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Alternative mechanisms of action of anti-oestrogens

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Summary

The molecular mechanism of action of anti-oestrogens such as tamoxifen appears to be a complex mixture of antagonism of the mitogenic action of oestradiol at the level of the oestrogen receptor, plus a range of other activities from enzyme inhibition to growth factor modulation. This article will concentrate on two specific areas: 1) the inhibition of protein kinase C and calmodulin-dependent cAMP phosphodiesterase; and 2) the regulation by tamoxifen of peptide regulators of breast cancer epithelial cell growth such as insulin-like growth factor I (IGF I) and transforming growth factor beta (TGF-β). The elucidation of these mechanisms is potentially important in the treatment and chemoprevention of breast cancer — the quantitative contribution of each individual mechanism of the overall antineoplastic action of anti-oestrogens is central to developing new and possibly more effective anti-oestrogens and optimizing strategies for their use.

Oestrogens, like the other members of the steroid hormone family, elicit their biological activities through their cognate receptor as a ligand-activated transcription enhancer [1–3]. Anti-oestrogens, as their name implies, are antagonists of oestrogen function which serve to competitively interact with the oestrogen receptor (ER) in the nucleus of oestrogen responsive cells [4]. The net result of this interaction is to abrogate the biological function of oestradiol. There are a number of known anti-oestrogens of both steroidal and non-steroidal origin, but this review will focus on the prototype anti-oestrogen as far as breast cancer therapy is concerned, the triphenylethylene tamoxifen. There are a number of excellent reviews dealing with the structure and transcriptional activation of ER as well as the species, organ, and cell specific pharmacology of tamoxifen [5]. This article will concentrate on two specific areas: i) the inhibition of protein kinase C and calmodulin-dependent cAMP phosphodiesterase; ii) the regulation by tamoxifen of peptide regulators of breast cancer epithelial cell growth such as insulin-like growth factor 1 and transforming growth factor beta.

The search for alternative mechanisms of action of anti-oestrogens was originally stimulated by the finding that the growth inhibition of human breast cancer cells *in vitro* with micromolar concentrations of tamoxifen and its active metabolites 4-hydroxytamoxifen and N-desmethyltamoxifen could not be completely reversed by competition with large concentrations of oestradiol [6, 7]. These findings suggested that pharmacological levels of these compounds may be acting at a site other than the conventional ER. Research in this area was stimulated by the discovery of a novel low affinity microsomal

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binding protein specific for the triphenylethylene class of anti-oestrogens, termed the anti-oestrogen binding site (AEBS) [8]. However the universal distribution of AEBS, coupled with a failure to purify the protein/s to homogeneity, has led to the virtual abandonment of this line of research in recent years.

The original demonstration by O'Brian and colleagues that micromolar concentrations of tamoxifen inhibit protein kinase C activity were seen as being of great potential significance [9]. Protein kinase C (PKC) is a calcium and phospholipid-dependent protein kinase which can be activated by diverse agents such as tumour promoting phorbol esters and by diacylglycerol, a product of cellular inositol phospholipid hydrolysis [10]. Tamoxifen, 4-hydroxytamoxifen, and N-desmethyltamoxifen were shown to have IC50 levels for the phosphotransferase activity of PKC of 8–25 μM, as did the structurally related oestrogen agonist clomiphene [11, 12]. However, the calcium and phospholipid-independent phosphorylation of protamine sulphate also catalysed by PKC was not inhibited by any of the triphenylethylenes, suggesting that these compounds do not directly interact with the active site of the enzyme [9, 11].

Further experiments showed that the triphenyethylene-induced inhibition of PKC activity could be partially titrated by increasing the concentration of phospholipid in the phosphotransferase assays, suggesting that these drugs were interacting with the phospholipid rather then with PKC directly. The pharmacological importance of this enzyme inhibition was also demonstrated by the observation that the rank order for the growth inhibition of MCF-7 breast cancer cells by tamoxifen, 4-hydroxytamoxifen, and N-desmethyltamoxifen is the same as their potency in the inhibition of binding of radiolabelled phospholipid precursors in murine C3H/10T1/2 cells [9].

Another key enzyme known to be inhibited by tamoxifen and structurally related analogues is calmodulin-dependent cAMP phosphodiesterase (CDP) [13]. A structure-activity relationship has been established for the inhibition of CDP, and has indicated that tamoxifen binds to, and interferes with the action of, calmodulin, rather than to the phosphodiesterase itself [14, 15]. The oestrogen-irreversible cytotoxic effects of tamoxifen and a range of analogues correlates with the calmodulin inhibition seen in ER positive cell lines, but not in heterologous cell lines such as L1210 and Walker cells. This suggests that ER and calmodulin are involved in the same signalling pathway responsible for hormone-regulated cellular proliferation. This conclusion is supported by the finding that the calmodulin antagonist calmidazolium and tamoxifen block the MCF-7 cell cycle at the same point [16].

The notion that anti-oestrogens such as tamoxifen influenced the cellular microenvironment in a breast tumour via the stromal component of the tumour was stimulated by the counter intuitive clinical findings of the Nolvadex adjuvant tamoxifen trial (NATO) [17]. The results of this trial suggested that the clinical benefits of tamoxifen treatment as an adjuvant to surgical treatment were quite independent of the ER status of the primary tumour. The results of this trial were confirmed by the Scottish MRC trial [18] and the Early Breast Cancer Triallists' Collaborative Group [19]. Taken together these studies demonstrate that ER status does not predict a subgroup of breast cancer patients that will not respond to adjuvant tamoxifen treatment. At much the same time there were reports of pure mesenchymal tumours which showed spectacular clinical responses to tamoxifen and its chlorinated analogue toremifene [20].

In our early studies we used two human foetal fibroblast strains as an *in vitro* model for the stromal component of breast cancer [21]. These were chosen after a report demonstrating the foetal characteristics of fibroblasts taken from patients with a strong family history of breast cancer [22, 23]. These cell strains were found to lack ER by ligand binding, immunocytochemistry, and Northern analysis, although they responded to the addition of 0.5–1 μM tamoxifen or toremifene with an increased secretion of biologically active transforming growth factor beta (TGF-β), particularly the prototype peptide TGF-β1, with little or no involvement of TGF-β2 [21]. Metabolic labelling experiments followed by immunoprecipitation of TGF-β1 showed the *de novo* synthesis of this peptide in response to tamoxifen, although Northern analysis did not

demonstrate an increase in TGF-β1 mRNA, suggesting a post-transcriptional mechanism of action of tamoxifen in these cells. Similar post-transcriptional mechanisms were also postulated for the induction of TGF-β2 by retinoic acid in keratinocytes [24] and the induction of TGF-β1 and TGF-β2 by tamoxifen and synthetic progestins in breast cancer cells [25, 26].

However, such *in vitro* findings do not always correlate well with the situation *in vivo*, so we recently examined the regulation of TGF-β secretion by tamoxifen in breast cancer patients [27]. In this immunohistochemical study the secretion of each of the three mammalian TGF-β isoforms was examined in breast tumour sections taken from patients with histologically proven invasive ductal carcinoma prior to and after three months treatment with tamoxifen. These samples were obtained from the Royal Marsden Hospital primary medical therapy trial of tamoxifen [28]. Polyclonal antibodies to both the intracellular (LC) and extracellular (CC) forms of TGF-β1 as well as antibodies to TGF-β2 and TGF-β3 were used, with pre- and post-treatment sections stained simultaneously to avoid any artefactual differences.

The data obtained from these sections demonstrated a consistent increase in the extracellular form of TGF-β1, which is specifically recognised by the CC antibody. This staining was confined to the stromal extracellular matrix, with no evidence for immunoreactivity in or around the epithelial tumour foci [27]. Interestingly, the increase in CC-immunoreactive extracellular TGF-β1 did not correlate with the expression of ER, as it was evident in both ER positive and negative tumours. There was very little staining for TGF-β2 or TGF-β2 in any of the tumour sections and they were unaffected by three months of tamoxifen treatment. The only evidence for LC-reactive intracellular TGF-β1 was seen in the peritumoural fibroblasts after treatment with tamoxifen, suggesting these cells as the likely site of synthesis, and supporting our previous observations *in vitro*.

As well as these observed increases in negative growth regulators after tamoxifen treatment, there is now compelling evidence to suggest a tamoxifen-mediated reduction in positive growth regulators, such as insulin-like growth factors I and II. These are potent mitogens for breast cancer cells [29–31] which express type 1 IGF receptors for which both IGF I and II are functional ligands [32]. Levels of this receptor correlate loosely with levels of oestrogen receptor (ER) in cells [33], but ER negative cells still express significant levels of IGF receptor [34]. However, breast cancer cells *in vitro* generally do not express IGF I or IGF II mRNA, suggesting that these cells may participate in a paracrine rather than an autocrine pathway [35, 36]. This is supported by evidence that fibroblasts derived from breast tumour tissue express high levels of IGF I and II mRNA, with fibroblasts from benign tumours overexpressing IGF I mRNA whilst those from malignant tumours overexpress IGF II [37]. Therefore IGF's produced by fibroblasts may act upon neighbouring epithelial cells in a positive paracrine manner. Pollak and co-workers have demonstrated that tamoxifen treatment *in vivo* is associated with a reduction in serum IGF I levels [38] and proposed that as IGF I is not only a mitogen for breast cancer cells, but also stimulates the motility and metastatic potential of malignant cells [39, 40], the reduction in circulating IGF I might be an important component of tamoxifen's mode of action *in vivo*. Other experiments have suggested that tamoxifen may mediate this effect through a reduction in growth hormone secretion [41].

A recent *in vivo* study has addressed the relevance of the growth hormone reduction on circulating IGF I levels by examining IGF I expression in tissues which are common sites for breast cancer metastases (liver and lung). By using hypophysectomised rats with and without recombinant growth hormone replacement therapy, these authors demonstrated that the induction of IGF I was partly independent of pituitary function as significant reductions in IGF I expression after tamoxifen treatment were still seen in the hypophysectomised animals receiving growth hormone replacement [42]. This study did not allow any conclusions to be drawn as to whether the reduction in IGF I expression occurred in the parenchymal or stromal elements of these tissues.

The molecular mechanism of action of anti-oestrogens such as tamoxifen appears to be a complex

mixture of antagonism of the mitogenic action of oestradiol at the level of ER as well as a range of other activities from enzyme inhibition to growth factor modulation. The elucidation of these mechanisms is of potential importance in the treatment and chemoprevention of breast cancer. The move towards developing pure steroidal anti-oestrogens, devoid of any of the other beneficial effects of tamoxifen, continues apace, and determination of the quantitative contribution of each individual mechanism to the overall antineoplastic actions of anti-oestrogens is central to development of optimal pharmacological strategies.

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