs, norgetomet was included in Annex II, with the restriction that in veterinary medicine norgestomet is only allowed for zootechnical purposes.

3. GENOTOXICITY AND CARCINOGENICITY OF HORMONES

3.1. INTRODUCTION

The carcinogenic and genotoxic properties of a variety of natural and synthetic hormones have been repeatedly assessed and the human risks arising from exposure to these compounds evaluated. According to IARC compilations, long-term exposures to endogenous oestradiols, progesterones and testosterones, among others, are associated with carcinogenic effects in humans and in experimental animals (IARC, 1974, 1979, 1987). The mechanisms of tumour induction by these compounds have been related to their hormone (receptor-) mediated stimulation of growth and differentiation in cells of the target tissues (endometrium, mammary, testis, and prostate), rather than genotoxicity (IARC, 1987; see also FAO/WHO, 1988; EC, 1996). Hence, the cell growth stimulation effect might lead to enhanced cell division rates (mitogenesis), and subsequently mutagenesis in the affected tissues/cells, thereby contributing to the carcinogenic effects observed (Ames and Gold, 1991).

IARC re-evaluated recently the carcinogenic risks of hormones used for contraception and/or therapy purposes and identified again cell proliferation as the "most important receptor-mediated mechanism by which hormonally active compounds act in carcinogenesis at hormone-sensitive target tissues" (IARC, 1999); it was also noted that the genotoxic effects of hormones reported earlier in certain studies would occur at "appreciably higher concentrations than those at which receptor-mediated events are already saturated in vitro"(IARC, 1999). In a recent re-evaluation of certain residues of veterinary drugs in food, JECFA came to analogous conclusions about the mode of (carcinogenic) action of hormones, namely, that "estradiol-17β has genotoxic potential …(while)… the increased cancer incidence among women receiving … estrogen therapy is due to the hormonal effects of estrogens" (JECFA, 1999). Nevertheless, claims have been made of positive effects of certain hormones in genotoxicity test systems *in vitro* and *in vivo*, and further that hormonal carcinogenesis would be related to the genotoxic properties of these compounds (see Adlercreutz, 1997; Cavalieri, 1997; Epstein, 1997; Liehr, 1997c; Metzler, 1997; Pinter, 1997). In analogy with properties common to "genotoxic carcinogens" described earlier (see for example Ashby and Paton, 1993; Dybing et al., 1997), the above suggestions would mean that the carcinogenic effects of hormones observed so far are the direct consequence of mutagenic effects induced in specific genes and/or chromosomes of the target cells, thereby leading to the activation of proto-oncogenes or the inactivation of tumour suppressor genes in these cells (for recent review see Dragan, 1997). Such suggestions were also made recently by Roy and Liehr (1999) who reiterated their earlier claims that "estrogens are complete carcinogens capable of tumor initiation by mutation … (followed by) … the hormonal effects of estrogens". Based on the results of 2 recent studies on the mutagenic action of 17β-oestradiol and related compounds (Rajah and Pento, 1995; Thibodeau et al., 1998), similar conclusions were reached by SCVPH, namely, that "oestrogens … are DNA reactive and mutagenic, … suggesting that 17β-oestradiol acts as complete carcinogen by exerting tumour initiating and promoting effects" (SCVPH, 1999). The carcinogenic and genotoxic properties of the 5 compounds, 17β-oestradiol, progesterone, altrenogest, flugestone acetate and norgestomet have been reviewed and evaluated earlier by EMEA (1996, 1997, 1998a, 1998b). Therefore, the following paragraphs (i) summarise the more recent epidemiological and experimental evidence in favour or against a contribution of genotoxic events in the carcinogenic process of hormones, and (ii) re-evaluate the hazards and human risks associated with exogenous exposure to hormones. It should also be noted that the available new data concern primarily 17β-oestradiol and/or some of its (synthetic) analogues.

3.2. GENOTOXICITY

3.2.1. Genotoxicity studies *in vitro*

3.2.1.1. Chromosome aberrations, cell transformation *in vitro*

No new data on the five compounds were available. A number of synthetic analogues of estrogenic sex steroids, including ethinylestradiol, were tested for their ability to induce cytogenetic damage (metaphase chromosome aberrations) in human lymphocytes *in vitro*. In these series of experiments, the highest concentration evaluated was either clearly cytotoxic or it resulted in visible precipitates in the culture medium. Neither of the steroids induced chromosome aberrations in the presence or absence of mammalian metabolic activation. Evaluation of all data available so far indicated that the tested compounds do not induce chromosome aberrations *in vitro* (Reimann et al., 1996).

Tsutsui and Barrett reported on the induction of transformed foci in Syrian hamster embryo cells exposed to 17β-oestradiol *in vitro*. Treatment of the cells failed to induce DNA damage, chromosome aberrations and gene mutations, but aneuploidy was observed. The authors conclude that estrogen-induced cell transformation may be important in hormonal carcinogenesis, and proposed that multiple effects of estrogens acting together cause genetic alterations leading to cell transformation (Tsutsui and Barrett, 1997; Tsutsui et al., 1997).

3.2.1.2. DNA adducts and/or damage *in vitro*

Following earlier studies on the induction of DNA strand breaks and DNA adduct formation by 3,4-estroquinone, a putative metabolite of catechol estrogens (Nutter et al., 1991; Abul-Hajj et al., 1995), it was recently demonstrated that 3,4-estroquinone could form chemically stable adducts upon reaction *in vitro* with deoxyribonucleosides and/or the corresponding nucleic acid bases (Akanni and Abul-Hajj, 1997; Akanni et al., 1997; Roy and Abulhajj, 1997).

Anderson et al. (1997a; 1997b) tested 17β-oestradiol in the COMET assay, a recently developed *in vitro* test methodology enabling the detection at the cellular level of single- and double-strand breaks induced by genotoxic agents. Fresh and/or frozen human sperm samples were exposed *in vitro* to the compound; both specimens showed positive responses by comparison with untreated samples.

3.2.1.3. Genomic instability *in vitro*

Some evidence that 17β-oestradiol may enhance genomic instability, thereby accelerating the accumulation of mutations, was obtained by Paquette (1996) who reported the enhancement by 17β-oestradiol of the onset of genomic rearrangements in minisatellite sequences of transformed 10T1/2 mouse cells. Upon incubation of the cells with 17β-oestradiol (dissolved in ethanol) for 5 days and subsequent analysis with fingerprinting assay an elevated frequency of genomic rearrangements was observed in the subclones. After withdrawal of the compound from the transformed cells, no additional rearrangements were observed.

3.2.1.4. HPRT Gene mutations *in vitro*

The mutagenic activity of 17β-oestradiol was tested in an HPRT gene mutation test with V79 Chinese hamster cells by Rajah and Pento (1995), referred to in SCVPH (1999), who reported in a research communication that "estradiol caused a 2 fold increase over the control … (and therefore) … should be considered to have a mutagenic potential". The study was considered as significant by the SCVPH. However, it has several drawbacks in the way it is performed and/or reported: (i) No absolute HPRT mutation values were given, only transformed, relative mutant frequencies were presented; from the description of the methodological procedure, it can be inferred that the numbers of actually counted 6-thioguanine resistant clones must have been rather small; (ii) the experiment was obviously performed only once; (iii) only the lowest of the 3 tested exposure levels (10^{-8} M, 10^{-9} M, 10^{-10} M) gave an increased HPRT frequency. Terefore, one can only agree with the conclusions of the authors, namely, that "caution should be exercised in the interpretation of these results … (and) … further studies will be necessary before definite conclusions can be drawn concerning the relative genotoxic potential of these compounds" (Rajah and Pento, 1995).

3.2.1.5. Methotrexate-resistance *in vitro*

A recent publication by Thibodeau et al., as also considered significant by SCVPH (1999), reported increased frequencies of methotrexate-(MTX) resistant clones in human MCF-7 breast cancer cells

upon exposure to 17β-oestradiol, and 3 of its hydroxylated metabolites (Thibodeau et al., 1998). On the basis of their results, the authors conclude that 17β-oestradiol, and in particular the 4- and 16-hydroxy estrone metabolites, do enhance the rate of MTX-resistance per cell generation. As in the case of the preceding paper, however, the present study is carrying certain drawbacks which makes an interpretation of the results diffcult: First, the methodology originally devised by Luria and Delbrück (1943) was meant for demonstrating the random occurrence of spontaneous mutation in (bacterial) populations, making use of the so-called critical population size i.e. that at which roughly one mutation would occur in a series of parallel cultures. In order to determine accurately (chemically-)induced mutation rates, the present experiments should have been carried out with a much higher number of parallel cultures. Furthermore, the paper has a number of limitations which hamper a straightforward explanation of the results, namely, that the estrogens did indeed induce mutations (or permanent cellular changes) leading to MTX resistance: (i) a positive refence agent capable of inducing MTX resistance was not included; (ii) only one exposure concentration was applied; (iii) it was not clear whether the MTX-resistant phenotypes of the clones were genetically fixed; (iv) the hydroxy metabolites tested were different from the quinone derivatives postulated earlier as being DNA reactive (Akanni et al., 1997; Cavalieri et al., 1997). Taken together, the present study does need verification and refinement before a conclusion can be drawn about the ability of 17β-oestradiol and hydroxyl metabolites to induce genetic changes leading to MTX resistance in mammalian cells.

3.2.2. Genotoxicity studies in experimental animals

3.2.2.1. Cytogenetics, host-mediated assays

In studies reported and evaluated in the IARC compilations, steroid hormones were described as mostly devoid of the ability to induce chromosome type aberrations *in vitro* and *in vivo* (see IARC, 1987; see also Bartsch and Malaveille, 1989). More recently, however, Dhillon and Dhillon (1995) reported that the natural estrogen, oestradiol, was able to induce chromosome type aberrations and sister chromatid exchanges (SCEs) *in vitro* (human lymphocytes, up to 100 microgram/ml) and micronuclei (MN) and SCEs in bone marrow *in vivo* (male mice, up to 10 mg/kg bw, intraperitoneal route). In the same series of experiments, the compound appeared devoid of ability to induce gene mutations in bacteria (*Salmonella*) either *in vitro* or in the host-mediated assay. The purity of the compound used in this study was not reported.

In another evaluation of the ability of oestradiol to induce micronuclei (MN) in the bone marrow of exposed rats and mice, Ashby et al. (1997) failed to observe any MN induction in both species. The dose range extended from 3 daily doses of 20 µg/kg to the rat (which elicited a potent uterotrophic response in the animals), to single doses of between 10-150 mg/kg to the mouse. The latter dose regimen simulated and extended the test conditions used previously by others (see above) who had found oestradiol and 3 structurally-related synthetic estrogens, to be active at doses ranging between 1 and 10 mg/kg. On the basis of the present results one can agree with the authors' conclusions that oestradiol is not genotoxic to the bone marrow of exposed rodents. The authors also noted that the top dose-level used in the present, negative experiments (150 mg/kg) was 150000 times the minimum estrogenic dose of this chemical to rodents; they concluded that this was above the dose at which useful genetic toxicity data would be generated (Ashby et al., 1997).

A further evaluation of the ability of 17β-oestradiol to induce micronuclei in bone marrow of exposed rodents (male B6C3 mice and male F344 rats) was carried out by Shelby et al. (1997). In a standard 3-injection protocol, no induction of MN was observed even at extremely high exposure concentrations of 1250 mg 17β-oestradiol (purity determined as ± 98.7%) per kg body weight.

The mutagenic activity of a synthetic analogue of prostaglandin F-2 alpha, cloprostenol, was investigated on human peripheral blood lymphocytes *in vitro* and on bone marrow cells of animals (mice) exposed *in vivo* to the compound. No mutagenic effects were observed in either test system (Delic et al., 1997).

The ability of lynoral (ethinylestradiol) to induce chromosome aberrations and/or micronuclei was investigated in the bone marrow of mice exposed to the compound. Neither dose- nor time-dependent induction of chromosome aberrations or micronuclei were induced (Shyama and Rahiman, 1996).

A number of synthetic analogues of estrogenic sex steroids, including ethinylestradiol, were tested for their ability to induce cytogenetic damage (micronuclei) in the bone marrow of mice exposed *in vivo* to the chemicals. Neither of the steroids induced an increase of micronuclei. In these experiments, the highest concentration evaluated induced signs of toxicity in the animals; in case of non-toxic compounds, the highest concentrations tested were 2 g/kg bw. Evaluation of all data indicated that the tested compounds do not induce micronuclei in bone marrow *in vivo* (Reimann et al., 1996).

3.2.2.2. DNA adducts and/or damage *in vivo*

In female Sprague-Dawley rats treated by intramammary injection with 200 nmol of a putative carcinogenic metabolite of catechol estrogen, i.e. catechol estrogen-3,4-quinone, DNA depurination was observed as a result of the formation of unstable DNA adducts (Cavalieri et al., 1997).

3.2.3. Conclusions on mode of action genotoxicity

In confirmation of earlier studies provided in the MRL dossiers, most of the available recent data indicate that oestradiols and/or their synthetic analogues are devoid of the ability to induce gene mutations or chromosome aberrations *in vitro*. With regard to the studies of Rajah and Pento (1995) and Thibodeau et al. (1998), those are considered inconclusive, and therefore additional experiments are needed before making any statements that 17β-oestradiol induces MTX resistance and/or HPRT-deficient gene mutations. Tsutsui and Barrett and Tsutsui et al. hypothesised that oestradiols are capable of inducing aneuploidy, followed by malignant transformation, and the studies of Abul-Hajj et al., Paquette, and Anderson et al. may suggest that 17β-oestradiol and/or its metabolites induce DNA damage or genomic instability. However, the demonstration remains to be made that the observed indicator effects are representative of mutagenesis at the gene or chromosome level, and (ii) also occur in the somatic target cells *in vivo*. This is not likely in view of the following:

Earlier studies had mostly indicated that hormones do not induce micronuclei (MN) or other chromosome aberration types *in vivo*. With the exception of the study reported by Dhillon and Dhillon (see above), the recent data described above from the studies of Ashby et al., Shelby et al., Delic et al., and Reimann et al. confirm the earlier findings and clearly indicate that hormones and/or their synthetic analogues are not associated with genotoxic properties in the bone marrow micronucleus assay *in vivo*.

3.3. CARCINOGENICITY

3.3.1. Studies in experimental animals

ccording to IARC compilations (IARC, 1974, 1979, 1987) there is sufficient evidence for the carcinogenicity of 17β-oestradiol in experimental animals; depending on the route of administration, the compound induced tumours of the mammary gland, cervix, uterus, among others, in female mice. Subcutaneous implants of the compound induced mammary tumours in female rats and pituitary adenomas in male rats. Guinea pigs as well as Turkish and Syrian hamsters also are sensitive to the tumorigenic action of 17β-oestradiol. In its recent re-evaluation, IARC concludes that there is sufficient evidence for the carcinogenicity of 17β-oestradiol in experimental animals (IARC, 1999) Analogous effects were observed with progesterone, which increased the incidence of ovarian, uterine and mammary tumours in mice; neonatal treatment also enhanced the occurrence of precancerous and cancerous lesions of the genital tract and resulted in increased mammary tumorigenesis in female mice (IARC, 1979; 1987). The carcinogenic properties of altrenogest, flugestone acetate and norgestomet have not been studied.

The studies summarised above also had indicated that the carcinogenic potential of steroid hormones is associated with their endogenous estrogenic potential, the physiological role of which would lead to

increased cell proliferation in the hormone-responsive target tissues. More recent studies confirm and extend these earlier findings, some examples are described below.

The correlation between estrogenicity and carcinogenicity in animals exposed to oestradiol-17alpha (E_2-17α) was studied by Hajek et al. (1997) who also tested the hypothesis whether neonatal exposure to E_2-17α was tumorigenic. The results indicated a strong correlation between estrogenic and carcinogenic effects and also confirmed that neonatal exposure to E_2-17α was tumorigenic as well. In studies reported by Hilakiva-Clarke et al., support was found for the hypothesis of a causal correlation between the number of target epithelial mammary cells and the risk for hormone-receptor mediated tumorigenesis (Hilakiva-Clarke et al., 1997). Studies reported earlier by Holland and Roy (1995) also showed an increase in the number of proliferative cells by more than 2 fold and a perturbation in cell kinetics in the mammary gland of female Noble rats exposed to estrone, a steroid known to play an important role as precursor of 17β-oestradiol. Analogous conclusions were drawn by Li et al. (1995) who observed a strong correlation between carcinogenic potency of steroidal and nonsteroidal estrogens and their hormonal (estrogenic) and cell proliferative capacity in the hamster kidney as target organ.

3.3.2. Studies in humans

According to IARC compilations, the evidence for carcinogenic effects in people exposed to 17β-oestradiol alone is inadequate (IARC, 1979; 1987; see also Tomatis, 1990). However, since there is sufficient evidence for the carcinogenic effects of steroid hormones in humans, i.e. an association with increased incidences of endometrium carcinoma, 17β-oestradiol should be regarded as a human carcinogen. Data on effects of progesterone, altrenogest, flugestone acetate and norgestomet in humans were not available. Recent epidemiological studies have confirmed and extended the tumorigenic effects of 17β-oestradiol, in particular the association between endogenous estrogens and tumorigenic effects, as described below.

In confirmation of several earlier epidemiological studies, reviewed by Beral et al. (1997), Dorgan and colleagues reported of a nested case-control study the results of which lended considerable support to the hypothesis that elevated serum concentrations of estrogens (and androgens) over a long period of time are related to an increased incidence of breast cancer in postmenopausal women (Dorgan et al., 1997). Analogous observations were made by Thomas et al. (1997a) who concluded from prospective study data that there is evidence for a (strong) positive correlation between endogenous serum oestradiol concentrations in postmenopausal women and breast cancer risk (see also Thomas et al. 1997b).

3.4. STRUCTURE-ACTIVITY RELATIONSHIPS

Cunningham et al. (1996) analysed the structure of a synthetic estrogen, diethylstilbestrol (DES), and concluded that there is an absence of structural alert for potential mutagenicity of this compound and any of its metabolites. A further analysis indicated that the carcinogenic action of DES in animals is due to the presence of a molecule moiety which is related to an estrogen receptor ligand, and not to genotoxicity. In structure-activity studies within a series of non-genotoxic mouse carcinogens, Rosenkranz et al. (1996) extended the results of Cunningham et al. (see above) to natural estrogens such as β-oestradiol, estriol and estrone, and some of their metabolites, among others (see also Cunningham et al., 1998). Taken together, the studies on structure-activity relationships indicate that the carcinogenic properties of 17β-oestradiol and related chemicals are not associated with Functional Structural Alerts (FSA) for genotoxicity in the molecule.

3.5. CONCLUSIONS ON MODE OF ACTION CARCINOGENICITY

As already demonstrated earlier, the recent studies show that hormonal carcinogens in humans and experimental animals are characterized by (i) tumorigenic action typically in various endocrine-responsive organs and/or tissues, and (ii) the need for a prolonged exposure to high concentrations before tumorigenic effects become apparent. The studies are also consistent with the notion of hormone-receptor mediated increase in cell division and proliferation in epithelial cells of the target tissues. This points to a non-genotoxic mode of action, which is in concurrence with (i) the negative

results of both earlier and recently performed genotoxicity tests, and (ii) the absence of structural alerts for genotoxicity in the molecule.

As cited in the introduction, the recent extensive reviews by IARC and JECFA also confirmed that the tumorigenic action of hormones, in particular 17β-oestradiol, in animals and man are the consequence of the receptor-mediated, cell division stimulating activity of these compounds in somatic target cells, and that the potential genotoxic properties of the compounds would not be expressed *in vivo* and/or not play a role in the tumorigenic activity (IARC, 1999; JECFA, 1999).

4. METABOLIC AND MECHANISTIC STUDIES ON HORMONES

4.1. STUDIES IN EXPERIMENTAL ANIMALS

Following suggestions that the organ-specific carcinogenic effects of oestradiol in Syrian hamsters were the reflection of organ-specific metabolism, i.e. the production of 4-hydroxylated species, Hammond and colleagues analysed the enzymes responsible for the hydroxylation reactions. Their results indicate that hydroxylation of oestradiol in the liver appears to be catalysed by cytochrome P450 enzymes (CYP), in particular a member of the CYP3A family, while in the Syrian hamster kidney 2-hydroxylation appears to be catalysed by members of the CYP1A and CYP3A families, which also contribute to 4-hydroxylation (Hammond et al., 1997). No information is yet available as to the possible role of CYP1B family in the formation of 4-hydroxy oestradiol in the kidney.

Another hypothesis of organ-specific carcinogenic effects of chronic administered oestradiol in Syrian hamsters involves the formation of free (hydroxy) radicals and subsequent reaction with DNA to form hydroxylated bases and/or deoxynucleotides, e.g. 8-OH-dGua, as reported by Han and Liehr (1994a). The same authors reported the formation of 8-OH-dG *in vitro* upon incubation of DNA with a liver microsomal activation system (so-called S9 mix) and with catechol estrogens, among others. The results indicate the formation of 8-hydroxylated guanine residues and the authors conclude that their data support the mechanism of hydroxyl radical generation from estrogens (Han and Liehr, 1994b). In another series of experiments from the same group of investigators (Han et al., 1995), and using the DNA post-labelling methodology, the presence of DNA adducts was detected in the dorsolateral prostate of NBL/Cr rats exposed to 17β-oestradiol and testosterone via separate Silastic tubing implants. The presumptive DNA adduct detected by 32P-postlabeling, the chemical nature of which rermains to be elucidated, was observed in prostate tissue of rats exposed for 16 or 24 weeks, but not in rats treated for 8 weeks and not in other tissues of the exposed animals.

Mechanistic studies showing a similarity between estrogens as risk factors for human breast cancer and Syrian hamster kidney tumors (Liehr, 1997a) prompted Liehr to postulate a common ground for organ-specific tumorigenesis in the two species, namely, via (i) the biotransformation of steroidal estrogens to 4-hydroxylated catechol metabolites and subsequent formation of DNA damage, combined (ii) with a hormone receptor-mediated response in cell division and proliferation (Liehr, 1997b; see also Liehr, 1998; Zhu and Conney, 1998). Regardless of the fact whether the assumed DNA adduct formation indeed leads to mutagenesis in the affected somatic cells, the second part of the postulated process involves a non-genotoxic parameter, previously referred to as “epigenetic” mechanism in earlier studies, namely, hormone-receptor mediated mitogenesis.

In order to determine whether the induction of mouse endometrial tumors induced by 17β-oestradiol was associated with mutations in the *ras* proto-oncogene or the p53 tumor suppressor gene, a PCR-SSCP analysis was performed by Murase et al. (1995) in 13 adenocarcinomas and 11 other preneoplastic lesions. The overall results did not indicate an involvement of *ras* mutations, and inactivation of p53 appears to occur with low frequency in this particular mouse endometrial carcinogenesis model.

4.2. STUDIES IN HUMANS

Following earlier suggestions that catechol metabolites of oestradiol may contribute to the development of estrogen-induced cancers in humans, while O-methylation catalyzed by catechol-O-methyltransferase (COMT) would inactivate catechol estrogens, Lavigne and colleagues analysed COMT genotype

polymorphism (COMTLL versus COMTHL) in a cohort of 112 matched, nested case-control samples. The results clearly indicated a correlation between COMTLL (and GST-M1 null or GSTP1 genotypes) and an increased risk for developing postmenopausal breast cancer. In turn, this would indicate that catechol metabolites of oestradiol indeed contribute to the development of breast cancer (Lavigne et al., 1997).

The role of metabolism in another type of hormone-related human cancer, namely prostate cancer, has been recently further elucidated. In studies involving the analysis of genotypic differences (constitutional DNA polymorphism) in the enzyme SRD5A2, which converts testosterone into the more bioactive dihydrotestosterone, Makridakis et al. (1997) found that a germline mutation (missense substitution of valine with leucine at codon 89) which leads to a reduced *in vivo* SRD5A2 activity, is particularly common among Asians and may explain the low risk for prostate cancer in this population (as compared to highest risk in African-Americans, intermediate risk in Caucasians, and slightly lower risk in Latinos (see also Reichardt et al., 1995; Devgan et al., 1997).

The hypothesis, that hormone-associated cancer may be due to interactions with cell cycle regulation processes, arises from mutation studies in two major human breast cancer susceptibility genes, BRCA1 and BRCA2. As reviewed by Marcus et al. (1997) it appears that BRCA1 is an estrogen-inducible cell cycle-associated protein which possesses antiproliferative and tumor suppression action, and that the release of cells from its control through germline mutations would give rise to genetically evolved breast carcinoma with characteristic features and proliferation phenotype.

4.3. CONCLUSIONS ON MECHANISTIC/METABOLIC MODE OF ACTION

The above studies add evidence to the earlier suggestions of receptor-mediated increased proliferation in epithelial cells as the mode of action of hormonal carcinogens (or their metabolites) in humans. The studies also show that endogenous factors modulate the incidence of hormone-related cancer in man (via differences in metabolism and/or regulation of cell cycle kinetics), but the magnitude by which humans vary in their susceptibility to hormone-related cancer remains yet to be determined.

An alternative hypothesis has been put forward on the basis of earlier and recent studies and involves a combination of two processes in hormone-related carcinogenesis, namely, (i) DNA damage and subsequent mutagenesis in target cells and (ii) estrogen-receptor mediated increased cell division and/or proliferation in the target cells. This hypothesis implies that components of normal physiological metabolism would specifically induce genotoxic events in somatic cells of humans and animals, and remains to be confirmed in independent studies. It has to be noted, however, that the hypothesis also includes the commonly accepted receptor-mediated mode of action of hormonal carcinogens, which implies that a direct genotoxic effect in the target cells is not involved.

It should also be noted here that endogenous as well as exogenous susceptibility factors appear to play a role as modulators of hormone-related cancers in certain human sub-populations. At the endogenous level, two major susceptibility phenotypes emerge, namely, (i) those related to an altered metabolism of hormones (or precursors), and eventually leading to an increased production of estrogenic metabolites (Hammond et al., 1997; Makridakis et al., 1997; see also Devgan et al., 1997), and (ii) those related to an alteration in cell cycle control, eventually leading to increased cell turn-over and proliferation (Marcus et al., 1997). Genetic predisposition, together with environmental factors, might also be responsible for the anomalous sexual development observed within certain human sub-populations (cf. Perez-Comas and Saenz, 1997). At the exogenous level, extensive epidemiological studies have indicated the role of smoking and alcohol consumption on breast cancer incidence in humans (for review see Zumoff, 1997).

5. HAZARD AND RISK ASSESSMENT

5.1. HAZARD IDENTIFICATION

5.1.1. General considerations

As reviewed in the foregoing, there is epidemiological and experimental evidence suggesting that both steroid and nonsteroid hormones are associated with the incidence of certain cancer types in man and laboratory animals, e.g. cancer of the breast, endometrium, testis, and prostate, with some variation in the strength of evidence in humans. Hence these compounds should (continue to) be considered as presenting a carcinogenic hazard.

With regard to genotoxicity, the current evidence prevails that the compounds are devoid of genotoxic activity in the currently available standardized test systems *in vivo*. This is further substantiated by the lack of Fundamental Structural Alerts for genotoxicity in the molecules.

5.1.2. 17β-Oestradiol

On the basis of the foregoing, the same conclusions as those reached in the previous hazard assessment can be followed, namely, that the compound (i) is mainly devoid of genotoxic activity and (ii) exerts its carcinogenic action after prolonged exposure and/or at levels considerably higher than those required for a physiological (estrogenic) response.

5.1.3. Progesterone

Only few recent data were available for a re-evaluation of the carcinogenic and/or genotoxic properties of progesterone. In view of previous data (already evaluated), and in view of a mode of action expected to be analogous to that of 17β-oestradiol, the same conclusions as those of the previous hazard assessment can be followed, namely, that the compound (i) is not genotoxic in most of the tests performed, and (ii) increases tumour incidences in animals at exposure levels clearly above the physiological levels.

5.1.4. Altrenogest

With regard to carcinogenic and/or genotoxic properties, no recent data were available for altrenogest. In view of its mode of action expected to be analogous to that of the other hormones, 17β-oestradiol and progesterone, the conclusions of the previous evaluation can be maintained, namely, that the compound (i) is not genotoxic and (ii) additional carcinogenicity tests are not necessary.

5.1.5. Flugestone acetate

With regard to carcinogenic and/or genotoxic properties, no recent data were available for flugestone acetate. In view of its mode of action expected to be analogous to that of the other hormones, 17β-oestradiol and progesterone, the conclusions of the previous evaluation can be maintained, namely, that the compound (i) is not genotoxic and (ii) additional carcinogenicity tests are not necessary.

5.1.6. Norgestomet

With regard to carcinogenic and/or genotoxic properties, no recent data were available for norgestomet. In view of its mode of action expected to be analogous to that of the other hormones, 17β-oestradiol and progesterone, the conclusions of the previous evaluation can be maintained, namely, that the compound (i) is not genotoxic and (ii) additional carcinogenicity tests are not necessary.

5.2. RISK ASSESSMENT

5.2.1. General considerations

Whether exogenous exposure to natural and/or synthetic hormones would present an additional cancer risk is subject of the following considerations: (i) Is the carcinogenic mode of action of the compound a direct consequence of its genotoxic activity, i.e. results from the induction of mutations at the gene, gene segment, or chromosome level in the affected somatic target cells, or (ii) is the carcinogenic mode of action due to a non-genotoxic mechanisms, e.g. increasing cell proliferation provoked by mitogenesis and resulting in an increased probability for spontaneous mutations to arise in the target cells (see

Preston-Martin et al., 1990, 1993; Ames and Gold, 1991). The distinction between genotoxic and non-genotoxic carcinogens has consequences for the risk assessment, namely the presence or absence of exposure "thresholds" below which no carcinogenic effects would be expected.

The previous data on the carcinogenic and genotoxic properties of the compounds (see IARC, 1979; 1987; see also previous CVMP evaluations), as well as the recent studies described here, support the notion that the five compounds of interest here belong to the group of non-genotoxic carcinogens, and imply a hormone-receptor-mediated mode of action resulting in increased cell division/proliferation in the target tissues. Whether additional genotoxic effects are needed for the tumorigenic activity of (endogenous) hormones is still subject of discussion; in any case, the studies indicate that the presumed genotoxicity alone would not be sufficient to elicit the carcinogenic effects observed in the target organs.

In addition, epidemiological evidence suggests that an exogenous exposure to hormones would need to be substantial (i.e. in the order of post-menopausal therapy levels) before carcinogenic effects would be detectable in humans. From the data reported previously for the two endogenous compounds, it appears that the hormone concentration levels resulting from therapeutical and/or zootechnical treatment are within the endogenous range in these animals. Hence, and in view of the poor biological availability of the compounds, the contribution from exposure to therapeutic and zootechnical doses of natural hormones was considered negligible. No new data were provided on the exposure to the five hormones under evaluation after therapeutic and/or zootechnical use, so this conclusion is still considered valid. With respect to the use of 17β-oestradiol, progesterone and testosterone as growth promotors, JECFA reached practically the same conclusion (JECFA, 1999) by saying "The Committee noted that the hormone concentrations found in individual populations of treated animals – despite the fact that they were typically higher than the corresponding values of the concurrent controls – were within the physiological range of these substances in cattle and that the calculated excess intakes contributed only in a small additional hormonal burden to the background dietary intakes resulting from the consumption of other normal foods of both animal and plant origin. Taking into consideration that the available data on the identity and concentrations of residues of the approved veterinary drugs in animal tissues indicate a wide margin of safety for consumption of residues in food when the products are used according to good practice in the use of veterinary drugs the Committee concluded that there would be no need to specify numerical MRLs for the three hormones…". Of course this conclusion is related to the use as growth promotor. For the zootechnical use of natural and synthetic hormones, one should also consider the low frequency of treatment, the low number of animals that are treated, and the fact that treated animals are not intended for slaughter during or immediately after treatment.

In an earlier evaluation, CVMP had based its risk assessment on the relation between any possible excess of hormones from zootechnically treated animals in the diet and the endogenous daily production of oestradiol in prepurbertal boys, the latter value being estimated as 6 ug per day. It was noted that the report by Klein et al. (1994) indicated much lower plasma levels of oestradiol when measured with a new method, based on β-galactosidase gene expression in genetically modified yeast, compared to the classical RIA measurements (Klein et al., 1994). However, (i) the measure was made only in plasma and needs to be carried out in other tissue(s) in order to enable the comparison between the intake of residual oestradiol and the endogenous levels, (ii) the methodology needs validation and is not (yet) generally accepted.

5.2.2. 17β-Oestradiol

A new risk assessment for 17β-oestradiol needs not to be performed in view of the facts that (i) the hazard assessment remained unchanged in the light of recent literature (see 5.1.2. above), and (ii) that no new exposure data were provided which would indicate a modification of the previously reported exposure levels after therapeutic and/or zootechnical use.

5.2.3. Progesterone

Only a few new literature data were available for progesterone. They did not lead to a modification of the previous hazard assessment (see 5.1.3. above). A new risk assessment for progesterone needs not be

performed, since no new exposure data were provided which would indicate a change in the previously reported exposure levels after therapeutic and/or zootechnical use.

5.2.4. Altrenogest

Only very few new literature data were available for altrenogest. As mentioned above (cf. 5.1.4.), they did not motivate a change of the previous hazard assessment. Since no new exposure data were provided which would indicate a change in the previously reported exposure levels after therapeutic and/or zootechnical use of altrenogest, a new risk assessment needs not be performed.

5.2.5. Flugestone acetate

No new literature data were available for flugestone acetate. As mentioned above (cf. 5.1.5.), data on the other hormones did not motivate a change of the previous hazard assessment. Since no new exposure data were provided which would indicate a change in the previously reported exposure levels after zootechnical use of flugestone acetate, a new risk assessment needs not be performed.

5.2.6. Norgestomet

No new literature data were available for norgestomet. As mentioned above (cf. 5.1.6.), they did not motivate a change of the previous hazard assessment. Since no new exposure data were provided which would indicate a change in the previously reported exposure levels after zootechnical use of norgestomet, a new risk assessment needs not be performed.

6. CONCLUSION

On the basis of the foregoing, it is concluded that the previous recommendations with regard to ADI and MRLs, are still applicable for the five compounds, 17β-oestradiol, progesterone, altrenogest, flugestone acetate and norgestomet.

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