Estetrol review: profile and potential clinical applications

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ABSTRACT
In this review paper, the existing information on the human fetal steroid estetrol (E4) has been summarized. In the past, E4 was considered as a weak estrogen and interest disappeared. However, recent new research has demonstrated that E4 is a potent, orally bioavailable, natural human fetal selective estrogen receptor modulator, since it acts in the rat as an estrogen on all tissues investigated except breast tumor tissue, where it has estrogen antagonistic properties in the presence of estradiol. Based on its safety data, its pharmacokinetic properties, its pharmacological profile and the results of first human studies, E4 may be suitable as a potential drug for human use in applications such as hormone replacement therapy (vaginal atrophy, hot flushes), contraception and osteoporosis. Additional areas worth exploring are the treatment of breast and prostate cancer, hypoactive sexual desire disorder and topical use (wrinkles) in women, autoimmune diseases, migraine, cardiovascular applications and the treatment of selected obstetric disorders.

HISTORY
Estetrol (E4) was discovered by Diczfalusy and co-workers in 19651 and was a topic of preclinical pharmacological research thereafter for a period of about 20 years. Studies during that period showed that this estrogenic steroid molecule has four hydroxyl groups2. Isolation and identification of this novel estrogen were achieved by extracting 200 liters of late pregnancy urine3. On the basis of physical and chemical characteristics, it was concluded that the compound was identical with 15α-hydroxyestradiol (15α-OHE3) or estra-1,3,5(10)-triene-3,15α,16α,17β-tetrol3. It was further concluded that E4 is synthesized exclusively by the fetal liver during human pregnancy, reaching the maternal circulation through the placenta. This conclusion was based on previous work, which showed that the liver is the exclusive site of 15α- and 16α-hydroxylation4–6. The structural formulae of E4 and other estrogenic steroids are presented in Figure 1, demonstrating that these estrogenic steroids differ in the number of hydroxyl (OH) groups only.

Several ADME (absorption/distribution/metabolism/excretion) properties of E4 have been studied in postmenopausal and last-trimester pregnant women using parenteral administration of steroids labeled with radioisotopes7,8. Estetrol was minimally, if at all, metabolized and was not reconverted to estriol (E3) or estradiol (E2). When injected intravenously to adults, it was rapidly and completely excreted in urine as a Ring D monoglucuronide, but otherwise metabolically unaltered9–11. According to these data,
Estetrol does not appear to enter the enterohepatic circulation. Estetrol was detected in maternal urine as early as 9 weeks of pregnancy. It was found at high levels in maternal plasma during the second trimester of pregnancy, with steadily rising concentrations of unconjugated E4 to about 1 ng/ml (3 nmol/l) toward the end of pregnancy. Conjugated E4 levels were seven times higher than unconjugated levels. The levels of unconjugated E4 in fetal plasma at parturition were about 12–19 times those in maternal plasma. Amniotic fluid levels were about one-third of fetal plasma levels and five to six times higher than maternal plasma levels. Maternal urinary excretion in late pregnancy varied between 0.5 and 2.3 mg/day.

For follow-up and survey of pregnancy pathology, E4 levels were not appropriate due to the large intra- and inter-individual variations in plasma levels. Consensus at that time was that, first, E4 is a weak estrogen and, second, E4 cannot be used as a marker of fetal well-being during pregnancy due to the high inter- and intra-individual variations in plasma levels.

After 20 years of experimental work, E4 research was virtually abandoned and ended in the mid-1980s. Consensus at that time was that, first, E4 is a weak estrogen and, second, E4 cannot be used as a marker of fetal well-being during pregnancy due to the high inter- and intra-individual variations in plasma levels.

However, it seems unlikely that an estrogenic steroid produced in such significant quantities by the male and female human fetal liver during pregnancy would have no physiological significance. Therefore, in 2001, a project was started at Pantarhei Bioscience to investigate the properties of E4 with state-of-the-art technologies.

**SYNTHESIS AND PHARMACEUTICAL PROPERTIES**

A new route of synthesis has been developed for E4 by Pantarhei, starting with the commercially available E1. This new route results in accep-
table yields of E₄ of very high purity (>98%) without contamination with E₂. It permits the synthesis of E₄ on a (semi)-industrial scale suitable for GMP (Good Manufacturing Production) for human use. This new method overcomes the disadvantages of previous methods. Pharmaceutical studies revealed that E₄ is chemically very stable even under non-optimal storage conditions. It has high water solubility and might be slightly hygroscopic. Estetrol has an octanol–water partition coefficient (Pow) of about 1.5, making it about a 100-fold less lipophylic than E₂ or ethinylestradiol. As a Pow of 2 is considered optimal for passage through the blood–brain barrier, E₄ might be expected to have effects on the central nervous system.

It is concluded that, with this new method, E₄ can be synthesized consistently with high purity and without important contaminations. Estetrol appears to have very favorable properties for the development of a pharmaceutical product.

**RECEPTOR BINDING AND TARGET INTERACTION**

Estetrol has a moderate affinity for human estrogen α receptor (ERα) and estrogen β receptor (ERβ), with Kᵢ values of 4.9 ± 0.567 nmol/l and 19 ± 1 nmol/l, respectively, demonstrating a four-to-five-fold preference for the ERα (lower Kᵢ value).

Estetrol has high selectivity for the estrogen receptors. Binding at the glucocorticoid, progesterone and testosterone receptors was only 11–15% at a concentration of 10 μmol/l and further profiling of E₄ in a set of 124 receptors and enzymes demonstrated inactivity towards 123 molecular targets. The single target showing interaction with E₄ was the adrenergic x₁β receptor (weak binding).

It is concluded that genomic clinical effects of E₄ will most likely occur through the estrogen receptors. The high selectivity of E₄ suggests a low risk of unexpected side-effects.

**LIVER CELL METABOLISM AND PROTEIN BINDING**

The rate of metabolism of E₄ was studied in rat and human hepatocytes and was found to be slow in both in vitro systems, complying with the observed slow elimination and long half-life in vivo.

The metabolites found after incubation of E₄ with rat and human hepatocytes were completely different. Metabolites produced by rat hepatocytes were not found with human hepatocytes and vice versa. In rat hepatocytes, phase I metabolism is most important. This may result in active metabolites in the rat, whereas inactivation by glucuronidation and sulfation, i.e. phase II metabolism, are the pathways observed in human hepatocytes. This confirms that, in the human, E₄ is an end-stage product of metabolism and has no active metabolites.

Estetrol at a high concentration of 10 μmol/l did not inhibit the major cytochrome P450 enzymes CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP 3A4. Estradiol and ethinylestradiol significantly inhibited CYP2C19. Ethinylestradiol had a strong inhibitory effect on CYP3A4, whereas E₂ stimulated this enzyme significantly and E₄ had some stimulatory effect. These results suggest that E₄ may exhibit less interference with concomitantly administered drugs (drug–drug interaction) compared to ethinylestradiol and E₂.

The ERα-dependent effect of the steroids E₂, E₃, E₄ and ethinylestradiol on sex hormone binding globulin (SHBG) production was investigated using the HepG2 and Hep89 cell lines. Estetrol did not stimulate the production of SHBG in both cell lines, suggesting that E₄ may not influence the plasma levels of SHBG. The estrogens E₂, E₃ and ethinylestradiol all show a dose-dependent ERα-mediated increase in the production of SHBG. This increase in SHBG production is most prominent for E₂, while addition of E₃ and ethinylestradiol resulted in a lower and comparable increase in SHBG. Binding of E₄ to SHBG was also studied in vitro. There was no detectable binding of E₄ to the estrogenic and androgenic human SHBG steroid-binding sites (Figure 2). By contrast, testosterone and E₂ were bound with high affinity, whereas the synthetic estrogen ethinylestradiol binds to SHBG with low affinity. These data indicate that SHBG has no influence on the plasma distribution of E₄ or its availability to target tissues, contrary to other natural steroid ligands such as E₂ and testosterone and several synthetic progestins that all bind to SHBG.

**ADME AND ORAL BIOAVAILABILITY**

Earlier ADME studies were performed using parenteral administration of radiolabeled steroids, but oral administration of E₄ was never considered in the past. Since oral treatment was considered crucial for the potential use of E₄...
as a drug for human use, its oral bioavailability and pharmacokinetics were studied as the very first stage of the new E4 research in the rat. When E4 appeared to have favorable oral kinetics in the rat, at a later stage pharmacokinetic studies were performed in the human.

In the bioavailability study in the rat, E4 was administered as a single dose of 0.05, 0.5 or 5.0 mg/kg orally or subcutaneously to female rats. Plasma was analyzed using an LC-MS/MS method. Oral bioavailability of E4, relative to that of subcutaneous dosing, was 70% and above at the 0.05 and 0.5 mg/kg doses, based on the AUC. Subcutaneous dosing provided significantly higher E4 levels at the 1-h time point only, and was comparable to oral dosing after 0.5, 2, 4 and 8 h (Figure 3). So, rather surprisingly, E4 was found to have a high oral bioavailability in the rat. Also the elimination half-life observed after 2–3 h is relatively long, since the rat liver is known to be very efficient in metabolizing steroids.

These findings had at least two implications. First, the oral bioavailability enabled once-daily oral treatment with E4 in further studies in the rat, a species considered to be relevant and predictive for the human. Second, the pharmacokinetic data obtained in the rat suggested that oral treatment with E4 might be possible also in the human.

A pharmacokinetic study with E4 was performed in healthy early postmenopausal women. Four single doses of 0.1, 1, 10 and 100 mg E4 were administered to six women each. It was shown that E4 is absorbed orally very effectively with high dose–response relationship and low inter-subject variability (Figure 4). The elimination half-life of E4 was found to be 28 h, suggesting slow metabolism of E4 and suitability for once-a-day oral administration. The pharmacokinetic pattern suggested enterohepatic recirculation. Pharmacokinetic simulations with special emphasis on AUCs were performed using these human pharmacokinetic data and E4 levels during pregnancy (see also History). Based on these calculations, the term fetus synthesizes about 3 mg E4 per day, whereas the term fetal exposure to E4 is more than 50 mg/day when compared to oral administration of E4 to postmenopausal women.

In summary, the old and new ADME data obtained with E4 support its potential as an oral once-a-day drug for human use.

**BONE**

The bone-sparing effect of oral E4 compared to that of ethinylestradiol was studied in ovariectomized rats. The use of the ovariectomized rat model for the preclinical evaluation of drugs intended for prevention and treatment of osteoporosis is recommended by the US Food and Drug Administration. Once-daily oral treatment with E4 by a dose of 0.1, 0.5, or 2.5 mg/kg/day, or by 0.1 mg/kg/day of ethinylestradiol as positive control, was given for 4 weeks. The following measurements were performed:

1. Serum osteocalcin,
2. Bone mineral density, bone mineral content and bone mineral area of lumbar vertebrae L3–L6,
3. Peripheral quantitative computed tomography of the left tibiae, and
4. The biomechanical properties (strength) of the distal femora.
Estetrol dose-dependently and significantly inhibited the ovariectomy-related increase in osteocalcin levels, increased bone mineral density and content and increased bone strength (Figure 5), all attenuated by ovariectomy. In this rat model, the relative potency of the highest dose of E₄ of 2.5 mg/kg/day was comparable to the 0.1 mg/kg/day ethinylestradiol dose, used as positive control. It was concluded that oral administration of E₄ conveys dose-dependent bone-sparing effects of high-quality bone in estrogen-depleted ovariectomized rats.

Based on its bone-sparing effects and its oral bioavailability in this highly predictive and
well-validated animal model, E4 is a potential drug for the prevention of osteoporosis in postmenopausal women. It seems worthwhile also to investigate the potential efficacy of E4 for the treatment of osteoporosis and osteoporotic fractures.

HOT FLUSH

The efficacy of E4 in alleviating hot flushes was studied in an experimental rat model considered representative for menopausal vasomotor symptoms\(^\text{41}\). In this model, the thermal responses in the tail skin of morphine-dependent ovariectomized rats are recorded after administration of naloxone. Six groups of rats were treated orally for 8 days as follows: vehicle (negative) control; E4: 0.1, 0.3, 1.0 and 3.0 mg/kg/day; and as active (positive) control ethinylestradiol: 0.3 mg/kg/day. On day 8, tail skin temperature was recorded at baseline and for 60 min at 5-min intervals following naloxone administration. In control animals, tail skin temperature increased sharply by about 4.5° C after naloxone treatment and reverted to baseline by 60 min. Estetrol suppressed the tail skin temperature increase in a dose-dependent fashion (Figure 6). The highest dose of E4 tested (3 mg/kg/day) was equipotent to a 10-fold lower dose of ethinylestradiol. Both fully suppressed tail skin temperature changes\(^\text{41}\).

It is concluded that E4 is effective in preventing temperature rises in an experimental animal model considered representative for studying the effect of drugs on the menopausal hot flush (vasomotor symptoms). In this model, the potency of E4 was 10-fold lower compared to ethinylestradiol.

These results suggest that E4 may be effective for the treatment of hot flushes and other vasomotor symptoms in peri- and postmenopausal women.

VAGINA, UTERUS AND ENDOMETRIUM

The effect of E4 on vaginal cornification and uterine weight was studied in ovariectomized rats\(^\text{42}\). Six groups of rats were treated orally once daily for 7 days as follows: vehicle (negative) control; E4: 0.1, 0.3, 1.0 and 3.0 mg/kg/day; and ethinylestradiol 0.05 mg/kg/day as active (positive) control. Vaginal lavages were obtained daily and, on day 7, uterine wet weight was determined. Vaginal cornification was observed by day 5 in all rats at all E4 doses and in the animals receiving ethinylestradiol, but not in the control rats (Figure 7). The onset of cornification with E4 was dose-dependent. After 7 days treatment, the two highest E4 doses (1.0 and 3.0 mg) induced statistically significantly higher uterine wet weight (myometrium) compared to vehicle\(^\text{42}\).

In the pharmacological study with E4 to investigate oral bioavailability and prevention of bone loss\(^\text{37}\), the uterus of the ovariectomized rats was excised after 4 weeks of treatment. Wet uterine weight (myometrium) was estimated and histological investigation of the endometrium was performed. Four weeks of E4 treatment induced dose-dependent increases in uterine weight of ovariectomized rats. In this model, the potency of ethinylestradiol in increasing uterine weight was 5–25 times higher than that of E4.
Estetrol appeared to have a dose-dependent proliferative estrogenic effect on the rat endometrium after 4 weeks’ treatment. Estetrol was found to be less potent than ethinylestradiol, since the order of increasing potency per mg/kg/day was estimated as follows: 0.1 mg E₄ < 0.5 mg E₄ < 0.1 mg ethinylestradiol < 2.5 mg E₄.

In summary, estrogenic activity of E₄ was demonstrated in three tissues in ovariectomized rats: vaginal epithelium, myometrium and endometrium. The potency of E₄ was approximately 20-fold lower compared to ethinylestradiol.

It is concluded that E₄ may be suitable for the treatment of urogenital atrophy and the accompanying clinical complaints such as vaginal dryness and dyspareunia in estrogen-deficient women. Since E₄ has a proliferative effect on the endometrium, in women with a uterus, measures...
to protect against endometrial hyperplasia and cancer should be taken.

**BREAST**

Rats treated with DMBA (7,12 dimethylbenz(a)-anthracene) develop estrogen-responsive breast tumors. Two prevention studies and one intervention study were performed in this animal model with $E_4^{43}$. In the prevention studies, the effect was investigated of oral doses of $E_4$ over a dose range of 0.5–3.0 mg/kg. The intervention study used oral doses of 1, 3 and 10 mg/kg $E_4$.

The antiestrogen tamoxifen was used as reference compound in all three studies; ovariectomy and ethinylestradiol, at doses pharmacologically equipotent to $E_4$, acted as control treatments in one prevention study and in the intervention study.

When DMBA-induced rats were co-treated with $E_4$ for 8 weeks, this resulted in a dose-dependent reduction in the number and size of tumors, an effect that appeared equally effective as tamoxifen treatment or ovariectomy and was not seen with ethinylestradiol. When $E_4$ was administered to rats in which tumors had already developed, a significant decrease in the number and size of tumors was observed after 4 weeks. This decrease was dose-dependent, comparable to tamoxifen-treated animals, and, at high dose levels, $E_4$ was as effective as ovariectomy (Figure 8).$^{43}$

It was concluded that $E_4$, dose-dependently, prevents the growth of chemically induced mammary tumors in female rats and has the potential to reduce the number and size of such mammary tumors when already present.

Based on these results, one may hypothesize that $E_4$ could be a natural antagonist of the abundantly available estrogens during human pregnancy. These findings provide the basis for the potential clinical development of $E_4$ as a natural selective estrogen receptor modulator (natural SERM) and antagonist for the treatment of breast cancer. The estrogen antagonistic effect on breast tumor tissue would be a major advantage compared to presently used estrogen-containing drugs.

**OVULATION INHIBITION**

The effectiveness of $E_4$ as an ovulation inhibitor was studied in regularly cycling female rats and compared to ethinylestradiol.$^{44,45}$ The animals were treated orally twice daily for 4 consecutive days, starting on the day of estrus, with $E_4$ (0.03, 0.1, 0.3, 1.0 or 3.0 mg/kg), or ethinylestradiol (0.0003, 0.001, 0.003, 0.01 or 0.03 mg/kg) or vehicle control. The primary endpoint was the number of ovulated oocytes in the genital tract. Estetrol at the twice-daily dose of 0.3 mg/kg and above inhibited ovulation. This effect was statistically significant ($p < 0.05$). The comparator, ethinylestradiol, significantly inhibited ovulation ($p < 0.05$) at the highest dose (0.03 mg/kg) administered twice daily. The ED$_{50}$ for the ethinylestradiol and the $E_4$ dose–response curves show that ethinylestradiol is 18 times more potent than $E_4$ (Figure 9)$^{44}$. In summary, twice-daily administration of $E_4$ effectively inhibits ovulation in cycling rats. The effect is dose-dependent. The relative potency of $E_4$ is about 18 times less compared to that of ethinylestradiol.
In the human pharmacokinetic study performed with single doses of 0.1, 1, 10 and 100 mg E4, the effect on plasma levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH) was also studied. LH levels were suppressed dose-dependently. A profound and sustained inhibition of FSH levels, lasting over 7 days, was observed in the 100 mg dose group (FSH was not measured in the other dose groups). It was concluded that E4 has a profound central inhibitory and dose-dependent effect on gonadotropins, expected to contribute to the contraceptive effect of E4.

In summary, based on these and other results reported in this review, E4 might be a potential candidate to replace ethinylestradiol in combined oral contraceptives.

CONCLUSIONS

Estetrol is a steroid synthesized exclusively by the human fetal liver during pregnancy. After its discovery in 1965 by Egon Diczfalusy and co-workers at the Karolinska Institute in Stockholm, Sweden, basic research was performed on E4 until about 1984. At that time there was consensus that E4 is a weak estrogen and interest in this steroid discontinued. The judgement of low potency of E4 was primarily based on low ER binding affinity, ranging from 0.3% (rat) to 6.25% (human) and 2–3% potency compared to E2 in a series of in vitro and in vivo experiments. Efforts to use E4 as a marker of fetal well-being during pregnancy failed due to the high inter- and intra-individual variations in plasma levels.

In 2001, Pantarhei Bioscience started its research activities on E4. The motivation was the simple consideration that it seems unlikely that nature would endow the human fetus with the ability to produce this steroid in such significant amounts without a sound reason.

The new research has revealed that, first of all, E4 seems a safe compound with selective binding to both estrogen receptors and some preference for the ERα above the ERβ. No toxicity was observed at high dose levels in pharmacological studies in the rat, with maximum doses of 10 mg/kg/day for 4 weeks and in single-dose studies in the human, with a maximum dose of 100 mg. Based on its chemical, pharmaceutical and metabolic properties, it seems possible to develop E4 as a drug for human use. Especially its high and dose-related oral bioavailability in the rat and the human, the absence of binding to SHBG and the long elimination half-life of 28 h in the human may enable its use as an oral once-a-day drug.

In well-validated and predictive rat models, E4 behaves as an estrogen agonist in all tissues investigated, i.e. bone, vagina, myometrium, endometrium and brain (hot flush and ovulation inhibition), except for breast tumor tissue where this steroid acts as an estrogen antagonist in the presence of E2. Interaction with liver function in in vitro models demonstrates, first, slow metabolism, explaining the long half-life; second, absence of cytochrome P450 inhibition.

Figure 9  Estimation of ED50 for ovulation inhibition in 4-day cycling rats treated twice daily with the indicated oral doses of ethinylestradiol (EE) or estetrol (E4)
which may implicate less drug–drug interaction; and, third, no stimulation of SHBG synthesis\textsuperscript{39}, which suggests a potentially lower risk of venous thromboembolism when used as a drug in the human, a serious side-effect of all known estrogens and synthetic SERMs.

Based on its pharmacological profile, E\textsubscript{4} can be classified as a natural human fetal SERM. Contrary to the conclusion in the past, E\textsubscript{4} seems to be a potent steroid. In the pharmacological studies, E\textsubscript{4} was 10–20 times less potent compared to ethinylestradiol, the most potent estrogen available. Single doses of E\textsubscript{4} strongly suppressed LH and FSH in postmenopausal women. The difference between the past and the present conclusions can be explained by (lack of) knowledge of the metabolic properties of E\textsubscript{4}. Past studies were all in vitro or short in vivo experiments and adequate ADME studies demonstrating the favorable pharmacokinetics of E\textsubscript{4} were performed only recently\textsuperscript{37,38}.

Estetrol may be useful for a series of potential clinical applications including the prevention and treatment of osteoporosis and hormone replacement therapy in women, especially for the treatment of vaginal atrophy and hot flushes. Estetrol seems also suitable as the estrogenic component in oral contraceptives. The effect of E\textsubscript{4} on breast cancer seems worthwhile of being investigated in view of the results in the rat DMBA model. All these possible applications should be explored in clinical proof-of-concept studies. Furthermore, it seems interesting to study the effect of E\textsubscript{4} in autoimmune diseases that are related to Thymocyte-1 (Th-1) function such as multiple sclerosis, rheumatoid arthritis and Sjögren’s syndrome, since Th-1-related diseases are known to improve considerably during pregnancy when E\textsubscript{4} is present. Studies with E\textsubscript{4} in animal models for multiple sclerosis (EAE model) and rheumatoid arthritis (CIA model) have shown a significant and dose-dependent favorable effect (data not shown). Additional areas worth exploring are the treatment of prostate cancer, hypoactive sexual desire disorder and topical use (wrinkles) in women, migraine, cardiovascular applications and the treatment of selected obstetric disorders.

Although there are sound reasons to test the use of E\textsubscript{4} in all these conditions, it seems unlikely that E\textsubscript{4} will be efficacious in all these disorders and diseases. However, the present data on E\textsubscript{4} allow the conclusion that the pharmacological profile of E\textsubscript{4} is not a weak estrogen but a potent steroidal SERM with potential applications in the human.

The question remains about the physiological role of E\textsubscript{4} during human pregnancy since this has not been studied and is unknown. The facts are that E\textsubscript{4} is a steroid synthesized exclusively by the human fetal liver during pregnancy\textsuperscript{1–6}. Data on file show that E\textsubscript{4} is not synthesized by pregnant rats and mares. Estetrol is already present in urine of pregnant women at 9 weeks of gestation\textsuperscript{12,13}. Estetrol plasma levels increase exponentially during pregnancy and the term fetus synthesizes a high amount of E\textsubscript{4} up to 3 mg/day. Unanswered questions are, for example:

1. Why is E\textsubscript{4} present during pregnancy?
2. How is 15\textalpha;-hydroxylation during pregnancy regulated?
3. Why is the expression of this enzyme restricted to pregnancy?
4. How is the fetus protected against the estrogenic activity of E\textsubscript{4}?

These and other questions may be elucidated by further estetrol research dedicated to unravel the raison d’être of this intriguing natural human fetal steroidal SERM.

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