4. Other Data Relevant to an Evaluation of Carcinogenicity and its Mechanisms

4.1 Absorption, distribution, metabolism and excretion in humans

The metabolism and disposition of various formulations of oral contraceptives used in humans differ. After entering the small intestine, estrogenic and progestogenic compounds in combined oral contraceptives undergo metabolism by bacterial enzymes and enzymes in the intestinal mucosa to varying extents. The mixture of metabolized and unmetabolized compounds then undergoes intestinal absorption, and thus enters the portal vein blood, which perfuses the liver. In the liver, the compounds can be metabolized extensively, which leads to variations in the amount of active drug. A fraction of the absorbed dose of ethinylestradiol and some progestogens is also excreted in the bile during its first transit through the liver. Although some of these compounds are partially reabsorbed via the enterohepatic circulation, a fraction may also be excreted in this ‘first pass’, which reduces overall bioavailability.

Since steroids penetrate normal skin easily, various systems have also been developed that deliver estrogens and progestogens parenterally, e.g. transdermal patches, nasal sprays, subcutaneous implants, vaginal rings and intrauterine devices (Fanchin et al., 1997; Dezarnaulds & Fraser, 2002; Meirik et al., 2003; Sarkar, 2003; Wildemeersch et al., 2003; Sturdee et al., 2004). These different modes of administration have been described previously (IARC, 1999). In general, all parenteral routes avoid loss of the drug by hepatic first-pass metabolism and minimally affect hepatic protein metabolism.

The absorption rates of orally administered estrogens and progestogens are usually rapid; peak serum values are observed between 0.5 and 4 h after intake. Serum concentrations rise faster with multiple treatments than with single doses and achieve higher steady-state levels, which are still punctuated by rises after each daily dose. The rise in steady-state levels with multiple doses may reflect the inhibitory effect of both estrogens and progestogens on cytochrome P450 (CYP) metabolic enzyme activities. Alternatively, estrogens may induce the production of sex hormone-binding globulin (SHBG), which may increase the capacity of the blood to carry progestogens. The metabolism of progestogens and ethinylestradiol typically involves reduction, hydroxylation and conjugation. In some cases, metabolism converts an inactive pro-drug into a hormonally active compound. Hydroxylated metabolites are typically conjugated as glucuronides or sulfates and most are eliminated rapidly, with half-lives of 8–24 h (IARC, 1999).

Little is known about the effect of hormonal therapy on aromatase (CYP 19), which is responsible for the synthesis of estrogens. Aromatase is expressed in both normal and malignant breast tissues and its activity in the breast varies widely. However, the mechanisms and extent of regulation of aromatase in breast tissues have not been fully established.
and, to investigate the potential role of estrogen in this regulation, studies were carried out in an in-vitro model (Yue et al., 2001) in which MCF-7 cells were cultured in long-term estrogen-deprived medium (LTED cells). It was found that long-term estrogen deprivation enhanced aromatase activity by three- to fourfold compared with that in wild-type MCF-7 cells. Re-exposure of LTED cells to estrogen reduced aromatase activity to the levels of wild-type MCF-7 cells. The authors also measured aromatase activity in 101 frozen breast carcinoma specimens and compared tumour aromatase activities in premenopausal patients with those in postmenopausal patients and in postmenopausal patients who did or did not take hormonal therapy. Although not statistically significant, a trend was observed that paralleled that in the in-vitro studies. Aromatase activity was higher in breast cancer tissues from patients who had lower levels of circulating estrogen. These data suggest that estrogen may be involved in the regulation of aromatase activity in breast tissues.

Two epidemiological studies examined the association of a common aromatase polymorphism (intron 4 TTTA repeat) and osteoporosis in postmenopausal women who did or did not take hormonal menopausal therapy. The Danish Osteoporosis Prevention Study showed an increase in bone mineral density in women who had long TTTA repeats and who received therapy (Tofteng et al., 2004). In untreated women, no association was observed between bone mass or bone loss and the TTTA polymorphism. In contrast, a Finnish study found that the TTTA polymorphism did not influence bone mineral density, which is a risk for fracture, or circulating levels of estradiol in treated or untreated women (Salmen et al., 2003).

4.1.1 Ethinylestradiol and mestranol

Structural modification of the estradiol molecule by insertion of an ethinyl group at carbon 17 yields ethinylestradiol, which is considerably more potent than estradiol and has high activity following oral administration. This compound is used frequently as the estrogenic component of oral contraceptives.

Modification of ethinylestradiol by formation of a methyl ether at carbon 3 gives rise to mestranol, which was widely used in the early years of oral contraception, but is now rarely employed. Mestranol binds poorly to the estrogen receptor and its estrogenic effect is due to its rapid demethylation in the liver to form ethinylestradiol; however, demethylation is not complete and more mestranol must be administered than ethinylestradiol to achieve similar effects.

Goldzieher and Brody (1990) studied the pharmacokinetics of doses of 35 μg ethinylestradiol (24 women) and 50 μg mestranol (27 women) in combination with 1 mg norethisterone. Serum concentrations of ethinylestradiol were measured after treatment with either estrogen, each of which produced an average serum concentration of approximately 175 pg/mL ethinylestradiol, with wide inter-individual variation. The maximal serum concentrations were achieved within about 1–2 h, and the half-life for elimination ranged from 13 to 27 h. The oral bioavailability of ethinylestradiol was 38–48%, and a 50-μg dose of mestranol was shown to be bioequivalent to a 35-μg dose of ethinylestradiol.
Hümpel et al. (1990) obtained serum samples from one group of 30 women who were taking a cycle of a combined oral contraceptive that contained ethinylestradiol and desogestrel and from another group of 39 women who were taking ethinylestradiol and gestodene. The serum concentrations of ethinylestradiol reached mean maximal levels of 106–129 pg/mL 1.6–1.8 h after intake. The mean serum concentrations of SHBG were 186–226 nmol/L, those of cortisol-binding globulin were 89–93 mg/L and those of cortisol were 280–281 μg/L.

Kuhnz et al. (1990a) compared the pharmacokinetics of ethinylestradiol administered as a single dose in combination with either gestodene or desogestrel to 18 women. In contrast to previous reports (Goldzieher & Brody, 1990; Hümpel et al., 1990), which showed that the bioavailability of ethinylestradiol differed according to the associated progestogen, this study showed no significant difference.

The major pathway of ethinylestradiol metabolism in the liver of humans and animals is 2-hydroxylation, which is presumably catalysed by CYP 3A4 (Guengerich, 1988; see Section 4.1 of the monograph on Combined estrogen–progestogen menopausal therapy).

4.1.2 Norethisterone

Most of the data that pertain to the pharmacokinetics of norethisterone derive from a study (Back et al., 1978) in which 1 mg norethisterone acetate and 0.05 mg ethinylestradiol were administered orally and intravenously as a single dose or at 4-weekly intervals to a group of six premenopausal women. The results show that the absolute bioavailability of norethisterone after administration ranged from 47 to 73% (mean, 64%) compared with intravenous administration. The half-life [of the β phase of a two-component model] of elimination ranged from 4.8 to 12.8 h (mean, 7.6 h) with no significant differences between oral and intravenous administration.

Data from two different studies (Odlind et al., 1979; Stanczyk et al., 1983; Stanczyk, 2003) showed a dose–response in circulating levels of norethisterone following oral administration to premenopausal women of 5 mg, 1 mg (combined with 0.12 mg ethinylestradiol), 0.5 mg and 0.3 mg norethisterone. Mean peak plasma levels were approximately 23, 16, 6 and 4 ng/mL, respectively, within 1–2 h after treatment.

Norethisterone undergoes extensive ring A reduction to form dihydro- and tetrahydro-norethisterone metabolites that undergo conjugation; it can also be aromatized. Low serum levels of ethinylestradiol have been measured in postmenopausal women following oral administration of relatively large doses of norethisterone acetate or norethisterone (Kuhnz et al., 1997). On the basis of the area-under-the-curve (AUC) values that were determined for ethinylestradiol and norethisterone, it was shown that the mean conversion ratio of norethisterone to ethinylestradiol was 0.7 and 1.0% at doses of 5 and 10 mg, respectively. The authors calculated that this corresponds to an oral dose equivalent of about 6 μg ethinylestradiol/mg of norethisterone acetate. Similarly, it was shown that a dose of 5 mg norethisterone administered orally was equivalent to about 4 μg ethinylestradiol/mg norethisterone. On the basis of these calculations, it was estimated that lower doses of norethistere-
rone or its acetate (e.g. 0.5–1.0 mg) in combination with ethinylestradiol would add between 0.002 and 0.01 mg ethinylestradiol to that already present. [The estimations for these lower doses were extrapolated from high doses of these compounds, which were not combined with ethinylestradiol. Nevertheless, it appears that significant amounts of ethinylestradiol are formed from norethisterone, and that the amount formed appears to be highly variable.]

No information on the pharmacokinetics of ethinylestradiol or mestranol via the dermal route (patch) was available to the Working Group.

4.1.3 Norethisterone acetate, ethynodiol diacetate, norethynodrel and lynestrenol

It is generally considered that progestogens that are structurally related to norethisterone are pro-drugs and that their progestational activity is due to their conversion to norethisterone. After oral administration, norethisterone acetate and ethynodiol diacetate are rapidly converted to norethisterone by esterases during hepatic first-pass metabolism. Although less is known about the transformation of lynestrenol and norethynodrel (Stanczyk & Roy, 1990), it appears that lynestrenol first undergoes hydroxylation at carbon 3 and then oxidation of the hydroxyl group to form norethisterone. Although there is no convincing evidence for the in-vivo transformation of norethynodrel to norethisterone, data from receptor-binding tests and bioassays suggest that norethynodrel is also a pro-drug.

4.1.4 Levonorgestrel

Stanczyk and Roy (1990) reviewed the metabolism of levonorgestrel in women treated orally with the radioactively labelled compound. Levonorgestrel was found mostly untransformed in serum within 1–2 h after administration, but the concentrations of conjugated metabolites increased progressively between 4 and 24 h after ingestion. Most of the conjugates were sulfates and glucuronides. In addition to the remaining unconjugated levonorgestrel, considerable amounts of unconjugated and sulfate-conjugated forms of 3α,5β-tetrahydrolevonorgestrel were found; smaller quantities of conjugated and unconjugated 3α,5α-tetrahydrolevonorgestrel and 16β-hydroxylevonorgestrel were also identified (Sisenwine et al., 1975a). Approximately 45% of radioactively labelled levonorgestrel was excreted via the urine and about 32% via the faeces. The major urinary metabolites were glucuronides (the most abundant was 3α,5β-tetrahydrolevonorgestrel glucuronide) and smaller quantities of sulfates were found (Sisenwine et al., 1975b).

A dose–response has been demonstrated for circulating levels of levonorgestrel (Stanczyk, 2003) following administration of single oral doses of 0.25, 0.15 and 0.075 mg levonorgestrel to six, 24 and 24 women, respectively (Humpel et al., 1977; Goebelsmann et al., 1986; Stanczyk et al., 1990). When the three doses were combined with 30–50 μg ethinylestradiol, mean peak levonorgestrel levels of 6.0, 3.5 and 2.5 ng/mL were attained at 1–3 h with the decreasing order of doses. At 24 h, the mean levonorgestrel level was 1.2 ng/mL with the highest dose and less than 0.5 ng/mL with the other two doses.
The bioavailability of levonorgestrel is generally accepted to be 100%. This generalization is based on two studies that used only a small number of women (Humpel et al., 1978; Back et al., 1981). In one of the studies (Back et al., 1981), absolute bioavailabilities were determined for doses of 0.25 and 0.15 mg levonorgestrel, each of which was administered to five women in combination with ethinylestradiol (0.05 mg). The results show that the bioavailability for the 0.15-mg dose of levonorgestrel ranged from 72 to 125% (mean, 99%); that for the 0.15-mg dose ranged from 63 to 108% (mean, 89%). When the individual bioavailabilities for the 0.25-mg dose were examined, it was noted that 60% of the subjects had bioavailabilities greater than 100%. [This demonstrates that, for each of these subjects, the AUC for levonorgestrel obtained by the oral route was greater than that obtained intravenously, and implies that there was a methodological problem in the study.] In the same study, the mean half-life of elimination was found to be 13.2 h and 9.9 h for the 0.15-mg and 0.25-mg doses of levonorgestrel, respectively, when administered intravenously. These values were similar after oral dosing.

Carol et al. (1992) evaluated the pharmacokinetics of levonorgestrel in groups of 11–20 women who were given single or multiple treatments with combined oral contraceptive preparations that contained 0.125 mg levonorgestrel plus 0.03 or 0.05 mg ethinylestradiol. The serum concentrations of levonorgestrel reached a maximum of about 4 ng/mL at 1–2 h after a single treatment with either preparation. After 21 days of treatment, the peak and sustained concentrations of levonorgestrel were about twice as high as those after a single treatment. The serum concentration of SHBG increased after treatment with both contraceptives (but to a greater extent with the contraceptive containing 0.05 mg ethinylestradiol), which indicates the important role of estrogen in the induction of this protein.

Kuhnz et al. (1992) treated a group of nine women with a single dose of a combined oral contraceptive that contained 0.15 mg levonorgestrel plus 0.03 mg ethinylestradiol; eight of these women received the same regimen for 3 months after an abstention period of 3 months. The peak concentrations of levonorgestrel were found 1 h after single or multiple treatments. The peak serum concentrations of levonorgestrel were 3.1 and 5.9 ng/mL, respectively. The AUC concentration for total and free levonorgestrel increased by two- to fourfold after a single dose compared with multiple treatments. The distribution of free, albumin-bound and SHBG-bound levonorgestrel was similar in women who had received one or multiple treatments, but the serum concentration of the globulin increased significantly after multiple treatments.

Kuhnz et al. (1994a) treated 14 women with a combined oral contraceptive that contained 0.125 mg levonorgestrel plus 0.03 mg ethinylestradiol as a single dose and for 3 months as a triphasic regimen (Triquilar®) after an abstention period of 1 week. The serum concentration of free levonorgestrel reached a peak of 0.06–0.08 ng/mL about 1 h after treatment with a single dose or on day 1 of the first or third cycle. In contrast, the calculated values of the AUC more than doubled, from 0.32 (single dose) to 0.75 (multiple treatment, first cycle)–0.77 (multiple treatment, third cycle) ng × h/mL. The serum concentrations of cortisol-binding globulin and SHBG more than doubled after multiple treatments with the contraceptive. After a single dose, 1.4% of the levonorgestrel in serum was free, 43% was
bound to albumin and 55% was bound to SHBG. After multiple treatments, only 0.9–1.0% levonorgestrel in serum was free and 25–30% was bound to albumin and the amount that was bound to SHBG increased to 69–74%. The concentrations of free and total testosterone decreased from 3 and 460 pg/mL, respectively, before treatment to 1 and 270 pg/mL, respectively, at the end of one treatment cycle and increased again to 2 and 420 pg/mL, respectively, by the 1st day of the third cycle. Thus, the treatment-free interval of 1 week was sufficient to restore pretreatment values for testosterone.

4.1.5 Desogestrel

Desogestrel is a pro-drug and its progestational activity is mediated by one of its metabolites, 3-ketodesogestrel. In a study in which 10 women ingested single doses of 0.15 mg desogestrel combined with 0.03 mg ethinylestradiol and another group of women ingested single doses of 0.15 mg 3-ketodesogestrel combined with 0.03 mg ethinylestradiol (Hasenack et al., 1986), serum 3-ketodesogestrel levels were essentially the same in both groups of women, whereas desogestrel was not found in significant amounts.

Following administration of a single oral dose of 0.15 mg desogestrel combined with 0.03 mg ethinylestradiol to a group of 25 women, the mean maximum concentration of 3-ketodesogestrel was 3.69 ng/mL, which was reached at a mean time of 1.6 ± 1.0 h (Bergink et al., 1990). The mean bioavailability of 3-ketodesogestrel in two cross-over studies, in which women received either a single oral or intravenous dose of 0.15 mg desogestrel combined with 0.03 mg ethinylestradiol, was reported to be 76 and 62%, respectively (Back et al., 1987; Orme et al., 1991). The mean half-life of elimination for 3-ketodesogestrel, calculated from two studies (Back et al., 1987; Bergink et al., 1990) in which women were given a single oral dose of 0.15 mg desogestrel combined with 0.03 mg ethinylestradiol, was not consistent (23.8 h versus 11.9 h); the longer half-life was calculated from serum 3-ketodesogestrel levels that were obtained up to 72 h (Bergink et al., 1990) whereas frequent blood sampling was carried out only up to 24 h in the other study. [The lack of data beyond 24 h is a deficiency of most studies of progestogen pharmacokinetics in which the half-life of elimination is calculated.]

A multiple dosing study (Kuhl et al., 1988a) was carried out in 11 women who ingested 0.15 mg desogestrel in combination with 0.03 mg ethinylestradiol daily for 12 continuous treatment cycles and whose blood was sampled at frequent intervals on days 1, 10 and 21 of cycles 1, 3, 6 and 12. The results showed that 3-ketodesogestrel levels were relatively low on day 1 but rose progressively and were higher on day 21 of the treatment cycles. This increase was attributed to the elevated serum levels of SHBG induced by the estrogenic component of the pill.

A group of 19 women were given three cycles of a triphasic oral contraceptive that contained combinations of desogestrel and ethinylestradiol at doses of 0.15, 0.05 and 0.035 mg for the first 7 days, 0.10 and 0.03 mg for days 8–14 and 0.15 and 0.03 mg for days 15–21, respectively, followed by 7 days without hormone. Multiple blood samples were taken from the women throughout this interval, and serum concentrations of 3-keto-
desogestrel, ethinylestradiol and SHBG were determined, together with the elimination half-life and dose proportionality. The concentration of 3-ketodesogestrel reached steady-state levels at each desogestrel dose, and the pharmacokinetics was proportional to the dose. The concentration of ethinylestradiol also reached a steady state, and the pharmacokinetics was constant thereafter. The concentration of SHBG was significantly increased between days 1 and 7 of the cycle but not between days 7, 14 and 21 (Archer et al., 1994).

4.1.6 Gestodene

Gestodene is metabolized primarily in the liver by CYP 3A4 and is a strong inducer of this enzyme. Although ethinylestradiol is also metabolized by CYP 3A4, gestodene does not appear to inhibit its metabolism. Known metabolites of gestodene include dihydrogestodene, 3,5-tetrahydrogestodene and hydroxygestodene.

Gestodene is not a pro-drug. Following administration of a single oral dose of 0.025, 0.075 or 0.125 mg gestodene in combination with or without 0.03 mg ethinylestradiol to six women, a dose–response was observed. The half-life of elimination of gestodene was shown to range from 12 to 14 h for the three doses studied (Taub et al., 1989). Mean maximum concentration values of 1.0, 3.6 and 7.0 ng/mL gestodene were attained between 1.4 and 1.9 h, respectively. The mean absolute bioavailability was calculated to be 99% (range, 86–112%) for the commonly prescribed dose of 0.075 mg gestodene. In another similar study (Orme et al., 1991), the mean absolute bioavailability was reported to be 87% (range, 64–126%).

The same experimental design that was used for the multiple dosing studies with desogestrel (Kuhl et al., 1988a) was also used for gestodene (Kuhl et al., 1988b). The results showed a dramatic rise in mean gestodene levels between day 1 and day 10, and a further rise between day 10 and day 21 in all study cycles. These findings were attributed to increased levels of SHBG and were similar to those obtained when multiple dosing was performed with desogestrel.

Following oral administration of 0.075 mg gestodene combined with 0.03 mg ethinylestradiol, either as a single dose or as multiple doses, the circulating levels of gestodene were relatively high compared with similar treatments with other progestogens in combination with ethinylestradiol (Fotherby, 1990). [This finding was surprising because the 0.075-mg dose of gestodene is the lowest dose of any progestogen used in a combined oral contraceptive pill. The major factor responsible for the elevated levels of gestodene appears to be the high affinity of SHBG for gestodene, which results in a lower metabolic clearance and consequently a higher concentration of this progestogen in the blood. It has been reported that approximately 75% of gestodene is bound to SHBG after oral treatment with gestodene/ethinylestradiol, which is considerably higher than that observed with other progestogens combined with ethinylestradiol.]

Kuhnz et al. (1990b) studied the binding of gestodene to serum proteins in 37 women who had taken a combined oral contraceptive that contained gestodene plus ethinyl-
estradiol for at least 3 months: 0.6% was free, 24% was bound to albumin and 75% was bound to SHBG.

Kuhnz et al. (1991) examined the effects of a single administration followed by multiple administrations over one cycle (after an abstention period of 1 week) of a triphasic combined oral contraceptive that contained gestodene and ethinylestradiol on the concentrations of ethinylestradiol and testosterone in 10 women. After a single oral dose of 0.10 mg gestodene plus 0.03 mg ethinylestradiol, the serum ethinylestradiol concentration reached 100 pg/mL in about 1.9 h; thereafter, the concentration declined, with a half-life of 11 h. On day 21 of the treatment cycle, the maximum concentrations reached 140 pg/mL 1.6 h after intake. In comparison with day 21 after the single dose treatment, the levels of total and free testosterone were reduced by about 60% on day 21 of the treatment cycle.

Kuhnz et al. (1993) treated 14 women with a combined oral contraceptive that contained 0.10 mg gestodene plus 0.03 mg ethinylestradiol as a single dose and for 3 months as a triphasic regimen after an abstention of 1 week. The maximum serum concentrations of gestodene 30 min after dosing were 4.3 ng/mL after a single dose, 15 ng/mL at the end of the first cycle and 14.4 ng/mL at the end of three cycles. A half-life for clearance of 18 h was observed after a single treatment, with a volume of distribution of 84 L. Multiple treatments increased the clearance half-life to 20–22 h and reduced the distribution volume to about 19 L. The serum concentration of SHBG increased with multiple treatments, presumably as an effect of ethinylestradiol, which is thought to account for the observed change in the distribution of gestodene (from 1.3% free, 69% bound to SHBG and 29% bound to albumin after a single treatment to 0.6% free, 81% bound to SHBG and 18% bound to albumin after multiple treatments).

Heuner et al. (1995) treated 14 women with a combined oral contraceptive that contained 0.075 mg gestodene plus 0.02 mg ethinylestradiol by a single administration or for 3 months as a triphasic regimen. The serum concentrations of gestodene, ethinylestradiol, cortisol-binding globulin, SHBG and testosterone were followed after the single treatment and through cycles 1 and 3. The concentration of gestodene reached a maximum of 3.5 ng/mL within 0.9 h after a single dose and 8.7 ng/mL within 0.7 h after multiple doses. The clearance half-time for a single dose of gestodene also increased from 12.6 h to nearly 20 h after multiple treatments. There was a large increase in the concentration of SHBG with time after multiple treatments. After a single dose, 1.3% of gestodene in serum was free, 30% was bound to albumin and 68% was bound to SHBG.

4.1.7 Norgestimate

Very little is known about the pharmacokinetics of orally administered norgestimate except that it is a relatively complex pro-drug. After its oral administration, the acetate group at carbon 17 is rapidly removed during hepatic first-pass metabolism. The product formed — levonorgestrel-3-oxime — has progestational activity. It has also been referred to as deacetylated norgestimate and, more recently, has been assigned the common name
norelgestromin. Rapid formation of norelgestromin from norgestimate was demonstrated by McGuire et al. (1990); serum levels of norelgestromin were measured after administration of single and multiple oral doses of 0.36 mg norgestimate combined with 0.07 mg ethinylestradiol to 10 women. Mean peak serum levels of 17-deacetyl-norgestimate (norelgestromin) were approximately 4 ng/mL and were attained after about 1.4 h; the levels remained elevated up to 36 h after treatment. In contrast, peak levels of norgestimate were only ∼100 pg/mL 1 h after treatment; the concentration declined rapidly thereafter and none was detectable 5 h after treatment.

Norgestimate is converted to levonorgestrel. In a randomized, comparative pharmacokinetic study by Kuhnz et al. (1994b), 12 women received single oral doses of 0.25 mg norgestimate combined with 0.035 mg ethinylestradiol and 0.25 mg levonorgestrel combined with 0.05 mg ethinylestradiol. The levonorgestrel AUC ratios were determined after administration of both formulations and were used to calculate the bioavailability of norgestimate-derived levonorgestrel: on average, about 22% of the administered dose of norgestimate became systemically available as levonorgestrel.

In addition to norelgestromin and levonorgestrel, a third progestationally active metabolite of orally administered norgestimate is formed, which is probably levonorgestrel-17-acetate (Kuhnz et al., 1994b).

4.1.8 Newly developed progestogens

In recent years, new progestogens have been synthesized that may improve the performance of combined hormones. Two of these that are currently in use (Sitruk-Ware, 2004a) are discussed below. Other members of this group, e.g. nesterone, nomegestrol acetate and trimegestone, are used much less frequently, although their pharmacological profile is similar with respect to receptor binding (see Tables 17 and 18 in Section 4.2 of the monograph on Combined estrogen–progestogen menopausal therapy) (Kuhl, 1996; Couthino et al., 1999; Kumar et al., 2000; Tuba et al., 2000; Lepescheux et al., 2001; Shields-Botella et al., 2003).

(a) Dienogest

Dienogest (17α-cyanomethyl-17β-hydroxyestra-4,9-dien-3-one) is a derivative of 19-nortestosterone. It has progestational activity but no androgenic, estrogenic, anti-estrogenic or corticoid activity. It strongly suppresses endometrial proliferation and does not antagonize the beneficial effects of estrogens. Dienogest binds highly selectively to the progesterone receptor, but does not bind to SHBG. As a result, it does not compete with testosterone for binding, and thereby helps to minimize the free serum levels of the androgen. Relatively high levels of the compound (approximately 9%) are free in the serum. After oral intake, a maximum serum concentration of dienogest is reached after about 1 h and does not accumulate after repeated dosing. The compound has an elimination half-life of 9.1 h. Studies of receptor binding have shown that the anti-androgenic activity of dienogest is similar to that of cyproterone acetate and progesterone (Teichmann, 2003).
Studies on the pharmacokinetics of dienogest have been carried out following oral and intravenous administration of different doses (Oettel et al., 1995). A dose–response in serum dienogest levels was observed in 12 women after oral administration of four single doses (1, 2, 4 and 8 mg) in randomized order during four consecutive menstrual cycles. Following administration of the 1-mg dose, the mean maximum concentration was 23.4 ng/mL and the time to reach this level was 2.2 ± 1.1 h; half-life of elimination was 6.5 h. The absolute bioavailability of dienogest was determined in 16 healthy male volunteers who ingested a single dose of two tablets, each of which contained 2 mg dienogest and 0.03 mg ethinylestradiol; the average bioavailability value was 96.2%. In the same study, the average terminal half-life was reported to be 10.8 and 11.6 h after oral and intravenous doses, respectively.

From the two studies described above and other related studies by Oettel et al. (1995), it can be concluded that circulating levels of dienogest are relatively high compared with those found with similar doses of other progestogens. The clearance of dienogest appears to be lower than that of other progestogens, although most of it is weakly bound to albumin in the blood (Oettel et al., 1995). No significant accumulation of dienogest was observed in serum during its daily intake (Oettel et al., 1995).

(b) Drospirenone

The pharmacokinetic characteristics of drospirenone (3 mg) combined with ethinylestradiol (0.03 mg) were assessed in 13 women during 13 continuous cycles, each of which consisted of 21 continuous days of treatment followed by a 7-day treatment-free interval (Blode et al., 2000). Frequent blood sampling was carried out on day 21 of treatment cycles 1, 6, 9 and 13. After administration of the first tablet, the mean maximum concentration of drospirenone was 36.9 ng/mL, which rose to 87.5 ng/mL on day 21 of the first cycle and ranged from 78.7 to 84.2 ng/mL on day 21 of the next three sampling cycles. The corresponding time to reach peak levels ranged from 1.6 to 1.8 h, and the half-life of elimination values were 31.1–32.5 h.

Other pharmacokinetic characteristics of drospirenone, based on data obtained by the manufacturer of an oral contraceptive that contained 3 mg drospirenone combined with 0.03 mg ethinylestradiol, have been reviewed (Krattenmacher, 2000). It was reported that a steady-state in circulating drospirenone levels is achieved after 1 week of treatment, and a dose–response in circulating drospirenone levels is obtained following oral administration of doses ranging from 1 to 10 mg. In addition, the absolute bioavailability was reported to be on average 76%.

4.1.9 Interactions of other drugs with oral contraceptives

Kopera (1985) reviewed the drug interactions associated with the administration of progestogens to patients who received other medications. Progestogens adversely affect the metabolism of certain drugs and, in turn, the metabolism of progestogens is affected by other drugs. These effects presumably occur as a consequence of the induction of
metabolic enzymes, or competition for metabolic pathways or for binding to serum carrier
proteins (Shenfield et al., 1993).

Data on the effects of some oral contraceptive estrogens and progestogens in animals
were reviewed previously (IARC, 1999) (see also Section 4.2 of this monograph and
Sections 4.1 and 4.2 of the monograph on Combined estrogen–progestogen menopausal
therapy in this volume).

4.2 Receptor-mediated effects

There is evidence that not all the effects of estrogens and progestogens that are used in
combined hormonal (oral) contraceptives are mediated through nuclear or other receptors.
In addition, the effects of these steroids probably involve several molecular pathways and
cross-talk between receptor- and/or non-receptor-mediated pathways. During the past
decade, research on the mechanisms of hormonal action and on hormones and cancer has
grown immensely. Two different subtypes of the progesterone receptor, subtypes A and B
(Kazmi et al., 1993; Vegeto et al., 1993), and several estrogen receptors that have different
functions — the nuclear estrogen receptors-α and -β and their subtypes — have been iden-
tified (Kuiper et al., 1996; Mosselman et al., 1996; Kuiper et al., 1997). In addition, estro-
gen receptors-α and other estrogen-binding proteins that are located in the plasma mem-
brane appear to be responsible for rapid non-genomic estrogen responses (Pietras et al.,
2001; Song et al., 2002; Razandi et al., 2003) and may activate signal transduction path-
ways by estrogens (Razandi et al., 2003; Song et al., 2004). There is also some evidence to
suggest that a non-genomically acting progesterone receptor is responsible for rapid proges-
togen responses (Castoria et al., 1999; Sutter-Dub, 2002; Sager et al., 2003). However, the
literature on specific interactions of constituents of combined oral contraceptive prepara-
tions with these receptor subtypes is still limited.

Increased attention to the various components of combined oral contraceptives in
recent years has resulted in the availability of more information on the progestogens used
with respect to their hormonal activities and binding to various receptors and other
binding proteins. This information is summarized in Tables 35 and 36.

4.2.1 Combined oral contraceptives

(a) Humans

(i) Breast epithelial cell proliferation

It was concluded previously that exposure to combined oral contraceptives increases
breast epithelial cell proliferation and that, when ethinylestradiol is the estrogen component,
this effect is dose-dependent (IARC, 1999). Increased breast epithelial cell proliferation
may be associated with an increased risk for breast cancer (Russo & Russo, 1996; Pihan
et al., 1998). Isaksson et al. (2001) confirmed and extended these conclusions which are
consistent with an increase in risk for breast cancer: all 53 women who had taken combined
Table 35. Overview of the spectrum of hormonal activities of progestogens used in combined oral contraceptives

<table>
<thead>
<tr>
<th>Progestogen</th>
<th>Progestogenic</th>
<th>Anti-estrogenic</th>
<th>Estrogenic</th>
<th>Androgenic</th>
<th>Anti-androgenic</th>
<th>Glucocorticoid</th>
<th>Antimineralocorticoid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlormadinone acetate</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Cyproterone acetate</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+, +</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Desogestrel</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>±, –</td>
<td>–</td>
</tr>
<tr>
<td>Dienogest</td>
<td>+</td>
<td>+, ±</td>
<td>–, ±</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Drospirenone</td>
<td>+, +</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>±, – ?</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Gestodene</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>±, +</td>
<td>+</td>
</tr>
<tr>
<td>Levonorgestrel</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Norethisterone acetate</td>
<td>+, +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Adapted from Wiegratz & Kuhl (2004); second value for progestogenic activity only from Sitruk-Ware (2002); second value, except for progestogenic activity from Schindler et al. (2003)

+, effective; ±, weakly effective; -, ineffective; ?, unknown

Data are based mainly on animal experiments. The clinical effects of progestogens are dependent on their tissue concentrations. No comparable data were available for ethynodiol diacetate or lynestrenol.

Note: This information should be viewed as only an indication of the hormonal activity (and its order of magnitude) of the various progestogens.
Table 36. Relative binding affinities of progestogens used in combined oral contraceptives to steroid receptors and serum binding globulins

<table>
<thead>
<tr>
<th>Progestogen</th>
<th>PR</th>
<th>AR</th>
<th>ER</th>
<th>GR</th>
<th>MR</th>
<th>SHBG</th>
<th>CBG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlormadinone acetate</td>
<td>134</td>
<td>5</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cyproterone acetate</td>
<td>180</td>
<td>6</td>
<td>0</td>
<td>6</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Desogestrel (as 3-ketodesogestrel)</td>
<td>300</td>
<td>20</td>
<td>0</td>
<td>14</td>
<td>0</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Dienogest</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Drospirenone</td>
<td>70, 19</td>
<td>65, 2</td>
<td>0, &lt; 0.5</td>
<td>6, 3</td>
<td>230, 500</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gestodene</td>
<td>180, 864</td>
<td>85, 71</td>
<td>0, &lt; 0.02</td>
<td>27, 38</td>
<td>290, 97</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>Levonorgestrel</td>
<td>300, 323</td>
<td>45, 58</td>
<td>0</td>
<td>1, 7.5</td>
<td>75, 17</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Norethisterone acetate</td>
<td>150, 134</td>
<td>15, 55</td>
<td>0.015</td>
<td>0, 1.4</td>
<td>0, 2.7</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Reference compounds (100%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Progesterone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metribolone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estradiol-17β</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dexamethasone (or cortisol)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aldosterone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5α-dihydrotestosterone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortisol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Wiegrazt & Kuhl (2004); second value from Sitruk-Ware (2004)

AR, androgen receptor; CBG, corticoid-binding globulin; ER, estrogen receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; PR, progesterone receptor; SHBG, sex hormone-binding globulin

*a Values were compiled by these authors by cross-comparison of the literature. Because the results of the various in-vitro experiments depend largely on the incubation conditions and biological materials used, the published values are inconsistent. These values do not reflect the biological effectiveness, but should be viewed as only an indication of the order of magnitude of the binding affinities of the various progestogens. No comparable data were available for ethinodiol acetate or lynestrenol.
oral contraceptives had used preparations that contained ethinylestradiol combined with levonorgestrel, desogestrel, lynestrenol or norethisterone; however, the actual dose levels were not provided. Data from these women were compared with those from 54 women who had not used hormonal contraception. Fine needle aspirates were obtained between days 16 and 21 on the first cycle of treatment or, for control women, during the second half of the menstrual cycle. The mean percentage of breast epithelial cells that stained for Ki-67 (using the MIB-1 antibody that reacts with a human nuclear antigen present in proliferating cells) was 4.8% (median, 3.0%; range, 0–50%) in women exposed to combined oral contraceptives and 2.2% (median, 1.5%; range, 0–8%) in control women, a difference that was statistically significant. In 37 women who had taken ethinylestradiol plus levonorgestrel, the correlation between serum levonorgestrel levels and breast epithelial cell proliferation was found to be statistically significant in a positive direction (Spearman $r = 0.43$; $p = 0.02$). For 16 women who had taken ethinylestradiol plus levonorgestrel, fine needle aspirates were obtained both before the start of the oral contraceptive treatment and 2 months afterwards. The mean percentage of breast epithelial cells that stained for Ki-67 was 1.4% (median, 0.5%; range, 0–5%) before treatment and 5.8% (median, 0.8%; range, 0–50%) after 2 months of treatment; this change was of borderline significance ($p = 0.055$).

(ii) Effects on the endometrium

Four independent studies have investigated the effects of combined oral contraceptives on the endometrium. Moyer and Felix (1998) obtained endometrial biopsies from two groups of nine women who were exposed for an unreported duration to a regimen that consisted of oral treatment with either 0.06 mg mestranol plus 5 or 10 mg norethisterone acetate or 0.075 mg mestranol plus 5 mg norethynodrel for 21 days, followed by 7 days with no treatment. Biopsies were taken during the 7 days with no treatment. Mitotic counts were significantly reduced from 12.3/1000 glandular cells in untreated women to 1.6 and 0.01/1000 cells in women exposed to mestranol plus norethynodrel and norethisterone acetate, respectively. [The Working Group noted that the timing of biopsy in the treated and untreated women probably does not permit an adequate comparison.]

Archer (1999) studied 11 women who took a triphasic oral regimen of 0.02 mg ethinylestradiol plus 0.05 mg desogestrel for 21 days, placebo for 2 days and 0.01 g ethinylestradiol for the last 5 days of each 28-day cycle. Endometrial biopsies were taken after 13–14 cycles. A progestational effect on endometrial histology was observed with secretory changes in samples obtained between days 11 and 21 of the treatment cycle. However, this was benign and no endometrial hyperplasia or metaplasia was observed.

Oosterbaan (1999) reported a study of women who took an oral preparation that consisted of 0.05 mg ethinylestradiol plus 0.06 mg gestodene for 24 days followed by 4 days with no treatment. Endometrial biopsies were taken during the late luteal phase before treatment (baseline biopsies) and between days 15 and 24 of the treatment cycle during cycle 3 or 6. Histological evidence of endometrial atrophy was observed in three of nine subjects
during cycle 3 and in four of nine subjects during cycle 6, whereas 11 of 13 baseline biopsies showed a secretory endometrium.

A regimen that consisted of 21 days of 0.03 mg ethinylestradiol plus 3 mg drospirenone followed by 7 days of no treatment was studied by Lüdicke *et al.* (2001). Endometrial biopsies were taken at baseline (26 women) and after 3 (11 women), 6 (10 women) and 13 cycles (26 women); endometrial thickness was also assessed by ultrasound at these four time-points (*n* = 26). Morphometry and ultrasound showed endometrial atrophy following the treatment: after 3, 6 and 13 cycles, 41, 44 and 63% of subjects, respectively, had an atrophic endometrium. Glandular mitotic activity (normally 0.3/1000 cells) was also absent at these three time-points.

Collectively, these four studies indicate atrophic and anti-proliferative endometrial effects of progestogen-containing combined oral contraceptives, apparently regardless of the regimen and the actual progestogen used. This anti-proliferative effect may be associated with a reduction in the risk for endometrial cancer.

**(iii) Effects on the colon**

In addition to a range of growth factor receptors, the human colon has both estrogen and progesterone nuclear receptors and expresses known estrogen-inducible genes (*pS2* and *ERD5*) (Di Leo *et al.*, 1992; Hendrickse *et al.*, 1993; Singh *et al.*, 1998). However, some studies did not confirm these findings; in addition, there are conflicting results on the expression of these receptors in stromal versus epithelial cells which are perhaps related to differences in the methodology used between studies (Waliszewski *et al.*, 1997; Slattery *et al.*, 2000). Nevertheless, these observations suggest that there may be a link between exposures to hormones in combined oral contraceptives and menopausal therapy and colon cancer, but they do not predict the nature of such a relationship.

***(iv) Effects on hormonal systems***

Several new studies have investigated the relationship between combined oral contraceptives and hormonal parameters. In four of these, serum levels of SHBG and free testosterone were measured. Levels of SHBG were increased by two- to fourfold and free testosterone levels were reduced by 40–80%, regardless of the combined oral contraceptive regimen used (Piérard-Franchimont *et al.*, 2000; Boyd *et al.*, 2001; Isakson *et al.*, 2001; Wiegratz *et al.*, 2003). Wiegratz *et al.* (2003) studied women who were taking four different contraceptive regimens for 21 days followed by 7 days with no treatment: 0.03 mg ethinylestradiol plus 2 mg dienogest, 0.02 mg ethinylestradiol plus 2 mg dienogest, 0.01 mg ethinylestradiol plus 2 mg estradiol valerate plus 2 mg dienogest or 0.02 mg ethinylestradiol plus 0.10 mg levonorgestrel. In addition to reducing free testosterone and increasing the levels of SHBG, all treatments reduced levels of dehydroepiandrosterone sulfate and increased those of corticoid-binding globulin by approximately twofold and of thyroxin-binding globulin by approximately 50%, while prolactin was not affected. All four regimens had comparable effects, except that ethinylestradiol plus levonorgestrel increased levels of SHBG by only 50–100% and ethinylestradiol plus estradiol valerate plus dienogest
increased levels of prolactin by up to 40%. In the study by Isaksson et al. (2001), levels of androstenedione and total testosterone were also reduced as well as that of serum progesterone by 10-fold; levels of insulin-like growth factor-1 in the serum were not affected. Chatterton et al. (2005) found an even stronger reduction in nipple aspirate fluid progesterone from the breast (98%) and in serum progesterone (96.5%) in women who took a variety of triphasic contraceptives, but observed no effect on dehydroepiandrosterone or its sulfate or androstenedione, which are the potential precursors of 17\(\beta\)-estradiol. Levels of 17\(\beta\)-estradiol and estrone sulfate were also substantially reduced. These data suggest the possible involvement of reduced androgenic and estrogenic stimulation of responsive tissue, e.g. the breast. However, all combined oral contraceptives contain estrogens and several of the progestogens used, such as levonorgestrel and norethisterone, have androgenic activity. These studies highlight the possibility of complex interactions with other hormonal systems.

(b) Experimental systems

Rodriguez et al. (1998, 2002) examined the effects of a triphasic oral contraceptive regimen on the ovary of cynomolgus macaque monkeys. The treatment was equivalent to human doses of 0.03 mg ethinylestradiol plus 0.05 mg levonorgestrel per day for 6 days, followed by 0.04 mg ethinylestradiol plus 0.075 mg levonorgestrel for 5 days, followed by 0.03 mg ethinylestradiol plus 0.125 mg levonorgestrel for 10 days, followed by 7 days with no treatment. Parallel groups received ethinylestradiol only, levonorgestrel only or no treatment (control). This cyclic regimen was repeated every 28 days continuously for 35 months. The percentage of ovarian epithelial (surface) cells that stained positively for a reaction indicative of apoptosis was reduced by ethinylestradiol alone to a mean value of 1.8% compared with the mean control value of 3.9%. In contrast, apoptosis was increased more than sixfold by levonorgestrel alone and almost fourfold by the contraceptive combination of ethinylestradiol and levonorgestrel (Rodriguez et al., 1998). The treatments also affected the protein (immunohistochemical) expression of isoforms of transforming growth factor-\(\beta\) (TGF-\(\beta\)) that are known to be associated with apoptosis. TGF-\(\beta1\) expression was reduced in epithelial cells, whereas expression of TGF-\(\beta2/3\) was increased (Rodriguez et al., 2002). These findings may be consistent with a protective effect of combined oral contraceptives against the risk for ovarian cancer.

In cell culture, 0.01 nM, 1 nM and 100 nM gestodene, levonorgestrel and the active metabolite of gestodene, 3-ketodesogestrel, given together either for 7 days or for the last 4 days of a 7-day estrogen treatment, all inhibited the cell proliferation induced by 10^{-10}\text{M} 17\(\beta\)-estradiol in estrogen receptor-positive MCF-7 breast cancer cells (Seeger et al., 2003). The inhibition was dose-dependent for the combined 7-day treatment, but was similar regardless of dose when given on the last 4 days of estrogen treatment. A concentration of 10 \(\mu\text{M}\) had a lesser (continuous progestogen) or no (sequential progestogen) inhibitory effect. This study suggests that the progestogen components of combined oral contraceptives may reduce the stimulation of breast cell proliferation by the estrogen component, but no human studies are available.
4.2.2 Oral contraception and HPV

(a) Humans

Both estrogen and progesterone receptors are expressed in normal human uterine cervix epithelium; in many cases, estrogen receptors, but not progesterone receptors, are lost during the development of cervical carcinoma in situ and invasive cancer (Nonogaki et al., 1990; Monsonego et al., 1991). Expression of progesterone receptors is increased in carcinoma in situ and greatly diminished in invasive cancer (Monsonego et al., 1991). Infection with HPV is an essential causative component of human cervical cancer in more than 90% of cases (see IARC, 2007). HPV has more than 100 genotypes, some of which are associated with high risk for cancer and others with lower risk (see IARC, 2007). The loss of estrogen receptors may be associated with infection with specific types of HPV (Nonogaki et al., 1990). These observations suggest that estrogen and progesterone may be involved in cervical carcinogenesis (de Villiers, 2003).

(b) Experimental systems

Transcription of HPV is regulated by the long control region of the viral genome (see IARC, 2007). Expression of the E6 and E7 genes of HPV is affected by progesterone (and glucocorticoids for which there are also receptors in cervical epithelium) through hormone response elements on the long control region (Chan et al., 1989). This appears to occur in both high-risk (HPV 16 and 18) and low-risk HPV (HPV 11) types. There is also evidence that E6 and E7 expression is regulated by estrogen but there are no known estrogen-response elements on the long control region. Plasmids for the expression of chloramphenicol acetyl transferase (CAT) in the HPV 16 and 18 long control regions that were transfected into HeLa cells that contain progesterone but no estrogen receptors responded differentially to different estrogen and progestogens, but combinations of these were not tested (Chen et al., 1996). 17β-Estradiol and estriol, but not estrone, induced HPV 16 CAT expression (2.3- and 2.7-fold, respectively) and, to a lesser extent, HPV 18 CAT expression (1.3- and 1.5-fold, respectively) at concentrations of 100 nM. HPV 18 CAT expression was only minimally increased by some progestogens (cyproterone acetate, norethynodrel and ethynodiol diacetate), hardly increased by progesterone itself and not increased by norethisterone acetate or norgestrel (all tested at concentrations of 100 nM). HPV 16 CAT expression was increased by all of these progestogens (except norgestrel); progestosterone and norethisterone acetate were the least active (1.7- and 1.8-fold increase, respectively) and ethynodiol diacetate, cyproterone acetate and norgestrel were the most active (2.3-, 2.5- and 2.5-fold increase, respectively).

In cervical epithelial cells transfected with and immortalized by HPV 16, 17β-estradiol was metabolized to a greater extent to 16α-hydroxy metabolites than to 2-hydroxy metabolites; 4-hydroxy metabolites were not detected (Auborn et al., 1991). 17β-Estradiol and 16α-hydroxyestrone stimulated cell proliferation and caused increased growth in soft agar of cervical epithelial cells transfected with and immortalized by HPV 16 (Newfield et al., 1998). In both studies, human foreskin epithelial cells (which do not express estrogen recep-
tors) transfected with and immortalized by HPV 16 did not metabolize or respond to estrogen. Fifty women who had moderate or high-grade carcinoma in situ of the cervix and an HPV infection but not 29 women who had carcinoma in situ and no HPV infection had serum estrone levels twofold higher \((p < 0.05)\) than those found in women who did not have cervical carcinoma in situ \((n = 45)\) (Salazar et al., 2001). [The hypothesis that 16α-hydroxyestrone is a major factor in estrogen-induced carcinogenesis in general is not supported by recent data (see Sections 4.1 and 4.3 in the monograph on Combined estrogen–progestogen menopausal therapy of this volume), but it may play a role in the HPV-infected cervix. However, in the study by Salazar et al. (2001), only serum estrone and not 16α-hydroxyestrone was measured and other estrogen metabolites have not been considered systematically in the studies reviewed. Therefore, support for the hypothesis that 16α-hydroxyestrone is a major factor in HPV 16-induced cervical carcinogenesis (de Villiers, 2003) is uncertain.]

In transgenic mice that carry the β-galactosidase gene under the control of the HPV 18 long control region (Morales-Peza et al., 2002), ovariectomy suppressed gene expression in the vagina–cervix, but not in the tongue (used as a non-estrogen-responsive control). Treatment with 17β-estradiol, but not progesterone, restored gene expression in the vagina–cervix, and combined treatment with these hormones did not further increase expression. The effect of estrogen was partially blocked by the anti-estrogen tamoxifen. The anti-progestogen RU486 markedly blocked the effect of both hormones combined and also that of 17β-estradiol alone.

In transgenic mice that carry the HPV 16 early region, which contains the E6 and E7 genes, under the control of the human keratin 14 promotor (Arbeit et al., 1994), E6/E7 gene expression was increased by treatment with 17β-estradiol, which ultimately resulted in the development of cervical (and vaginal) squamous-cell carcinomas (Arbeit et al., 1996). These genes were not shown to be estrogen-responsive (Arbeit et al., 1996), and a direct effect of estrogen on their expression is unlikely. In contrast with estrogen-treated wild-type mice, 17β-estradiol caused a substantial increase in proliferating cells in the cervical epithelium in the K14-HPV 16 transgenic mice, an effect that is known to be mediated by the estrogen receptor (Lubahn et al., 1993). When these K14-HPV 16 transgenic mice were compared with other transgenic mice that carry either HPV 16 E6, HPV 16 E7 or HPV 16 E6/E7 genes under the control of the keratin 14 promotor, the estrogen-induced increase in cell proliferation appeared to be confined to the HPV 16 E7 and the HPV 16 E6/E7 mice, whereas estrogen-induced up-regulation of transgene expression was confined to the HPV 16 E6 mice. After 6 months of treatment with estrogen, the HPV 16, HPV 16 E7 and HPV 16 E6/E7 mice all developed cervical cancer at a high incidence, but no such cancer occurred in the HPV 16 E6 mice (Riley et al., 2003). In another study, continuous treatment with estrogen for 9 months resulted in the development of cervical cancer in 100% of HPV 16 E7 and HPV 16 E6/E7 mice (HPV 16 E6 mice were not studied). However, when the estrogen treatment was discontinued 3 months before the end of the experiment, only the HPV 16 E6/E7 mice developed cervical cancer at a high incidence, whereas the HPV 16 E7 mice had a 50% lower cancer incidence, tumours were smaller and multiplicity was lower (Brake & Lambert, 2005). Estrogen appears to act in
these models as a receptor-mediated stimulus of proliferation that is related to the neoplastic transforming activity of HPV to which genotoxic estrogen metabolites possibly contribute (Arbeit et al., 1996; Riley et al., 2003; Brake & Lambert, 2005). [The results of the discontinuation study by Brake and Lambert (2005) and the observation that \( E6 \) and \( E7 \) expression is enhanced by estrogen via a mechanism that apparently does not involve the estrogen receptor (Arbeit et al., 1996) indicate a complex interaction between \( E6 \) and \( E7 \) in the estrogen-enhanced causation of cervical cancer in these mouse models that carry parts of the HPV genome. In addition to acting as a mitogen via the estrogen receptor, the effects of estrogen probably involve progesterone, progesterone receptors and/or other cellular factors that act on HPV gene expression in a manner that is poorly understood. The effects of progestogens or estrogen–progestogen combinations were not examined in any of these studies.]

4.2.3 Individual estrogens and progestogens

(a) Humans

Pakarinen et al. (1999) studied 28 premenopausal women (mean age, 31–32 years) who had used either 0.03 mg per day levonorgestrel orally, an intrauterine device that released levonorgestrel or an intrauterine device that contained copper for 3 months. The only statistically significant change from baseline was a slight reduction in serum levels of SHBG in the women who took oral levonorgestrel. Levels of serum testosterone and insulin growth factor-binding protein-1 were not changed and no changes occurred in women who used the two intrauterine devices.

(b) Experimental systems

No new studies of the estrogens ethinylestradiol or mestranol or the progestogens chlormadinone acetate, ethynodiol diacetate, lynestrenol or norethynodrel that are relevant to the evaluation of the carcinogenic risk of combined contraceptives via the oral or other routes have been published since the last evaluation (IARC, 1999).

(i) Estrogens

17\( \beta \)-Estradiol stimulates the growth of human colon cancer CaCo-2 cells directly in vitro via the estrogen receptor which is blocked by anti-estrogens (Di Domenico et al., 1996) and by anti-sense-mediated inhibition of estrogen receptor expression in mouse colon cancer MC-26 cells (Xu & Thomas, 1994). This stimulation appears to be mediated via estrogen receptor-\( \alpha \), since growth is inhibited in colon cancer cells when estrogen receptor-\( \beta \) is expressed (Arai et al., 2000; Nakayama et al., 2000). Moreover, estrogen receptor-\( \beta \) appears to be the predominant form of estrogen receptor in colon cancer and colon cancer cells (Fiorelli et al., 1999; Campbell-Thompson et al., 2001; Witte et al., 2001). Mediation of the stimulatory action of 17\( \beta \)-estradiol on the proliferation of colon cancer cells may also involve a non-genomic mechanism via the protein kinase C pathway (Winter et al., 2000). Male rats treated with colon carcinogens develop more colon...
cancers than female rats (Di Leo et al., 2001). Treatment of ovariectomized female rats with 17β-estradiol and the colon carcinogen dimethylhydrazine leads to a significant inhibition of the development of colon tumours, from 8.1 ± 1.9 tumours per rat in those treated with carcinogen only to 2.3 ± 1.1 tumours per rat ($p < 0.001$) (Smirnoff et al., 1999). Thus, the available evidence indicates that estrogens inhibit colon carcinogenesis in animal experiments, and experimental studies strongly suggest that the estrogen receptor-β plays an inhibitory role in colon carcinogenesis. These observations support the protective effect of hormonal oral contraceptives and menopausal therapy against colon cancer that has been observed in epidemiological studies (Nanda et al., 1999; Grodstein et al., 1999; Di Leo et al., 2001).

(ii) Progestogens

Cyproterone acetate stimulated the in-vitro production of growth hormone by explants of normal human breast tissue with an estrogen receptor-negative and progesterone receptor-positive phenotype and of insulin-like growth factor I by explants of normal and cancerous human breast tissue with this phenotype (Milewicz et al., 2002).

Recent studies have demonstrated that desogestrel activates the estrogen receptor-α at an activity of about 50% of that of 17β-estradiol but activates the estrogen receptor-β at an activity of only 20% (Rabe et al., 2000). Desogestrel and/or its metabolite 3-keto-desogestrel (etonogestrel) were strongly progestogenic (approximately twofold over progesterone), weakly or not androgenic in animal studies in vivo and in-vitro binding assays and weakly or not active on the glucocorticoid receptor (Deckers et al., 2000; Schoonen et al., 2000). The active metabolite of desogestrel, 3-ketodesogestrel, strongly bound to and activated progesterone receptor-A and, to a slightly lesser extent, progesterone receptor-B (Schoonen et al., 1998).

Dienogest has the same degree of progestogenic activity as progesterone; it is anti-estrogenic and anti-androgenic, and binds weakly to progesterone and androgen receptors (Kaufmann et al., 1983; Katsuki et al., 1997a). It has uterotrophic effects in rabbits that are stronger than those of norethisterone, medroxyprogesterone acetate and dydrogesterone and are blocked by the anti-progestogen RU486 but does not appear to have anti-mineralocorticoid activity (Katsuki et al., 1997a). Mammary hyperplasia but not neoplasia was observed in preclinical toxicological studies of dienogest in dogs (Hoffmann et al., 1983). Dienogest can inhibit neovascularization, including tumour cell-induced angiogenesis (Nakamura et al., 1999), which raises the possibility that it may counteract tumour progression.

The in-vivo anti-tumour activity and anti-uterotropic activity of dienogest were studied in mice and compared with those of several progestogens. At oral doses of 0.01–1 mg/kg bw per day, dienogest significantly suppressed the 17β-estradiol benzoate-dependent tumour growth of HEC-88nu cells, which express estrogen receptors but not progesterone receptors. These cells were unresponsive to known progestogens such as medroxyprogesterone acetate (100 mg/kg bw per day orally) and norethisterone (100 mg/kg bw per day orally). The suppressive effect of dienogest on tumour growth was not diminished in the presence of excess medroxyprogesterone acetate (100 mg/kg bw per day). Dienogest also suppressed the
estradiol-dependent tumour growth of Ishikawa cells (derived from a well-differentiated human endometrial carcinoma) and MCF-7 cells (derived from a human breast carcinoma), both of which express estrogen and progesterone receptors and respond to medroxyprogesterone acetate. However, the minimal effective dose of dienogest (0.01–1 mg/kg per day) was much lower than that of medroxyprogesterone acetate (100 mg/kg per day). Thus dienogest showed potent anticancer activity against hormone-dependent cancers at doses at which other progestogens show no activity. Dienogest showed no anti-uterotropic activity at tumour-suppressive doses (Katsuki et al., 1997b).

Drospirenone is a relatively new progestogen that is used in combined oral contraceptives (Keam & Wagstaff, 2003). It has anti-androgenic and anti-mineralocorticoid effects; it binds strongly to mineralocorticoid receptors, but weakly or not at all to the androgen, glucocorticoid or estrogen (α) receptors (Pollow et al., 1992), with the potential to decrease blood pressure (for reviews see Muhn et al., 1995; Krattenmacher, 2000; Keam & Wagstaff, 2003; Rübig, 2003; Oelkers, 2004).

Studies on the highly progestogenic compound gestodene have demonstrated that its progestogenic activity in an in-vivo system is far lower than those of its in-vitro binding or receptor activation (Deckers et al., 2000; Schoonen et al., 2000; Garcia-Becerra et al., 2004); however, its androgenic activity in vitro has been confirmed (Garcia-Becerra et al., 2004) and it appears to have weak binding activity to the glucocorticoid receptor (Schoonen et al., 2000). Its weak estrogenic activity (transactivation of estrogen receptor-mediated gene expression in model cells) appears to derive from its metabolism to the A ring-reduced metabolites, 3β- and 3α,5α-tetrahydrogestodene, and is probably mediated by the activity of 5α-reductase (Lemus et al., 2000, 2001). These metabolites appeared to be selective agonists of estrogen receptor-α but not of estrogen receptor-β (Larrea et al., 2001). The parent compound did not activate estrogen receptors-α or -β (Rabe et al., 2000).

Recent studies have demonstrated that the estrogenic activity (transactivation of estrogen receptor-mediated gene expression in model cells) of levonorgestrel appears to be derived from its metabolism to the A ring-reduced metabolites, 3β- and 3α,5α-levonor- gestrel, and is abolished by co-treatment with the pure steroidal anti-estrogen ICI 182,780 (Santillán et al., 2001). Levonorgestrel appears to activate estrogen receptor-β strongly (75–90% of the activity of 17β-estradiol) but estrogen receptor-α is only slightly activated (15–25% of the activity of 17β-estradiol) (Rabe et al., 2000). Its weak androgenic activity was confirmed in androgen receptor-binding and transactivation studies (Garcia-Becerra et al., 2004). Levonorgestrel weakly induced a decrease in insulin growth factor-I and an increase in growth hormone production in primary human breast cancer explants in vitro when the explants were progesterone receptor-positive and estrogen receptor-negative, but not in the presence of estrogen receptors or the absence of progesterone receptors (Milewicz et al., 2002).

Studies on norethisterone are summarized in the monograph on Combined estrogen–progestogen menopausal therapy.
4.3 Genetic and related effects

The extensive literature on direct genetic toxicological effects, or the lack of such effects, of the steroid hormones used in combined oral contraceptives has been reviewed previously (IARC, 1979, 1999), and the reader is referred to these tabular and textual considerations of the earlier genotoxicity data. Reports published since the previous evaluations are summarized below. Because many hormones are used in both combined oral contraceptives and hormonal menopausal therapy, synthetic hormones that are used widely in combined oral contraceptives are considered below, but several hormones relevant to this topic are listed exclusively in Section 4.4 of the monograph on Combined estrogen–progestogen menopausal therapy. New evidence has shown that, in aggregate with previous findings, there is a stronger case for the potential of some of these hormones to cause direct genetic damage that could result in genetic alterations of cells.

4.3.1 Ethinylestradiol

(a) Humans

Daily oral doses of 0.02 mg ethinylestradiol and 0.075 mg gestodene administered to 30 healthy women in a monthly cycle of 3 weeks with and 1 week without treatment for six consecutive menstrual cycles did not induce micronuclei in the peripheral blood lymphocytes (Loncar et al., 2004).

A significant increase in the number of lymphocytes with DNA fragmentation and an increased frequency of sister chromatid exchange per metaphase was observed in 18 women who took combined oral contraceptives (daily oral doses of 0.02–0.03 mg ethinylestradiol and 0.15 mg desogestrel) for 24 months compared with age-matched untreated controls ($p < 0.005$) (Biri et al., 2002).

In a population-based study of young women (< 45 years of age) in the USA, those who had started using oral contraceptives at least 20 years before the reference date had a twofold increased risk for breast cancer with cyclin D1 overexpression (odds ratio, 2.2; 95% CI, 1.2–4.0) but not for breast cancer without cyclin D1 overexpression (odds ratio, 1.1; 95% CI, 0.7–1.8) (Terry et al., 2002). The authors suggested that early oral contraceptive use may be associated with the induction of a subset of mammary tumours that overexpress cyclin D1.

Prolonged use of oral contraceptives is more strongly associated with p53-positive breast cancer (odds ratio, 3.1; 95% CI, 1.2–8.1) than p53-negative breast cancer (odds ratio, 1.3; 95% CI, 0.6–3.2) among younger women only (Furberg et al., 2003).

[In the above studies, women who were administered combined oral contraceptives appear to have sustained genetic alterations. It should be recognized that the observed effects of combined oral contraceptives could have been the result of a direct genotoxic effect of the hormonal preparation or could have been an indirect effect of hormonal influences on cellular functions, most notably cell proliferation, mediated by receptor- or
non-receptor-linked mechanisms. It is therefore appropriate not to overinterpret these observations as evidence for a direct genotoxic effect.

(b) Experimental systems

The tissue- and gender-specific patterns of DNA methylation in the promoter regions of the estrogen receptor and aromatase genes were analysed in adult male and female Japanese Medaka fish (Oryzias latipes) exposed to either 0 or 500 ng/L 17α-ethinylestradiol in the water for 14 days. The protein content of the estrogen receptor in exposed fish was significantly increased in all male and female tissues (liver, gonads and brain) compared with controls. Aromatase activity in the exposed fish was significantly increased in the male brain and gonads and female brain compared with controls (Contractor et al., 2004). The changes in DNA methylation of the estrogen receptor and aromatase genes observed indicated that the mechanisms that control gene expression could potentially be altered, as well as gender- and tissue-specific sensitivity. While differences in patterns of DNA methylation did not parallel the changes observed in protein expression, they may impact the regulation of normal gene expression and could be genetically imprinted and transmitted to offspring.

The formation of 8-dihydroxy-2′-deoxyguanosine, an indicator of oxidative DNA damage, has been shown to be increased in the testicular cells of Wistar rats 1 h or 4 h after intraperitoneal injection of 0, 2.8 or 56 mg/kg bw ethinylestradiol in vivo and after exposure to 0.1–10 nM 17α-ethinylestradiol for 30 min in vitro (Wellejus & Loft, 2002). In the total cell population and in round haploid rat testicular cells, oxidized purines show a bell-shaped concentration–response relationship with maximally increased levels at 10 nM. No significant effects were observed in diploid, S-phase or tetraploid cells. The mRNA level of rat 8-oxo-guanine DNA glycosylase was unaffected by ethinylestradiol (Wellejus et al., 2004).

Siddique et al. (2005) recently analysed the genotoxicity of ethinylestradiol in human lymphocytes by measuring chromosomal aberrations, mitotic index and sister chromatid exchange. Ethinylestradiol was genotoxic at 5 and 10 μM in the presence of a rat liver microsomal fraction (metabolic activation system) with nicotinamide adenine dinucleotide phosphate (NADP). Concomitant treatment with superoxide dismutase increased the frequency of chromosomal aberrations and sister chromatid exchange and decreased the mitotic index compared with levels induced by treatment with ethinylestradiol alone, whereas concomitant treatment with catalase decreased the frequencies of chromosomal aberrations and sister chromatid exchange and increased the mitotic index. Concomitant treatment with catalase in combination with superoxide dismutase also decreased the frequencies of chromosomal aberrations and sister chromatid exchange and increased the mitotic index, which suggests a possible role of reactive oxygen species in the induction of the genotoxic damage. Bukvic et al. (2000) reported that ethinylestradiol and norgestrel (1:5) had an aneuploidogenic effect on cultures of human fibroblasts and lymphocytes. Fluorescent in-situ hybridization (with pancentromeric alphoid probes) analysis of micronuclei from lymphocyte cultures and anaphase preparations from fibroblast cultures supported this conclusion.
In primary rat hepatocytes exposed to ethinylestradiol for 20 h at subtoxic concentrations in the range of 1–50 μM, DNA repair was induced in cells derived from both of two males and one of two females (Martelli et al., 2003).

4.3.2 Progestogens

Most progestogens have not been tested systematically for genotoxicity (see Table 20 in the monograph on Combined estrogen–progestogen menopausal therapy and Brambilla & Martelli, 2002). [It should be noted that the negative results for progestogens obtained with the standard battery of genotoxicity tests may be the consequence of using insufficiently sensitive assays, inappropriate target cells and/or suboptimal metabolic activation systems.]

(a) Cyproterone acetate and some structural analogues

The genotoxic potential of cyproterone acetate and some of its analogues has been established. Cyproterone acetate is metabolically activated in the liver of female rats to one or more DNA-damaging intermediates that may induce the formation of DNA adducts, DNA repair and increased levels of micronuclei and gene mutations (reviewed by Kasper, 2001; Brambilla & Martelli, 2002; Joosten et al., 2004). Most importantly, cyproterone acetate induced the formation of DNA adducts in primary cultures of human hepatocytes, which indicates that human liver cells can metabolically activate cyproterone acetate to genotoxic intermediates (Werner et al., 1996, 1997). Cyproterone acetate is activated by hepatocytes to reactive species with such a short half-life that they react only with the DNA of the cell in which they are formed. The response is similar in both men and women but is markedly greater in female than in male rats. The promutagenic character of DNA lesions in the liver of female rats is indicated by the increase in the frequency of micronucleated cells, mutations and enzyme-altered preneoplastic foci (reviewed by Brambilla & Martelli, 2002).

Two other synthetic progestogens, chlormadinone acetate and megestrol acetate, and an aldosterone antagonist, potassium canrenoate, share the 17-hydroxy-3-oxopregna-4,6-diene structure with cyproterone acetate. They all induce genotoxic effects that are qualitatively similar to those of cyproterone acetate (Brambilla & Martelli, 2002).

Chlormadinone acetate and megestrol acetate are genotoxic only in the liver of female rats and in primary human hepatocytes from male and female donors (Brambilla & Martelli, 2002). The metabolic activation of these molecules to reactive species and the consequent formation of DNA adducts occur only in intact hepatocytes.

In primary rat hepatocytes exposed for 20 h to subtoxic concentrations ranging from 1 to 50 μM, DNA repair was induced by drospirenone in both of two males and all of three females, by ethinylestradiol in both of two males and one of two females, by oxymetholone in one of two males and one of two females, by norethisterone in one of two males, by progesterone in one of four females and by methyltestosterone in one of four males (Martelli et al., 2003). A few inconclusive responses were observed in rat hepatocytes exposed to progesterone, medroxyprogesterone, norethisterone, methyltestosterone and oxymetholone. The authors of this small study assert that steroid hormones differ in their ability to induce
DNA repair, and that their genotoxicity may be: (a) different in rat and human hepatocytes; (b) dependent on the sex of the donor; and (c) affected by interindividual variability.

In rat hepatocytes, subtoxic concentrations of potassium canrenoate ranging from 10 to 90 μM consistently induced a dose-dependent increase in DNA fragmentation (Martelli et al., 1999; Brambilla & Martelli, 2002). In another study with other steroids, DNA fragmentation was greater in female than in male rat hepatocytes and DNA-damaging potency was decreased in the following order: cyproterone acetate > dienogest > 1,4,6-androstatriene-17β-ol-3-one acetate > dydrogesterone (Mattioli et al., 2004). Under the same experimental conditions, responses in an assay of DNA repair synthesis were positive or inconclusive in hepatocytes from female rats and were consistently negative in those from male rats.

Analysis of sister chromatid exchange and chromosomal aberrations in bone-marrow cells from mice exposed in vivo to chlormadinone acetate (5.62 mg/kg bw) showed that this dose is non-genotoxic (Siddique & Afzal, 2004). However, doses of 11.25 and 22.50 mg/kg bw chlormadinone acetate significantly increased the frequency of sister chromatid exchange (p < 0.001) and chromosomal aberrations (p < 0.01) compared with untreated controls (Siddique & Afzal, 2004). The authors suggested a genotoxic effect of chlormadinone acetate in mouse bone-marrow cells.

Administration of a single high dose of 100 mg/kg bw cyproterone acetate to female lacI-transgenic Big Blue™ rats induced a strong initial rise in mutation frequency in the liver over that in controls up to a maximum at 2 weeks after administration accompanied by a corresponding increase in cell proliferation and levels of DNA adducts (Topinka et al., 2004a). The mutation frequency decreased after 2 weeks to one-third of the maximum level and this was maintained for an additional 4 weeks. The levels of DNA adducts in the liver decreased by only 15% during this time, which suggests that most adducts were lost as affected hepatocytes were eliminated. When given as a single dose, 5 mg/kg bw cyproterone acetate did not produce significantly elevated levels of mutation. However, mutation frequencies increased 2.5-fold when female lacI-transgenic Big Blue™ rats received repeated daily doses of 5 mg/kg bw cyproterone acetate for 3 weeks (Topinka et al., 2004b).

(b) Norgestrel

A study on human lymphocytes showed that norgestrel induced chromosomal aberrations and significant levels of sister chromatid exchange and inhibited lymphocyte proliferation at concentrations of 25 and 50 μg/mL only. In the presence of a metabolic activation system, the values obtained for chromosomal aberrations, sister chromatid exchange and mitotic index were more significant. A time- and dose-dependent genotoxic effect of norgesterol was observed (Ahmad et al., 2001). The authors concluded that norgestrel itself, and possibly its metabolites, are potent mutagens in human lymphocytes.

(c) Norethisterone

A study on human lymphocytes that used chromosomal aberrations, sister chromatid exchange and cell-growth kinetics as end-points showed that doses of 20, 40 and 75 μg/mL
norethisterone were non-genotoxic either in the presence or in the absence of a metabolic activation system (Ahmad et al., 2001).

Gallmeier et al. (2005) applied a novel and particularly sensitive method to screen for DNA damage with special attention to double-strand breaks. They found that norethisterone is probably genotoxic and therefore potentially mutagenic. A \( p53 \)-reporter assay served as a first, high-throughput screening method and was followed by the immunofluorescence detection of phosphorylated H2AX (a variant of histone H2A protein) as a sensitive assay for the presence of double-strand breaks. Norethisterone at concentrations of 2–100 \( \mu \)g/mL activated \( p53 \) and induced phosphorylation of H2AX on Ser-139 in the vicinity of double-strand breaks. Phosphorylation of H2AX increased in a dose-dependent manner. Double-strand breaks were not detected with the neutral comet assay, a less sensitive method than H2AX phosphorylation. The authors suggested that, since norethisterone induced double-strand breaks in their experiments, this both complements and adds a new aspect to the existing literature on its genotoxic potential. However, they noted that, since the effective concentrations of norethisterone in these assays were approximately 100–1000-fold higher than therapeutic doses, the significance of these findings with regard to human exposure has yet to be determined.

In-vitro studies that analysed gene expression of isolated normal endometrial epithelial cells treated with estradiol and norethisterone acetate showed upregulation of the \( \text{Wnt-7a} \) gene; with estradiol only, \( \text{Wnt-7a} \) was expressed at very low levels (Oehler et al., 2002). \( \text{Wnt} \) genes are a large family of developmental genes that are associated with cellular responses such as carcinogenesis.