Novel agents to modulate oestrogen action

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As women enter the menopause, the majority suffers symptoms associated with a dramatic fall in circulating levels of 17β-oestradiol and oestrone. As a result, the oestrogen protective effect against coronary artery disease and osteoporosis is lost. To solve these problems, hormone replacement therapy is often used. However, there are a number of side-effects including increased risk from breast and uterine cancer that can limit compliance. New drugs, called selective oestrogen modulators (SERMs), have been developed to mimic oestrogen's effects on the liver, heart and bones but without its harmful effects on the breast and uterus. SERMs are structurally diverse compounds that bind to oestrogen receptors and elicit agonist or antagonist responses depending on the target tissue and hormonal milieu. The drugs are being used, or evaluated, for the prevention of hormone-responsive breast cancer, osteoporosis and cardiovascular disease in postmenopausal women. Tamoxifen is the endocrine treatment of choice for breast cancer, but it also has beneficial effects on bone density and serum lipids in postmenopausal women. Recently, tamoxifen was shown to decrease the risk of invasive breast cancer in women at high risk. However, tamoxifen has some stimulatory effects on the endometrium. Raloxifene is used to prevent osteoporosis and fractures. Raloxifene also lowers circulating cholesterol and the incidence of invasive breast cancer in postmenopausal women but does not stimulate the endometrium. The SERMs have evolved from mere laboratory curiosities into drugs that hold promise for preventing several major diseases associated with ageing in women.

Oestrogens and functions

Oestrogens are a class of sex hormones associated with the development and maintenance of secondary female sex characteristics, control of the menstrual cycle and the maintenance of pregnancy. Oestrogens also have an anabolic effect on protein metabolism and water retention. However, the major beneficial effect of oestrogen in a woman’s body is apparent when the dramatic fall in circulation levels of 17β-oestradiol occurs as women enter the menopause. During the menopause, ovulation and cyclical elevation in the concentration of progesterone ceases and...
pituitary gonadotrophin levels increase. These hormonal changes are associated with loss of menstrual cyclicity and a number of symptoms associated with the decline in oestrogen, like vasomotor instability (hot flushes, sweats, and frequent awakening from sleep with resulting daytime fatigue), and central nervous system problems (mood-swings, depression, memory loss, and sleep disorders). Urogenital atrophy is also observed in postmenopausal women, in particular dry vagina, dyspareunia, urinary incontinence, and increased risk of urinary tract infections.

Most importantly, however, chronic diseases such as heart disease and osteoporosis have been associated with oestrogen deficiency. It is known that oestrogen decreases LDL-cholesterol, an effect that may protect against heart disease, more specifically myocardial infarction. The beneficial effects of oestrogen may result from both lipid-lowering actions and by direct effects on the vasculature. Postmenopausal women also experience a sharp rise in bone turnover that leads to a net loss of bone mass due to excessive bone resorption by osteoclasts. In consequence, fractures of the hip, wrist, and spine may become more frequent as women age. The physician and patient need to identify which of these problems are specifically relevant to the individual patient. This approach requires assessment of specific symptoms or risk factors for heart and bone disease as part of a medical evaluation. Together, the physician and patient can tailor effective treatments for that specific patient and her particular problem.

Oestrogen replacement therapy (ERT) or hormonal replacement therapy (HRT), oestrogen in combination with one of several progestin regimens, are used to prevent the climacteric symptoms and diseases associated with the menopause. The most common oestrogen used for oral ERT or HRT is conjugated oestrogen, which contains water-soluble conjugated forms of mixed oestrogens obtained either wholly or partly from the urine of pregnant mares or synthetically from oestrone and equilin. The principal oestrogen present is sodium oestrone sulphate. The total oestrogenic potency of the preparation is expressed in terms of an equivalent quantity of oestrone sulphate. ERT and HRT normally are administrated orally or transdermally, which obviates the first pass effect, minimising the synthesis of several unwanted hepatic proteins and triglycerides. The regimens can be cyclical or sequential and are usually accompanied by recurrence of uterine bleeding in postmenopausal women with intact uteri. These withdrawal-bleeding episodes are unwelcome by a majority of women and may reduce compliance with HRT. Continuous, combined regimens were developed to produce amenorrhoea. However, many bleeding episodes continue in some women although these gradually decrease with increased treatment duration.

ERT and HRT arrest bone loss due to the menopause and may reduce the rate of major cardiovascular events, such as myocardial infarction.
and cerebrovascular accidents\(^5\). In addition, there is some evidence that oestrogen improves cognitive function\(^6\). Although ERT reduces the risk associated with several diseases, there are concerns about the increased risk of endometrial cancer. This concern has necessitated the development of therapeutic regimens in which the uterine effects of oestrogen are opposed by progestin treatment. Unfortunately, the side-effects of progestin treatment, such as resumption of menses, central nervous system disturbances, and the possibility of attenuated cardiovascular benefits, have significantly reduced patient compliance\(^7\).

Herein lies the problem. Oestrogen provides excellent physiological support for the postmenopausal women but oestrogen is also linked with cancer. Breast and endometrial cancers are important, hormone-dependent tumours that occur most frequently in postmenopausal women and the major risk factor for both cancers is associated with life-time exposure to unopposed oestrogens. Long-term use of HRT may be associated with a modest increase in the risk of breast cancer\(^8\). It is important to stress, however, that short-term use of HRT to solve menopausal symptoms is not associated with an increase risk for breast cancer. An increased risk of developing endometrial cancer can be decreased in postmenopausal women using HRT, depending upon the progestin schedule used and the duration of therapy\(^9\). Progestins, however, do not reduce breast cancer risk.

The increased risk of breast and uterine cancer associated with ERT has stimulated the search for treatment alternatives\(^9\). The goal now is to find an ideal oestrogen, which combats the pathologies associated with menopause states, such as osteoporosis and coronary disease, but without the side-effects of oestrogen replacement therapy (i.e. increased risk of breast and uterine cancer).

The search for more acceptable and safer postmenopausal HRT has led to the evaluation of anti-oestrogen compounds, e.g. tamoxifen and raloxifene, known now as selective oestrogen receptor modulators (SERMs). However, it is first important to consider how oestrogen produces its effects around the body.

**Oestrogen receptors and oestrogen action**

In 1962, Jensen discovered the oestrogen receptor (ER) and he subsequently correlated the presence of ER with the hormone responsiveness of a patient’s tumour to endocrine ablation\(^10\). The ERs are located in the cells of target tissues throughout the body, so that oestrogenic molecules can orchestrate the physiological functions of reproduction and the biochemical events specific to women. Oestrogen receptors are ligand-inducible nuclear transcription factors, which are physiologically
activated by steroids but which can also be activated by a large number of non-steroidal ligands\textsuperscript{11}.

The ER protein\textsuperscript{12} consists of six functional domains (A–F) transcribed by eight exons (Fig. 1). Two of these functional domains are highly conserved in the primary sequence of members of the nuclear hormone receptor superfamily. One of the domains, the DNA binding domain (DBD), contains two zinc fingers that mediate receptor binding to an oestrogen response element (ERE) in the promoter region of hormone-responsive genes. In the C-terminal region, the ligand-binding domain (LBD) has two regions of sequence homology with other hormone receptors and it is responsible for the hormone selectivity and specificity. Activating functions (AF), AF-1 and AF-2, present at the N-terminal and C-terminal (Fig. 1), respectively, activate transcription in a cell and promoter context specific manner. AF-1 is responsible for the promoter-specific transcriptional activation independent of the presence of ligand and AF-2 provides ligand-specific activation.

There are two types of oestrogen receptors: ER\textsubscript{\(\alpha\)} and ER\textsubscript{\(\beta\)}. The ER\textsubscript{\(\alpha\)} cDNA was cloned and sequenced from MCF-7 human breast cancer cells\textsuperscript{13}; its protein consists of 595 amino acids with a molecular weight of 66 kDa. Recently, ER\textsubscript{\(\beta\)} cDNA was cloned from a rat prostate complementary DNA library\textsuperscript{14} and the protein contains 485 amino acids with a molecular weight of 54.2 kDa. ER\textsubscript{\(\alpha\)} and ER\textsubscript{\(\beta\)} demonstrate interesting similarities in the DBD and significant differences in the LBD (Fig. 2).

Clearly, one possibility to explain the target site specificity and altered oestrogenicity of anti-oestrogens is a differential distribution of ER\textsubscript{\(\alpha\)} and ER\textsubscript{\(\beta\)} to different tissues\textsuperscript{15}. The mechanism of action of the differential

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**Fig. 1** The ER protein is derived from a 6.6 kb mRNA containing eight exons. The translated ER protein is 66 kDa and consists of six functional domains (AF). Arrowheads indicate the most important mutations of ER.
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![ERα and ERβ proteins](image)

**Fig. 2** Schematic representation of the ERα and ERβ proteins and their regions of homology. The mutations (540, 543, 547) can silence activating function (AF)-2 by preventing co-activator binding. Amino acid 351 (aspartate) is critical on the surface of the tamoxifen–ER complex to form oestrogen-like actions. Raloxifene does not bind co-activators in this position, which is why it is less oestrogen-like than tamoxifen.

pharmacology between ERα and ERβ may also involve different methods of gene activation. A novel signal transduction pathway has recently been identified as a protein–protein interaction between ERβ–anti-oestrogen complexes and AP-1 (fos and jun) that is capable of activating a reporter gene. Thus, multiple signal transduction pathways can potentially modulate oestrogen action around the body. However, the recognition of selective target site modulation by non-steroidal anti-oestrogens was the stimulus that opened the door to the current therapeutic developments.

**Selective oestrogen receptor modulators**

Oestrogen action can now be described in its basic form. Oestradiol-17β (E2) diffuses through the cellular membranes to the nucleus where it binds to the ER. Consequently, there is a conformational change, causing the ER to dimerize and interact with an ERE located in the promoter region of an oestrogen responsive gene. Transcriptional activation occurs, through a transcriptional complex that binds to the E2–ER complex, resulting in a phenotypic response which is specific for a particular target cell (Fig. 3). But how do SERMs work? Clearly they could produce different effects at ERα and ERβ, but the precise details are as yet unknown. Nevertheless, this is an area of intense research interest.

Several SERMs are marketed or are in clinical development, including triphenylethylenes (tamoxifen and its derivatives: toremifene, droloxifene and idoxifene), chromans (levormeloxifene, EM 652), benzothiophenes (raloxifene, LY353381) and naphthalenes (CP336, 156). Tamoxifen and its derivatives are all partial oestrogen agonists in the uterus that also inhibit DMBA-induced rat mammary tumour growth and the growth of...
Fig. 3 The ER signal transduction pathway. Oestradiol (E2) binds to oestrogen receptor (ER), in the nucleus, and induced a conformational change. The ER then forms a homodimer and interacts with an oestrogen response element (ERE) to initiate gene transcription.

ER positive MCF-7 breast cancer cell growth in vitro. However, these compounds are considered to be selective oestrogen receptor modulators, depending on the tissue target being investigated for agonist or antagonist action. For example, tamoxifen and raloxifene have oestrogen-like effects on bone and circulating cholesterol, but tamoxifen is also oestrogen-like on the growth of endometrial cancer.

To explain the selective actions of tamoxifen in different targets of the same host, it was suggested that the ER complex could be interpreted as a stimulatory or inhibitory signal at different sites. Experimental evidence suggesting this hypothesis, comes from studies in which it was shown that when human tumour cell lines were transplanted into athymic mice (immunologically tolerant), tamoxifen inhibited the growth of breast cancers, but stimulated cancers derived from the endometrium. Progress in understanding the molecular mechanisms involved has recently come from X-ray crystallography of SERM–ER complexes.

The crystallization of the ligand-binding domain of the ER with oestradiol and raloxifene has provided an important insight into the conformational changes that occur in the receptor. Oestradiol causes helix 12 to seal the ligand inside the hydrophobic pocket of the ligand-binding domain causing receptor activation through the binding of co-activators on the surface of helix 12 (Fig. 5A). By contrast, the binding of raloxifene prevents helix 12 from sealing the hydrophobic pocket and gene transcription cannot occur because co-activators cannot bind (Fig. 5B). In its basic form, this molecular model can be viewed as oestradiol binding in the jaws of a crocodile and then the jaws close to complete...
activation. Raloxifene acts like a stick in the jaws that prevents them closing.

The crystal structure also provides proof of the critical importance of a single amino acid (aspartate AA351) for the different oestrogen-like actions of the SERMs, raloxifene and tamoxifen. The alkylaminoethoxyside chain is the essential structural feature of non-steroidal anti-oestrogens. The distance between the nitrogen and the oxygen must be optimal, the conformations available to the side chain must not be restricted and the basicity of the nitrogen must be correct. The side chain of raloxifene has a very close interaction with AA351, the removal of the side chain results in an increase in oestrogenic properties. The discovery of an ER mutant (asp 351 tyr) in a tamoxifen-stimulated MCF-7 breast tumour and the finding that it can increase the oestrogenic properties of 4-hydroxytamoxifen, the active metabolite of tamoxifen, as well as the conversion of raloxifene from an anti-oestrogen to an oestrogen, is valuable biological proof that AA351 is important for the anti-oestrogenic activity of these specific compounds. This interaction, at the critical contact point of helix 3 and helix 12, prevents helix 12 from sealing the ligand into the binding pocket (Fig. 4B). Shiau et al demonstrated from the crystal structure of 4-hydroxytamoxifen and ER that there is a complex, but weak, interaction of the side chain with several amino acids including AA351. This subtle difference between the crystal structure of the raloxifene-ER complex and the 4-hydroxytamoxifen-ER complex may explain the promiscuous oestrogen-like nature of the 4-hydroxytamoxifen-ER complex. Recent experimental evidence illustrates the importance of the acidic charge at AA351 because substitution of glycine for aspartate retains anti-oestrogen properties for the ER complex but the oestrogen-like effects of tamoxifen disappear. It is proposed that this area could be a site for protein interaction involved in transcriptional regulation of genes.

The presence of co-activator and co-repressor proteins has been implicated in the construction of a transcription complex in target cells. The finding that an anti-oestrogen ER complex could become increasingly oestrogenic in different cell contexts raised the possibility that the differential distribution of co-activators or co-repressors could be responsible for changes in oestrogenicity between different tissues. The AF-2 region, in the LBD, is known to be repressed by tamoxifen and raloxifene but the AF-1 region is unaffected by tamoxifen binding. As a result, the shape of a particular complex of ligand and ER will be different for different drugs. Thus, co-activators could modulate oestrogenicity differentially in different target sites. The candidate proteins could, therefore, amplify the anti-oestrogen receptor complex into an oestrogenic complex. Alternatively, the anti-oestrogen receptor complex might recruit completely new proteins at a specific target site.
to induce or to suppress gene transcription. Only further basic research will be able to decipher all of the possible combinations. Nevertheless, if the molecular mechanism of SERMs could be understood, there is huge potential for targeted drug discovery. This promise is already becoming a reality with the emerging clinical data of tamoxifen and raloxifene.

Fig. 4 (A) Oestrogen binds to ER and helix 12 in the receptor folds across the oestrogen molecule. This rotation positions certain amino acids so that they can mesh with the co-activators needed to activate oestrogen-responsive genes. (B) When raloxifene binds to ER, this rotation does not occur. This change in shape prevents the receptor from interacting properly with co-activators (adapted from Brzozowski et al.)*
Tamoxifen as a preventive drug for breast cancer

Early animal experiments demonstrated that tamoxifen can prevent mammary carcinogenesis, but it has taken 20 years to demonstrate that breast cancer can be prevented by tamoxifen in woman. Tamoxifen is a relatively safe drug and the beneficial profile of oestrogen-like properties that maintain bone and decrease circulating LDL-cholesterol provides additional advantages for women who will not have an increase risk for osteoporosis and coronary heart disease during treatment.

The major concern about tamoxifen therapy has been the link with endometrial carcinoma. Tamoxifen does not cause endometrial carcinoma, but increases risk by 3-4-fold in postmenopausal women. However, the increase in the detection of endometrial cancer from 1 per 1000 women per year to 3 per 1000 women per year is consistent with the known rate of occult disease. Most importantly, the stage and grade of endometrial cancer observed in women taking tamoxifen is the same as the general population.

The fact that tamoxifen was already known to reduce the incidence of contralateral breast cancer made the drug the primary agent to test in high-risk women. The major clinical assessment of tamoxifen in North America was successful, so tamoxifen was approved in 1998 in the US for the reduction of risk in pre- and postmenopausal women with a high risk of breast cancer.

There are three studies (Fig. 5) that appear to provide some information about whether tamoxifen will or will not prevent breast cancer. However, it should be stressed that only the study completed by the National Surgical...
Adjuvant Breast and Bowel Project (NSABP) is a prospective randomized clinical trial with risk determined using a multiple risk factor evaluative profile\textsuperscript{28}.

The Royal Marsden Pilot Study was initially described as a pilot toxicology study that, subsequently, became a nation-wide clinical trial in Britain. A total of 2494 women in the pilot study were analyzed\textsuperscript{30} and the results did not demonstrate tamoxifen's efficacy as a preventative drug in high-risk women for breast cancer development. However, it is important to point out that the Marsden study was underpowered to detect a significant benefit for tamoxifen treated women\textsuperscript{17}.

The Italian Study was designed to determine whether tamoxifen was efficacious in preventing breast cancer in 20,000 young women. The study, however, only recruited and analyzed 5,408 hysterectomized women who were aged 35–70 years, and were planned to receive 20 mg of tamoxifen per day for up to 5 years\textsuperscript{31}. The critical short-coming of this trial was that the participants were not required to be at high risk for breast cancer development and less than 200 women completed the course of treatment. The incidence of breast cancer did not differ between the treatment and control groups. Nevertheless, women who were taking HRT had a higher incidence of breast cancer than those taking HRT and tamoxifen\textsuperscript{31}.

The NSABP clinical trial\textsuperscript{28} aimed to identify potential benefits of tamoxifen as a breast cancer preventive. The study recruited 13,388 high-risk participants between 35–75 years of age and analyzed the incidence of breast cancer. The result observed in this study established that the risk of breast cancer was reduced in the tamoxifen group by 50%. Incidence of ER positive tumours in the tamoxifen group demonstrated a reduction of 69% per year in comparison to the placebo group. In contrast, incidence of ER negative tumours did not change between the groups. This study also demonstrated that 5 years (69% reduction) of tamoxifen therapy is better than 1 year (33% reduction) for the prevention of invasive breast cancer.

The results of the NSABP prevention trial have established tamoxifen as the current standard care to reduce the risk of breast cancer in high-risk populations. As a result, all new drugs must be compared with tamoxifen if they are to be considered seriously as providing benefits as breast cancer preventives.

**Raloxifene concept to reality**

Raloxifene has strategically located phenolic groups that ensure high binding affinity for ER\textsuperscript{19}. Like tamoxifen, raloxifene behaves as a partial agonist in the uterus and an antagonist in the breast. Raloxifene inhibits
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the growth of DMBA-induced rat mammary cancer, but tamoxifen is more effective dose for dose. Raloxifene has a short biological half-life, so a larger dose than used for tamoxifen is necessary for clinical testing\textsuperscript{17}. Raloxifene maintains bone density in ovariectomized rats by reducing bone resorption\textsuperscript{32}. The effects on bone in animals appear to be equivalent to oestrogen treatment, but without increases in uterine weight. Preliminary studies in 251 postmenopausal women receiving raloxifene (200 or 600 mg daily) or premarin (0.625 mg daily) shows a positive effect (like oestrogen) of raloxifene related to the osteoporotic markers\textsuperscript{33}. Delmas et al\textsuperscript{34} showed that raloxifene (60 and 120 mg daily) increases bone density in the lumber spine and hip and raloxifene prevents fractures of the spine.

Raloxifene and its analogues are effective and potent inhibitors of the growth of breast cancer cell \textit{in vitro}, and also prevent mammary cancer in rats. Several different trials have been used to demonstrate the potential of raloxifene to prevent breast cancer. The largest trial, MORE (Multiple Outcomes of Raloxifene Evaluation) trial, is testing raloxifene (60 or 120 mg) against a placebo in 7704 postmenopausal women who had osteoporosis and no history of breast or endometrial cancer. Three-year findings from the MORE trial demonstrated that raloxifene reduces the risk of breast cancer (70\%) and may decrease the risk of endometrial cancer in postmenopausal women\textsuperscript{35}. The second database includes 10,553 women monitored for up to 3 years. The integrated data from these multiple double blind, randomized trials demonstrated that incident primary breast cancers are reduced (54\%) by raloxifene\textsuperscript{36,37}. Raloxifene reduces the incidence of ER positive cancer and has no effect on the incidence of ER negative breast cancer.

Overall, these preliminary clinical findings provide a rationale to test the worth of raloxifene to prevent breast cancer. The STAR (Study of Tamoxifen and Raloxifene) double-blind trial is recruiting 22,000 postmenopausal women to either daily tamoxifen (20 mg orally) or raloxifene (60 mg orally) therapy for 5 years. The primary goal of the trial is to establish the relative effectiveness of raloxifene compared to tamoxifen treatment in preventing invasive breast cancer and to determine the overall impact of the drugs on bone, coronary heart disease and endometrial cancer.

Raloxifene decreases total cholesterol because of a decline of LDL cholesterol\textsuperscript{38}. The effectiveness of raloxifene in reducing heart attacks is currently being addressed in the RUTH (Raloxifene Use for The Heart) trial. This trial is testing raloxifene (60 mg) against a placebo in 10,000 women at high risk for coronary disease. The results will be available in 5 years and, in addition, there will be further data on the incidence of breast and endometrial cancers.

Raloxifene has less oestrogenicity in the uterus than tamoxifen and in the laboratory raloxifene only increases the growth of human endometrial
carcinomas by about 50% of that noted with tamoxifen. In women, raloxifene unlike oestrogen does not increase endometrial thickness.

Overall raloxifene is advancing the goal of developing multifunctional drugs to prevent multiple diseases associated with ageing; however, it does not prevent vasomotor symptoms of the menopause.

Summary: multifunctional drugs

Tamoxifen is the endocrine treatment of choice for all stages of breast cancer. It is recommended as an adjuvant therapy for patients with node-positive and node-negative ER-positive disease. It is known that 5 years of adjuvant tamoxifen provides a superior survival advantage for women than shorter (1 or 2 years) adjuvant therapy. It is also clear from the Overview Analysis that longer duration of tamoxifen treatment reduces the risk of contralateral breast cancer. Based on a huge safety database and the recent results of the NSABP prevent trial, tamoxifen was approved by the Food and Drug Administration in 1998 for reduction of risk in premenopausal and postmenopausal women with a high risk of breast cancer. This was a landmark decision as it is the first time a drug has been proven to reduce the risk of any cancer. However, not all women who develop breast cancer have high levels of risk other than age.

In 1990, it was proposed that new drugs should be developed that could exploit the known oestrogen-like effects of tamoxifen on bones and lipids. The wide-spread use of the new SERMs to prevent osteoporosis and coronary heart disease would, as a side effect, prevent breast cancer in the general postmenopausal population. Raloxifene is the first drug to be developed that exploits the new SERM strategy.

Raloxifene can maintain bone density in rats and, as a result, the drug has been found to prevent osteoporosis in postmenopausal patients. Extensive clinical trials are underway to confirm the original hypothesis that multiple diseases can be prevented with SERMs in a broad range of populations at risk. The further development of novel agents to amplify the required actions of SERMs, but solve the requirement to have oestrogenic actions on menopause symptoms, will provide a menu of medicines to be used as preventives in the 21st century.

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