Pergamon

Biochemical Pharmacology, Vol. 49, No. 11, pp. 1545–1549, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(94)00528-1

COMMENTARY

USE OF PURE ANTIOESTROGENS TO ELUCIDATE THE MODE OF ACTION OF OESTROGENS

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Key words: pure antioestrogen(s); oestrogen(s); oestradiol; pharmacology; uterus; bone

With the advent of molecular biological techniques enabling the detailed description of the primary structure of the ER† and subsequent analysis of its effector domains, it is easy to imagine that a combination of this information with the extensive literature on the physiology and biochemistry of oestrogen action would provide a more or less complete account of the mode of action of oestrogens. This article will provide a challenge to that view with particular emphasis on recent *in vivo* work with a novel class of oestrogen antagonists, the so-called pure antioestrogens, and will briefly review how these new agents are aiding studies of the molecular mode of action of oestrogens.

There are important practical consequences to further investigations of oestrogen action related to the role of these hormones in disease processes, in particular their role in the incidence and progression of breast cancer. Directly related to the latter is the current controversy surrounding the long-term use of antioestrogens, like tamoxifen, to prevent breast cancer [1, 2]. Tamoxifen is representative of the most-studied class of antioestrogens; typically, such agents are non-steroidal in structure and manifest complex pharmacology encompassing variable tissue-, cell- and gene-specific effects from oestrogen-like agonist actions to complete blockade of oestrogen action characteristic of pure antagonism [3, 4]. The balance of agonist and antagonist activities in tamoxifen may be beneficial in clinical use since antagonist activity is dominant for tumour regression, whereas agonist activity appears important for effects on bone and lipid metabolism [5, 6]. However, agonist activity expressed in other tissues may be less desirable, for example in the stimulation by tamoxifen of the endometrium. Variations in tissue sensitivity to tamoxifen should not be too surprising since it is well established that similar differences of end organ sensitivity to oestrogen also occur [7, 8].

Antioestrogens with partial agonist activity were, until recently, the only pharmacological tools available with which to investigate the mode of action of oestrogens. Clearly, the utility of such mixed agonist/antagonists is limited, since one is unable to probe the consequences of complete inhibition of oestrogen action. Similarly, in physiological studies, ablation or surgical removal of the ovaries does not achieve total oestrogen ablation since oestrogens may still be synthesized from androgen precursors of adrenal origin. The advent of molecules that bind ER with a high affinity without activating receptor signalling provided for the first time the opportunity to study in an unequivocal manner the consequences of a full blockade of oestrogen action. These agents, typified by ICI 164384 and ICI 182780, are pure antioestrogens since they block oestrogen action *in vitro* and *in vivo* in a concentration (dose)-dependent and complete manner, whereas in the absence of endogenous oestrogens the agents alone produce no oestrogen-like effects [9, 10].

Pharmacological characteristics of antioestrogens

In this section we shall consider studies of the *in vivo* actions of antioestrogens and the insights provided to the mode of action of oestrogens. The broad range of oestrogen physiology will be illustrated by considering effects on the uterus, on bone, and on the brain.

Uterus. The simplest demonstration of the difference between pure and partial agonist antioestrogens takes advantage of a classical test for oestrogenic activity, that is the measurement of uterotrophic activity. Harper and Walpole [11] first demonstrated that tamoxifen increases uterine weight in immature female rats in a dose-dependent manner without attaining the same maximum effect as oestradiol, thus defining tamoxifen as a partial agonist. Administration of tamoxifen together with oestradiol provided a partial but incomplete antagonism of the uterotrophic action of oestradiol [11]. Other non-steroidal antioestrogens with differing intrinsic uterotrophic activity differ in the maximum degree to which they inhibit the trophic action of oestradiol, reflecting the net balance between inhibitory and stimulatory activities [3]. ICI 164384 and ICI 182780 do not stimulate the immature uterus, and they fully block the uterotrophic action of oestradiol. Co-administration of tamoxifen and either of the pure antagonists demonstrated dose-dependent and complete inhibition of the uterotrophic action of tamoxifen [9, 10]. As well as

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†Abbreviations: ER, oestrogen receptor; IGF-I, insulin-like growth factor-I; EGF, epidermal growth factor; and IGFBPs, IGF binding proteins.

providing a graphic demonstration of the difference between partial and pure antioestrogens, the latter experiment provides a compelling case that oestradiol, tamoxifen and the novel agents all act through the ER. Similar conclusions may be drawn from comparative studies of the effects of oestradiol and antioestrogens on the growth of mammary ducts in rats. Tamoxifen and the benzothiophene antioestrogens LY 117018 and LY 139481 promoted full ductal development equivalent to that supported by oestradiol, whereas ICI 164384 alone had no effect and completely antagonized the effect of tamoxifen [12]. Thus, at the organ level in rats, tamoxifen is a partial agonist for uterine growth and a full agonist for mammary duct growth, whereas ICI 164384 is a full antagonist in both tissues.

Since the consequences of tamoxifen stimulation of the endometrium are of intense current interest, it is worth examining in more detail. More intensive investigations of tamoxifen effects on the rat uterus revealed differential actions on epithelial and stromal components of the organ. The increased wet weight in tamoxifen-treated animals is largely due to hypertrophy of the luminal epithelium with little change in the stroma and myometrium [4]. The hypertrophic effect of tamoxifen on the epithelium was similar to that of oestradiol. This hypertrophic effect was not associated with any change in thymidine incorporation or cell division typical of uterine response to oestradiol stimulation [4]. These studies demonstrate that both qualitative and quantitative cell-specific actions underlie differential tissue responses to tamoxifen and account for its partial agonist action on the uterus. The molecular mechanisms underlying these cell-specific actions remain to be described.

Bone. The importance of oestrogens in bone metabolism is illustrated by osteoporosis, a major cause of morbidity and mortality in postmenopausal women precipitated by the loss of oestrogen following cessation of ovarian function. Osteoporosis can be prevented by oestrogen replacement therapy. The process of bone loss and its prevention with oestradiol can be modelled in ovariectomized rats [13]. Data in the literature show that tamoxifen can act on bone as antagonist and partial agonist, rather like its effects on the uterus [14, 15] and is, therefore, of limited utility in studying the mechanism of ER-mediated oestrogen action on bone. However, the oestrogenic effect of tamoxifen on bone in postmenopausal breast cancer patients is considered a particularly valuable adjunct to its antitumour action [5, 6].

Initial observations with ICI 182780, that long-term treatment of normal adult female rats did not affect bone density whereas the uterus underwent an ovariectomy-like regression, were counterintuitive [16]. The reasons for this apparent difference between the bone and uterus response to ICI 182780 are unclear but may reflect a differential threshold of sensitivity to oestrogen of the two tissues. More recent histiomorphometric studies have demonstrated an ovariectomy-like action of ICI 182780 on tibial cancellous bone volume in intact rats, consistent with its expected antioestrogenic action [17]. The discrepancy between the two studies may reflect the fact that cancellous bone volume represents a small (~20%) proportion of total bone. Unlike ovariectomy or oestrogen treatment, ICI 182780 had no effect on longitudinal or periosteal tibial growth. As well as preventing bone loss by blocking resorption, oestrogen stimulates bone formation in ovariectomized rats, an effect that was also prevented by ICI 182780 [17]. Thus, the effects of ICI 182780 on bone appear to be confined to the cancellous bone fraction and are consistent with blockade of ER-mediated bone resorption and formation.

Recent studies with ZM 189154, a non-steroidal pure antioestrogen, further illustrated the differential sensitivity of bone mineral metabolism to oestrogens compared with other tissues in the intact rat [18]. Daily doses of 0.6 mg/kg blocked ovulation, 2 mg/kg achieved maximum uterine atrophy without affecting bone density or growth rate, and 10 mg/kg reduced bone density but to a lesser extent than ovariectomy. Differential tissue actions have been reported for the non-steroidal antioestrogen raloxifene, which has little stimulatory action on the uterus [19] but prevents bone loss in ovariectomized rats [20]. Raloxifene demonstrates a bone-selective agonist effect and may, therefore, have advantages over conventional oestrogens in postmenopausal hormone replacement therapy.

Brain. The most important role of oestrogens in brain is feedback control of the menstrual cycle mediated by the control of gonadotropin secretion by ER in the hypothalamic-preoptic area of the brain and pituitary. In rats, oestrogens also profoundly influence food intake, body weight and body fat stores as well as controlling oestrous behaviour, effects that have both a central and peripheral component. Studies with non-steroidal anti-oestrogens have shown the anticipated mix of antagonist and agonist actions. For example, tamoxifen mimics the effects of oestradiol in reducing food intake, body weight and luteinizing hormone secretion of ovariectomized rats but can block oestrogen-induced oestrous behaviour and ovulation [9, 21]. Comparative studies of the effects of tamoxifen and ICI 164384 in intact rats showed that in contrast to the potent oestrogen-like actions of tamoxifen on body weight and serum LH concentration (both decreased), ICI 164384 at effective antiuterotropic doses did not appear to affect either parameter [9]. Similar observations were reported with ICI 182780 and ZM 189154 [10, 18]. One interpretation of these data is that the pure antioestrogens failed to penetrate the blood–brain barrier and, therefore, did not block centrally mediated actions of oestrogen on food intake and gonadotropin secretion. Confirmation of the peripherally selective action of ICI 182780 emerged from studies of the tissue uptake of oestradiol in rats pretreated with either tamoxifen or ICI 182780 [22]. Following injection of [^{3}H]oestradiol, specific uptake was readily measured in uterus, pituitary gland, brain and adipose tissue. Tamoxifen blocked uptake by all tissues, whereas ICI 182780 blocked all tissue uptake apart from that in the brain. *In vitro* receptor binding studies confirmed that ICI 182780 competes more effectively than tamoxifen with oestradiol for

binding to brain-derived ER, confirming that the absence of *in vivo* blockade of brain ER in ICI 182780-treated rats must be due to the inability of ICI 182780 to penetrate the blood–brain barrier [22]. Wade *et al.* [22] went on to show that ICI 182780 partially attenuated the oestrogenic effects of oestradiol and tamoxifen on body composition and sexual behaviour in ovariectomized rats in support of the thesis that these actions are mediated, in part, by non-neural target tissues.

Mode of action studies

The regulation of cellular events in oestrogen target tissues is mediated by oestrogen binding to intracellular receptors, which function as ligand-responsive transcription factors of oestrogen-regulated genes. Oestradiol binding to ER initiates a complex series of events including dissociation of heat shock proteins from ER, ER dimerization and binding to discrete DNA sequences termed oestrogen response elements in the regulatory regions of target genes [23]. It is assumed that the high-affinity ligand–ER interaction drives a series of conformational changes to facilitate these events, but these changes have not been characterized at the molecular level. Functional mapping of effector domains has defined core regions of the ER, which mediate binding to oestradiol, heat shock proteins and DNA, and regions essential for dimerization and transcriptional activation. Two major independent transcriptional activation domains of the ER have been defined, one region at the amino terminus and one in the carboxy terminus of ER, designated AF1 and 2, respectively [24]. It is assumed that these regions of the ER influence, either directly or indirectly, the assembly and efficient operation of the transcriptional complex. Since receptors do not function in the same way in all cells, it is further assumed that the particular spectrum of gene response to oestrogen is determined by both the differentiated nature of the cell and promotor context [23, 25].

Studies with antioestrogens have been useful in illuminating the role of AFs. Following an initial observation that 4-hydroxytamoxifen, unlike oestradiol, does not induce AF2 activity, Berry *et al.* [26] demonstrated that this ligand can activate AF1 and that the activity of AF1 is promotor context dependent. Thus, in individual cells and potentially for individual genes the relative strength of AF1 and 2 could determine the partial agonist activity of antioestrogens. Where AF2 is dominant, tamoxifen would be expected to act as an antagonist. The partial agonist activity observed *in vivo* would correspondingly depend on the activity of AF1 [26, 27]. It was also shown that ICI 164384 is unable to activate either AF1 or 2, consistent with its pure antagonist activity [26]. Independent confirmation of the importance of AF1 for tissue-specific agonist activity of non-steroidal antioestrogens has been published recently [28].

Oestrogen-induced cell growth

An enduring conundrum of oestrogen action is whether the hormone is a direct or an indirect mitogen, that is whether cell division in oestrogen-responsive tissues, such as the breast and endometrium, involves obligatory induction of, or interaction with, other growth factors. This question has been investigated intensively using cultured human breast cancer cells, and although it is clear that these cells secrete and respond to a number of growth factors and that oestrogen regulates the production of some of these factors, no clear answer has emerged [29]. Recent studies of interactions between oestrogen and IGF-I with tamoxifen and the pure antioestrogens provide some further insight into this issue. Both oestradiol and IGF-I alone stimulate the growth of breast cancer cells *in vitro* and added together have a powerful synergistic action, suggesting the presence of independent but interacting mitogenic pathways [30, 31]. The mitogenic action of IGF-I can be attenuated by anti-IGF-I receptor antibodies but also by antioestrogen treatment [30]. The latter inhibitory effect of antioestrogens can be seen in the complete absence of oestradiol but does require the presence of ER. This implies that the mitogenic action of IGF-I is mediated, in part, through the ER. Similar conclusions may be drawn from studies with other breast cell mitogens, for example EGF [30, 32]. It is not clear how the oestrogen- and growth factor-mediated mechanisms interact, but the "cross-talk" may involve modulation by growth factor-induced changes in phosphorylation of the ER itself or of other protein components of the promoter-transcriptional complex. Transcriptional activation of the ER in the absence of oestradiol can be induced by dopamine [33], EGF [34], cAMP [35] and IGF-I [35, 36]; ER-induced transcriptional activation in all cases was blocked by the pure antioestrogen ICI 164384 [33–36]. Protein kinase (PK) activators acting through PKA or PKC have been shown to act synergistically with oestradiol to activate ER [37]. The correlation between the phosphorylation status of ER and its transcriptional activity is, however, not simple because ICI 164384 was as effective as oestradiol in increasing ER phosphorylation but completely ineffective in transcriptional activation. ICI 164384 also did not block IGF-I-stimulated ER phosphorylation [35].

Physiological interactions between oestrogen and growth factor-mediated mitogenesis have also been studied using the uterus as a model system. In the rodent uterus, oestrogen stimulation of the expression of IGF-I and EGF is consistent with the view that these factors may mediate tissue growth. EGF administration to ovariectomized mice mimics oestrogen-induced DNA synthesis in the uterus, and this induction is blocked by ICI 164384 [38, 39]. The blockade of EGF action by ICI 164384 may be explained by the rapid loss of ER from the uterus following treatment with ICI 164384 [40]. A similar depletion of ER in ICI 164384-treated cells *in vitro* [41, 42] may account for the inhibition of growth factor stimulated cell division discussed above. Loss of ER reflects increased turnover of the protein [41] and disrupted nucleocytoplasmic shuttling of ER [43] in the presence of pure antioestrogens.

Like oestradiol, tamoxifen stimulates expression of the IGF-I gene in the rat uterus, consistent with its uterotrophic action, whereas ICI 182780 has a powerful inhibitory effect [44]. This stimulatory

effect of tamoxifen in the uterus was distinct from effects in the liver and lung where inhibition of IGF-I gene expression was reported [45] and from the reduced serum concentration of IGF-I in patients treated with tamoxifen [46]. The reason for this differential organ response of the IGF-I gene to tamoxifen is not understood but may be clinically important since uterine induction of IGF-I could contribute to the trophic alterations of the endometrium reported in some postmenopausal breast cancer patients treated with tamoxifen. The contrasting actions of tamoxifen also extend to effects on IGFBPs, which attenuate the mitogenic action of IGF-I. Tamoxifen and oestradiol suppress uterine IGFBP3 expression, but ICI 182780 significantly stimulates expression [47]. These contrasts between the trophic actions of tamoxifen and the inhibitory effects of ICI 182780 on the IGF-I/IGFBP system in the uterus have a parallel in tamoxifen-resistant human breast cancer cells. In tamoxifen-sensitive human breast cancer cells, tamoxifen decreases IGF-I binding [48] and both antioestrogens significantly increase IGFBP3 [49], whereas in cells selected for acquired resistance to tamoxifen, IGF-I binding was increased and tamoxifen-stimulated growth was dependent on IGF-I [50]. The growth of these tamoxifen-resistant cells could be inhibited by pure antioestrogen treatment [50]. Similarly in another resistant human breast cancer cell line, tamoxifen stimulated growth, and expression of oestrogen-regulated genes remained sensitive to inhibition by ICI 164384 and ICI 182780 [51].

Conclusions

The differential tissue and gene responses to oestrogens and antioestrogens discussed here have important practical implications in therapeutics, particularly with respect to the differences between partial agonists like tamoxifen compared with pure antagonists like ICI 164384 and ICI 182780. The latter compounds may hold out particular advantages in breast cancer therapy, particularly in the fact that tumours resistant to tamoxifen may not be cross-resistant to pure antioestrogens [16], and unlike tamoxifen pure antioestrogens do not stimulate the uterus, but those advantages must be balanced against the beneficial actions of the oestrogenic activity of tamoxifen on the bone. The recent description of non-steroidal (anti)oestrogens selective for bone [20, 52] may presage a new era of drug discovery directed to tissue- or gene-selective agents. Further mechanistic insights must be expected from the search for ER-associated proteins in the transcriptional complex whose expression might be tissue- or differentiation-stage specific [53, 54] and from continued analysis of the complex interactions between growth factor and steroid-induced gene expression.

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