Definition of the molecular mechanism of action of tissue-selective oestrogen-receptor modulators

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Introduction
The steroid hormone oestrogen manifests its biological activity through specific high affinity receptors located within target cell nuclei [1]. Whereas it was previously considered that all the actions of this hormone occurred through a single oestrogen receptor (ER\(_2\)), which was biochemically identical in all cells, it has recently been determined that a second receptor for oestrogen exists in some target tissues [2]. This receptor, called ER\(_\beta\), to distinguish it from the original ER\(_2\), was originally cloned from rat prostate tissue, however, the human homologue has recently been cloned [3]. The biological significance of this second ER remains enigmatic as studies published to date have not yet identified a target tissue where ER\(_\beta\), but not ER\(_2\), is expressed [4,5]. However, studies in the rat hypothalamus indicate that there are some nuclei within this organ in which ER\(_\beta\) mRNA is dominant over ER\(_2\). Although the two ERs have significant differences in primary sequence, it is generally considered that their mechanism of action is similar (Figure 1) [5]. In the absence of ligand, ER resides in the nuclei of target cells in a transcriptionally inactive form associated with heat-shock proteins and other cellular chaperones [6]. The receptor is ‘activated’ upon interaction with ligand, an event which permits homodimerization and its interaction with high-affinity oestrogen response elements (EREs).

Abbreviations used: ER, oestrogen receptor; ERE, oestrogen response element; AF, activation function; IL-6, interleukin 6; NF-\(\kappa\)B, nuclear factor kappa B; SERM, selective oestrogen receptor modulators.

Located within target gene promoters [7,8]. Depending on the cellular and promoter context, the DNA-bound receptor can either positively or negatively regulate target gene transcription [9].

Redefining the terms ‘oestrogen’ and ‘anti-oestrogen’
In the classical models of ER-action, the role of the agonist oestradiol was considered to be that of a ‘switch’ which, upon binding to the receptor, converted it from a latent to a transcriptionally active form [10]. Anti-oestrogens therefore, by definition, functioned merely by competitively inhibiting the binding of oestradiol to its receptor. However, it has recently become clear that this model is too simple and does not describe the observed clinical and preclinical pharmacology of the known ER-ligands (Figure 2). Some of the most useful information in this regard has come from studies of the anti-oestrogen tamoxifen. In a landmark paper published in 1992, Love et al. unveiled what is now known as the ‘tamoxifen paradox’ [11]. Specifically, they reported that postmenopausal women receiving tamoxifen as adjuvant chemotherapy for breast cancer had significantly less bone loss in the lumbar spine than appropriately matched women who did not receive the drug [11]. This finding suggested that, at least in some contexts, tamoxifen was not opposing, but mimicking, the actions of oestrogen. These initial studies have been confirmed and extended by others. The clinical data supporting these conclusions are supported by a wealth of studies from preclinical models which, likewise, have demonstrated that tamoxifen can function as an antagonist, an agonist or a partial...
The mechanism of action of oestrogen is similar to that described for all members of the steroid receptor subfamily of nuclear receptors. It mediates its effects on target gene transcription via specific intracellular receptor proteins (SR) located within target cell nuclei. Upon interaction with its cognate ligand, the latent receptor becomes "activated". This event permits the displacement of heat-shock proteins (HSP), facilitates receptor dimerization and promotes the interaction of the receptor with specific response elements located within the regulatory regions of target gene promoters. At this location, depending on the cellular and promoter context, the ligand-activated receptor can interact with the general transcription machinery (solid) directly, or indirectly, through adaptor proteins. Ultimately, these interactions stabilize the transcription preinitiation complex and enhance RNA polymerase activity. Although several rounds of phosphorylation of the receptor have been shown to occur, its role in ER signalling has yet to be determined. This Figure has been reproduced with permission from [38].

Figure 1
The mechanism of action of the human ER

[Diagram showing the mechanism of action of the human ER]

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Figure 2
Chemical structures of the ER ligands discussed

- Estradiol
- Tamoxifen
- ICI 182780
- Raloxifene
- GW 5638

The molecular mechanism of action of cell-selective ER modulators

One of the most significant findings with respect to ER pharmacology was the demonstration that structure of an ER-ligand complex is dependent on the nature of the bound ligand [20,28]. For example, it has been shown, using differential sensitivity to proteases as an assay, that the structure of ER in the absence or presence of an agonist is different and both of these structures are distinct from that observed in the presence of tamoxifen [20]. It was proposed, as a consequence of this observation, that it was the ability of the cell to distinguish between these structures which explained the different biological activities of oestrogen and tamoxifen. A series of studies followed which appeared to explain how information was transferred from these structurally altered receptors to the transcription apparatus. Specifically, it was determined that ER contains two sequences which are required for transactivation: activation function-1 (AF-1) located within the amino terminus and ER-AF-2 contained within the carboxyl terminus of the receptor [22,29–32]. The function of these activation sequences was highly dependent on the cell context in which they were assayed [20,22,31]. In some cells, activation sequence alone was sufficient for maximal ER efficacy, whereas in others both were required. Importantly, it was determined that in any cell where AF-2 was required, tamoxifen functioned as a pure antagonist [20–22]. However, if AF-2 was not required, or was inactive, and AF-1 alone was sufficient to support ER transcriptional activity, then tamoxifen functioned as a partial ER agonist [21,22,33]. Thus, considering our data and those of others, we proposed that AF-1 alone is sufficient for ER transcriptional activity in the bone and uterus where tamoxifen functions as an agonist, whereas AF-2 or both AF species must be required in the breast where tamoxifen functions as an antagonist. It now appears that this model is over simplified. However, it introduced the concept that factors downstream of ER, and not the receptor itself, were the critical determinants of the cell-selective actions of ER ligands.

Recently, the complexity of ER pharmacology has expanded even further following the extension of these in vitro analyses to other ER ligands [20,22,34]. The most interesting studies were performed with raloxifene, a benzothio-
Recombinant Antibodies and Receptors as Reagents and Drugs

...anti-oestrogen which is in late phase 3 clinical trials for the treatment and prevention of osteoporosis, and a new anti-oestrogen GW5638, which we have developed in our laboratory in collaboration with Glaxo-Wellcome [34].

The hallmark of both of these drugs is that they, like tamoxifen, function as AF-2 antagonists and block ER activity in cell contexts where ER-AF-2 is required. However, unlike tamoxifen, these compounds do not possess AF-1 activity in any of the available in vitro systems [20,34]. Furthermore, when assayed in rats these compounds are as effective as tamoxifen and oestrogen in protecting against ovariectomy-induced bone loss, but they do not demonstrate oestrogenic activity in the uterus [16,34–36]. Thus, the relationship between AF-1 function and bone protection does not appear to hold although AF-1 activity does seem to track with agonist activity in the uterus. This suggests that classical ER agonist activity, that requiring either AF-1 or AF-2, is not required for ER action in bone [34]. Cumulatively, these in vitro studies have identified several mechanistically distinct ER ligands which, when tested in vivo, display a spectrum of activities in the reproductive system but which all appear to mimic oestrogen in the bone. Interestingly, all compounds which prevent bone resorption in an ER-dependent manner appear also to suppress serum cholesterol, indicating that the bone protective and cardioprotective actions of these compounds are occurring in a similar manner [26,34,35,37]. The challenge, therefore, is to process the available data and derive a unifying model to explain ER action in bone and the cardiovascular system.

In searching for some common feature(s) of these compounds, we have noted that they all display high-affinity interactions with ER, promote receptor dimerization and permit a tight interaction of the dimer with a classical ERE [20,38] (Table 1). Whether the ability of a compound to deliver ER to DNA is required or just tracks with ER agonist activity in bone remains to be determined. It is noteworthy, however, that the steroidal pure anti-oestrogens (represented by ICI182,780) are not bone-protective [39]. Originally, it was believed that these compounds inhibited receptor dimerization and blocked DNA binding [40]. It now appears that the pure anti-oestrogens do permit high-affinity interactions with DNA in most cells [20]. However, in some cells these compounds induce ER turnover by shuttling the protein to the lysosomes [41,42]. Thus, the failure of ICI182,780 to protect against bone loss may be a consequence of its ability to induce ER degradation in bone cells and may not be related to its inherent anti-oestrogenic activity. It is possible therefore, that pure anti-oestrogens which can deliver ER to DNA, but which do not induce turnover, would also be active in bone.

**Models which explain the mechanism of action of cell-selective oestrogens**

Analysis of the in vitro and in vivo pharmacology of ER ligands has provided considerable insight into the role of ER in bone. From these observations a series of different models have evolved which appear to encompass available information. Clearly, the mechanism by which ER acts in this organ is not identical to the manner by which it operates in the reproductive tissues. One of the most likely and appealing explanations for this differential pharmacology is that the general

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mechanism of action of ER in all cells is the same and requires the interaction of ER with specific ERs in target gene promoters. However, subtle differences in transcription factor pools within specific target cells can permit different ER–ligand complexes to exhibit different biological activities. Thus, although raloxifene and GW5638 do not display agonist activity in our reconstituted in vitro transcription systems under conditions where tamoxifen and oestrogen do, it is possible that, in cells or on target gene promoters relevant to bone protection, all bone-protective ER ligands may be equally effective in activating AF-1 or AF-2 and that all of these compounds are equally effective as agonists [20,34]. It is also possible that activation domains within the receptor, in addition to ER AF-1 and AF-2, may be involved in ER transcriptional activity in some cells. Both variations of this model suggest that, in the appropriate cell(s) within bone, oestrogen, tamoxifen, raloxifene and GW5638 are equally effective agonists. This hypothesis is supported by the recent studies of Yang et al. that demonstrated that in rats transforming growth factor β-3 mRNA was positively up-regulated in bone by oestrogen and raloxifene suggesting that compounds, classically defined as ER-antagonists, can in fact function as agonists on specific target genes in bone [36].

A second model for which there is a considerable amount of supporting preclinical data is that activated ER can inhibit the actions of a positive regulator of a gene, an event requiring activated receptor but not DNA binding. One example of this activity is the regulation of the cytokine interleukin-6 (IL-6) [43]. It has been shown recently that in mice ovariectomy leads to an increase in serum IL-6 levels with a concomitant increase in the relative number of osteoclasts to osteoblasts [44]. The link between this activity and bone was established when it was shown that: (i) the administration of IL-6-neutralizing antibodies was sufficient to inhibit osteoclast formation in primary bone cell cultures derived from neonatal murine calvaria [44]; (ii) that mice bearing a genetic deletion of the IL-6 gene did not demonstrate ovariectomy-induced bone loss [45]. Although other events, in addition to IL-6 up-regulation, are likely to be involved in osteoporosis, the observation that the IL-6 promoter is down-regulated upon administration of oestrogen makes it a useful system with which to study ER action. Recently, the molecular basis of ER regulation of the IL-6 promoter has been determined [46–48]. Specifically, it has been shown that both nuclear factor kappa B (NF-κB) and C/EBPβ are required for IL-6 promoter activity. Interestingly, ER, in the presence of oestradiol, physically interacts with both the rel homology domain of NF-κB and the b-Zip region of C/EBPβ, blocking their ability to interact with DNA and subsequently lowering the transcriptional rate of the IL-6 promoter [46] (Figure 3). In support of this model, it has been shown that tamoxifen and raloxifene function as partial ER agonists in this system, and like oestrogen, suppress IL-6 promoter activity, whereas the pure ER antagonist, RU58668, is inactive [48]. It will be interesting to determine if tissue-selective ER agonists can be developed which can function as complete ER agonists in this system. Thus, given what we know about the mechanism of the bone-protective ER modulators and considering the data on IL-6 regulation, it is possible that ‘classical’ ER agonist activity is not required for bone protection.

Summary and perspectives

The recent advances in our understanding of the molecular mechanism of action of ER agonists and antagonists has indicated that the pharmacology of these compounds is complex and has highlighted the fact that not all oestrogens are the same. These advances have led to the conclusion that ER does not merely change from an inactive to an active form upon binding a ligand but is quite malleable and can exist in several different induced conformations. Importantly, the transcription apparatus within a cell is configured so as to be able to distinguish between these different complexes. Although it is going to be difficult to tease out, this complexity has made it possible to develop selective oestrogen receptor modulators (SERM); compounds which act as oestrogens in some tissues but not in others. The first generation of SERMs are currently being evaluated in the clinic and, if approved, will provide women with an additional option for the treatment and prevention of osteoporosis and cardiovascular disease. These drugs will be particularly appealing to those women who are at high risk for breast cancer or for those who, for one reason or another, do not want to take oestrogen. The SERMs currently being evaluated represent the first generation of novel ER-mediated therapies for the treatment of osteoporosis. They are not the last word but do...
Recombinant Antibodies and Receptors as Reagents and Drugs

Figure 3
Oestrogen-mediated repression of the IL-6 promoter; a model for ERE-independent regulation of gene transcription

The IL-6 promoter is negatively regulated by oestrogen tamoxifen and raloxifene but not by pure antagonists [43, 44, 46-48]. It is proposed that, upon activation, ER can physically interact with either C/EBP/ and/or NFκB (p65/p50 complex) and inhibit their positive activity on this promoter [46]. Interestingly, this activity does not require a direct interaction of ER with DNA. From this model it is predicted that any compounds which can promote ER-activation but which do not decrease the expression level of ER will mimic the action of oestrogen in this system. This Figure has been reproduced with permission from [38].


Received 22 September 1997

Volume 26