Tamoxifen metabolism as a mechanism for resistance

by C K Osborne

INTRODUCTION

Breast cancer progression is mediated in part by steroid hormones, such as estrogen, which stimulate target tissues via their interaction with specific cellular receptors. As a result, breast cancer therapies have been designed to either reduce or block the effects of estrogen. Tamoxifen, a nonsteroidal antiestrogen, is the most frequently used drug in breast cancer treatment today. The drug inhibits breast cancer growth by competitively blocking the estrogen receptor (ER) and thereby inhibiting estrogen-induced growth. Although tamoxifen is effective in delaying recurrence in and prolonging survival of breast cancer patients in the adjuvant setting, and in inducing remission in patients with advanced breast cancer, its use is limited by the inevitable development of tamoxifen resistance (Early Breast Cancer Trialists’ Collaborative Group 1992, Saez & Osborne 1989). Furthermore, 50% of patients fail to respond to the drug initially despite the presence of ERs, indicating that de novo resistance is also important clinically. The mechanisms for either intrinsic or acquired tamoxifen resistance are unknown, but they are probably multifactorial (Osborne & Fuqua 1994).

Understanding the mechanisms by which breast tumors develop resistance to tamoxifen would provide clues for new strategies for preventing or reversing the emergence of resistant cells. Since the major mechanism of action of tamoxifen is competitive blockade of ERs, selection of an ER-negative clone from an initially heterogeneous tumor cell population would result, eventually, in a tumor resistant both to estrogen and to tamoxifen. A secondary mechanism by which tamoxifen may inhibit breast cancer growth is through inhibition of the production of autocrine growth factors that are normally secreted in response to estrogen (Arteaga & Osborne 1991). These growth factors include transforming growth factor alpha and insulin-like growth factor-II, as well as members of the fibroblast growth factor family. Tamoxifen also induces the secretion of transforming growth factor beta, a potential growth inhibitor; thus, downregulation of the expression of growth factors or upregulation of the expression of growth inhibitors by tamoxifen could contribute to its growth suppression. Alterations in the expression of these growth factors or growth inhibitors, or of their specific cell membrane receptors, could provide the tumor cell with sufficient growth stimulation to overcome the tamoxifen block, resulting in tamoxifen resistance. Cross talk between polypeptide growth factor pathways and ER pathways could also theoretically result in tamoxifen resistance. It has been shown that increasing the level of cellular cyclic AMP alters the cellular response to tamoxifen, converting it from an antiestrogen to a weak estrogen agonist (Fujimoto & Katzenellenbogen 1994). Altered ER function could explain tamoxifen resistance in other patients either through mutations in the ER or through the altered expression of accessory proteins that interact with the ER and modify the transcriptional signal generated by ligands binding to it. Another possible mechanism for resistance involves the cytoplasmic antiestrogen-binding sites whose function is not yet clearly understood. Overexpression of these sites could theoretically work as a ‘sponge’ to soak up tamoxifen molecules, thereby preventing their binding to ERs. Finally, altered systemic metabolism of tamoxifen, or altered uptake or metabolism of tamoxifen by the tumor itself, could also theoretically contribute to tamoxifen resistance in some situations.
CLINICAL STUDIES IN TAMOXIFEN RESISTANCE

Clinical studies in tamoxifen-resistant patients have provided clues for mechanisms by which acquired resistance may develop. ER is lost from tumors in some patients treated with tamoxifen, presumably by the selection of an ER-negative tumor cell clone. ER loss would then result in an estrogen-independent tumor refractory to tamoxifen. Some patients with tamoxifen resistance do develop resistance to all forms of endocrine therapy via selection of an ER-negative tumor cell clone. We have recently reported a series of patients with acquired tamoxifen resistance in whom tumor ERs and progesterone receptors (PgRs) were measured both by ligand binding and by immunohistochemical assays to circumvent the problem of the receptor occupancy by the drug (Encarnacion et al. 1993). More than 60% of tumors continued to express ER and/or PgR even while progressing in the face of tamoxifen. Thus, although ER negativity may account for some cases of resistance, mechanisms of resistance other than receptor loss are more common.

If patients’ tumors remain ER positive after tamoxifen resistance develops, one would expect that these tumors might have retained their sensitivity to estrogen and would thus respond to other endocrine treatments. Clinical experience demonstrates, in fact, that patients who have initially responded to tamoxifen, and who later develop tumor progression, frequently respond to second-line or third-line endocrine therapies (Saez & Osborne 1989). Thus, acquired tamoxifen resistance does not necessarily indicate global hormonal unresponsiveness, but rather selective resistance to tamoxifen itself. Although it has not been studied systematically, anecdotal experience indicates that some patients with tamoxifen resistance do respond to a rechallenge with the drug after an interval in which they receive other treatments. Furthermore, patients who receive tamoxifen adjuvant therapy and whose tumors then later recur will not infrequently respond to a rechallenge with the drug. This suggests that tamoxifen resistance in some cases may not be a permanent phenotype but may be reversible when the drug is stopped. Anecdotes also suggest that some patients may respond to an increase in the tamoxifen dose after having developed progression with a lower-dose schedule. Finally, similar to reports of patients treated with high-dose estrogen therapy, some patients who have responded to tamoxifen will have a withdrawal response when the drug is stopped at the time of tumor progression (Taylor et al. 1986). The prolonged serum and tumor half-lives of tamoxifen make it difficult for clinicians to withhold alternative therapy while waiting for a withdrawal response to the drug, but 20-30% of such patients will respond. These data suggest that in some patients with acquired resistance, tamoxifen may actually be stimulating tumor growth when tumor progression develops.

Two previously published trials also suggest that tamoxifen resistance in some patients may be due to the acquired ability of the tumor to be stimulated rather than inhibited by tamoxifen (Pritchard et al. 1980, Hoogstraten et al. 1984). In both of these studies, premenopausal women with advanced breast cancer were treated initially with tamoxifen and then with second-line ovarian ablation after progression had occurred on tamoxifen. In one of the studies, a secondary response to ovarian ablation was common in patients who had previously responded to tamoxifen (Pritchard et al. 1980). However, in the other study opposite results were obtained and no patients responded to second-line ovarian ablation (Hoogstraten et al. 1984). In the second study, tamoxifen therapy was continued after surgical ovariectomy, whereas in the first study tamoxifen treatment was stopped. Secondary response to ovarian ablation would not be expected in the second study if tamoxifen itself was behaving like an estrogen agonist to stimulate tumor growth. Just how tamoxifen-stimulated tumor growth evolves is not yet clear.

LABORATORY MODEL OF TAMOXIFEN-STIMULATED GROWTH

We have developed an in vivo experimental model of tamoxifen resistance in which MCF-7 human breast cancer cells are inoculated subcutaneously into athymic mice (Osborne et al. 1987, Osborne et al. 1991). Tamoxifen treatment of these mice suppresses tumor growth for 4-6 months, and then tumor growth resumes despite continued treatment. Tamoxifen resistance in this case is not due to the emergence of
a receptor-negative clone or to alterations in serum levels of tamoxifen or of its major metabolites. Transplantation of fragments of these tamoxifen-resistant tumors into fresh mice demonstrates that their growth has not become estrogen independent but in fact is now stimulated by tamoxifen as well as by estrogen. Similar data have been reported by others (Gottardis & Jordan 1988). These data suggest that one form of acquired tamoxifen resistance may be due to the ability of the tumor cells to be stimulated rather than inhibited by tamoxifen. Since certain metabolites of tamoxifen have estrogenic properties, we first investigated pharmacologic explanations for tamoxifen-stimulated growth.

**Tamoxifen metabolism in tamoxifen-resistant tumors**

To investigate potential mechanisms for the tamoxifen-stimulated tumor phenotype, we first compared levels of tamoxifen and several of its metabolites both in serum and in tumor extracts (Osborne et al. 1991). We found no differences in the levels of tamoxifen or of its major metabolites in the serum from mice with tamoxifen-stimulated compared with tamoxifen-inhibited tumors. However, when we examined the tumors themselves, we found that extracts from tamoxifen-stimulated tumors had on average tenfold lower tamoxifen concentrations than did extracts from tamoxifen-sensitive tumors. The major metabolites of tamoxifen are n-desmethyl-tamoxifen and trans-4-hydroxytamoxifen. The latter is a potent antiestrogen which binds to the estrogen receptor with much greater affinity than does the parent drug itself. We also found reduced concentrations of these metabolites in tamoxifen-stimulated tumors; however, there was a relative increase in the **cis** isomer compared with the **trans** isomer of 4-hydroxytamoxifen. **Cis** 4-hydroxytamoxifen is a very weak antiestrogen compared with the **trans** isomer. Both in whole tumor as well as in nuclear extracts, there was a relative abundance of the **cis** isomer in tamoxifen-stimulated tumors. In other words, in these tumors there was an accumulation of a very weak antiestrogen at the expense of a very potent one.

We do not yet have an explanation for the reduced concentrations of tamoxifen and its metabolites in these tumors. The fact that serum levels of tamoxifen from mice with these tamoxifen-stimulated tumors remain normal while tumor levels are markedly reduced, suggests the possibility of an efflux pump that reduces net uptake of tamoxifen by the tumor. We do know that p-glycoprotein, an efflux pump that is known to bind to tamoxifen, is not over-expressed in these tumors.

To confirm the data from our experimental model, we also analyzed metabolites of tamoxifen in patients with tamoxifen resistance (Osborne et al. 1992). Extracts of tumors from these patients showed a similar pattern. Tamoxifen concentrations were reduced in the tumors of patients with acquired resistance and there was an increased ratio of **cis** to **trans** 4-hydroxytamoxifen. A more recent study with larger numbers of patients has confirmed that tumors with acquired resistance tend to have lower levels of tamoxifen (Johnston et al. 1993).

However, a reduction in tamoxifen concentration in the tumor and a relative increase in the **cis** isomer of 4-hydroxytamoxifen would not explain tamoxifen-stimulated tumor growth. Therefore, we next investigated the possibility that accumulation of estrogenic metabolites of tamoxifen, such as metabolite E and bisphenol, might account for the development of tamoxifen-stimulated tumor growth. With the aid of HPLC and mass spectrometry we identified both of these metabolites in tumors from our experimental model as well as in tumor specimens from patients on tamoxifen. This lends further support to the hypothesis that one potential mechanism for tamoxifen resistance is a reduction in intracellular tamoxifen concentration together with isomerization and metabolism of the parent drug to less potent antiestrogens, or to frankly estrogenic metabolites, resulting in stimulation of tumor growth (Wiebe et al. 1992). To further investigate this hypothesis, we quantified the levels of tamoxifen and its estrogenic metabolites in tumors from our nude-mouse model, and we employed analogues of tamoxifen that are resistant to isomerization and metabolism to determine the importance of tamoxifen metabolism in this experimental model of acquired resistance.

Similar to our findings in our previous report, we found a ten- to fifteenfold reduction in tamoxifen concentrations in extracts from tamoxifen-stimulated tumors (Osborne et al. 1994). Other metabolites, including n-desmethyla-
moxifen and the cis and trans isomers of 4-hydroxytamo-xifen, were also reduced in these tumors. Again, the cis to trans ratio of 4-hydroxytamo-xifen was higher in the tamoxifen-stimulated tumors. Metabolite E, the estrogenic metabolite lacking the dimethylaminooxy side chain, was present both in tamoxifen-inhibited and in tamoxifen-stimulated tumors. However, the concentration of metabolite E relative to tamoxifen itself, or relative to the antiestrogenic metabolite trans 4-hydroxytamo-xifen, was distinctly different in tamoxifen-stimulated tumors. Although the absolute level of metabolite E was reduced in these tumors, it was relatively more abundant compared with the parent drug or with the other metabolites. The ratio of tamoxifen to metabolite E in tamoxifen-inhibited tumors was 132:1 whereas that in tamoxifen-stimulated tumors was only 20:1. These data suggested the possibility that there would be sufficient metabolite E in tamoxifen-stimulated tumors to reverse the tamoxifen-mediated inhibition of tumor growth.

The effect of non-isomerizable analogues

If isomerization of tamoxifen and/or conversion to estrogenic metabolites are important for tamoxifen-stimulated growth, then tamoxifen analogues resistant to these metabolite conversions would not be expected to stimulate tumor growth. To investigate isomerization, we used two non-isomerizable, 7-membered-ring tamoxifen analogues fixed in the trans position. When fragments from a tamoxifen-stimulated tumor were transplanted into mice, both tamoxifen and estrogen stimulated tumor growth. No tumors formed in mice receiving no hormonal treatment. Toremifene, a triphenylethylene anti-estrogen similar to tamoxifen, also stimulated tumor growth. Interestingly, both the dimethyl and diethyl fixed-ring analogues had a similar stimulatory effect on tumor growth. Finally, nafoxidine, another nonsteroidal antiestrogen structurally different from tamoxifen, was just as potent as the other analogues in promoting growth of these tumors. Thus, isomerization of tamoxifen and its metabolites is not responsible for the evolution of tamoxifen-stimulated growth in this model.

The effect of a deoxytamoxifen analogue

Metabolite E and bisphenol are estrogenic metabolites of tamoxifen formed by cleavage of the dimethyl aminoethoxy side chain. By the elimination of the oxygen atom and the presence of a carbon:carbon bond, a deoxytamoxifen analogue relatively resistant to cleavage of this side chain was formed. When mice transplanted with tamoxifen-stimulated tumors were treated with the deoxytamoxifen analogue, tumor-growth stimulation identical to that seen with tamoxifen treatment was observed. HPLC analysis of tumor extracts revealed no evidence for conversion of this analogue to metabolite E. Thus the deoxytamoxifen analogue, which is resistant to side-chain cleavage, was still capable of stimulating growth of these tumors.

Effect of pure steroidal antiestrogens

The pure steroidal antiestrogen ICI 182,780 has no estrogen-agonist activity in experimental models and is structurally distinct from tamoxifen. This antiestrogen not only failed to stimulate growth of tamoxifen-stimulated tumors when used alone in our nude-mouse model, but it also inhibited the stimulatory effects both of estrogen and of tamoxifen. These data indicate that tamoxifen-stimulated tumor growth is mediated through the ER. In addition, by virtue of a different mechanism of action, pure steroidal antiestrogens may be useful in reversing this form of tamoxifen resistance. ICI 182,780 is now being found to have activity in the clinical setting in tamoxifen-resistant patients (DeFriend et al. 1994).

DUAL AGONIST/ANTAGONIST EFFECTS OF TAMOXIFEN

If tamoxifen-stimulated tumor growth were due to conversion of tamoxifen to a pure estrogen agonist, then combinations of tamoxifen with estrogen might be expected to have an additive effect. In transplant experiments, however, when tamoxifen was combined with estrogen, antagonistic rather than additive properties were observed (Osborne et al. 1994). Thus, tamoxifen in this setting has a dual effect, being capable of stimulating tumor growth when
used alone (agonist activity) but still able to antagonize estrogen when the drugs are used together.

The results of the tamoxifen-analogue experiments suggest that tamoxifen metabolism is unlikely to be responsible for the phenomenon of tamoxifen-stimulated growth in this model system. Whether the reduced concentrations of tamoxifen in tamoxifen-stimulated tumors are somehow related to this mechanism of tamoxifen resistance remains unknown. However, preliminary experiments with mice treated with low concentrations of tamoxifen, to mimic the lower tumor levels seen after the development of tamoxifen resistance, indicate that these low concentrations are not capable of stimulating tumor growth. Whether reduced tumor-tamoxifen concentrations are simply a marker of resistance or are related mechanistically to the development of resistance requires further study.

**ALTERED ER FUNCTION AS A MECHANISM FOR TAMOXIFEN RESISTANCE**

In our current efforts we are focusing on ER function as a mechanism for tamoxifen-stimulated growth. ERs contain several functional domains. Transcriptional activation functions are located both in the A/B region and in the E region of the receptor, which also serves as the hormone-binding domain. Mutations in the receptor might render it nonfunctional or they might even alter the transcriptional activating activity leading to tamoxifen resistance. The transcriptional activating activity in the A/B region of ERs (AF-1) is constitutive and is active even when the receptor is bound by tamoxifen. Mutations in this region, or the presence of other auxiliary proteins that interact with the ER, could potentially modify transcription leading to increased activity of AF-1 and to tamoxifen-stimulated growth. By cloning and sequence analysis, we have not yet been able to identify mutations either in the A/B region or in the hormone-binding domain of ERs extracted from our tamoxifen-stimulated tumors. Thus, the ER itself seems to be normal in these tumors. We are currently investigating the hypothesis that altered expression of accessory proteins that affect transcriptional activation through the ER might contribute to tamoxifen-stimulated growth, and we are now searching for ER-interacting proteins that might be differentially expressed in tamoxifen-stimulated tumors.

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**REFERENCES**


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