Anti-Cancer Drugs 1995, **6**, pp. 498–507

Review paper

Possible mechanisms in the emergence of tamoxifen-resistant breast cancer

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Tamoxifen (TAM), a non-steroidal antiestrogen, is the endocrine treatment of choice for all stages of breast cancer. However, despite a favorable initial response to therapy, most tumors will eventually exhibit TAM resistance resulting in disease recurrence. Several mechanisms of TAM resistance have been proposed, yet a single distinct mechanism has not been identified. We will systematically consider the following steps of the estrogen receptor (ER)-mediated signal transduction pathway to identify possible sites of alteration leading to tamoxifen-resistance: (1) ligand metabolism and availability, (2) loss or mutation of the ER, (3) defects in ER post-translational modification, and (4) alteration of the estrogen response element (ERE). In particular, the ERE will be discussed as a position in the signal transduction pathway with considerable potential, if altered, to confer TAM resistance.

Key words: Breast cancer, resistance, tamoxifen.

Introduction

Tamoxifen (TAM), a non-steroidal antiestrogen, has proven to be an effective drug in the treatment of both pre- and postmenopausal women with all stages of breast cancer.[1,2] A favorable response to TAM therapy can be expected in 70–80% of patients with estrogen receptor (ER)/progesterone receptor (PR) positive advanced breast cancer.[3,4] Moreover, in postmenopausal women with early stage disease, adjuvant TAM treatment was shown to prolong disease-free and overall survival.[1,4] TAM is a cytostatic agent which exerts its antiestrogenic activity primarily by binding to the steroid binding domain of the ER and blocking estradiol (E_2) action. In the breast, TAM exhibits antiestrogenic activity,[5] whereas in other estrogen target tissues, it acts as a partial agonist.[6,7] Despite the initial favorable response to TAM therapy, eventually tumors become refractory to treatment resulting in disease recurrence. Often these tumors will subsequently respond to alternative hormone therapy or chemotherapeutic regimens. In some instances, resistance occurs due to loss of ER^+ cells in the tumor; however, in most cases expression of the ER is maintained.[8]

The ER is a member of the family of steroid binding receptors which function as transcription factors. The pathway by which the ER mediates ligand induced changes in gene expression is illustrated in Figure 1. Initially E_2 diffuses through the cell membrane and interacts with the ER in the nucleus. The E_2/ER complex dimerizes, binds to the DNA at the estrogen response element (ERE) and transcriptional activation occurs resulting in the expression of estrogen responsive genes. In the presence of TAM, the sequence of events is similar. However, although the TAM/ER complex does not prevent binding to the ERE, it does appear to prevent transcriptional activation. [9]

In deciphering the mechanism of TAM resistance, each step of the signal transduction pathway should be considered as a putative target site of alteration resulting in the misinterpretation of the signal. Some mechanisms that have been explored but will not be addressed in this review include changes in autocrine and paracrine dynamics affecting the expression of growth factors, the role of antiestrogen binding sites, and the possible involvement of a drug efflux pump.[2,13] Mechanisms we will consider include (Figure 2): (1) ligand metabolism and availability, (2) loss or mutation of the ER, (3) defects in the post-translational modification of the ER, (4) alterations in the levels or function of other transcription factors or ER-associated proteins, and (5)

This work was supported by RO1-CA 56143 and a generous grant from the Susan G Komen Breast Cancer Research Foundation, Dallas, Texas.

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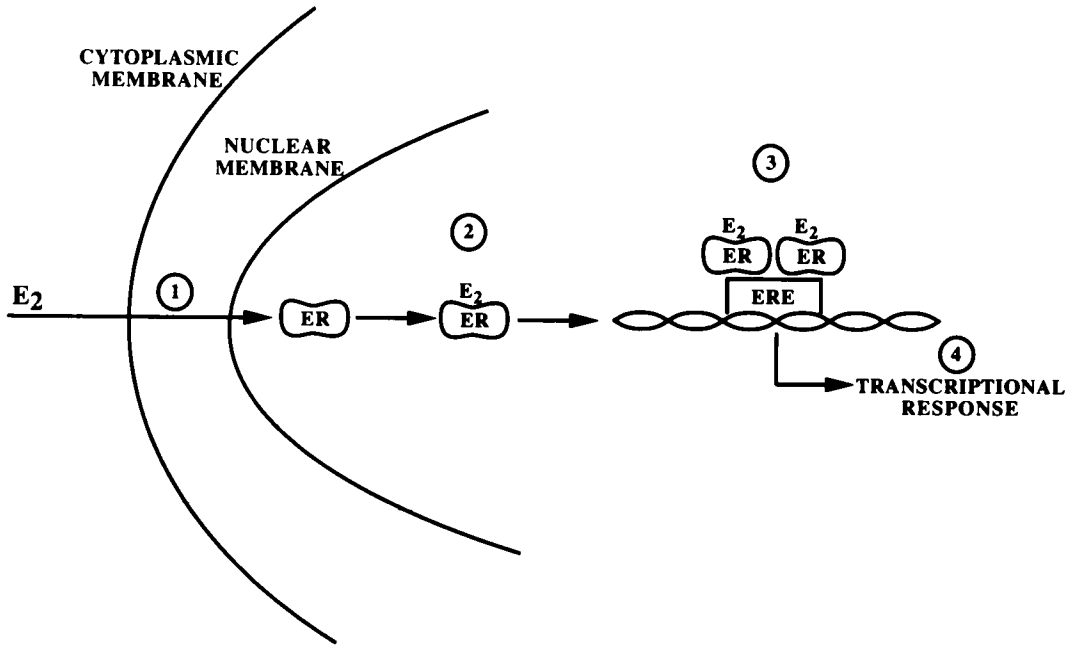

Figure 1. The ER-mediated signal transduction pathway mediated by E_2. 1. E_2 diffuses through the cytoplasmic and nuclear membrane. 2. E_2 complexes with the ER. 3. The ER/ligand complex binds to the ERE as a homodimer. 4. A transcriptional response is elicited.

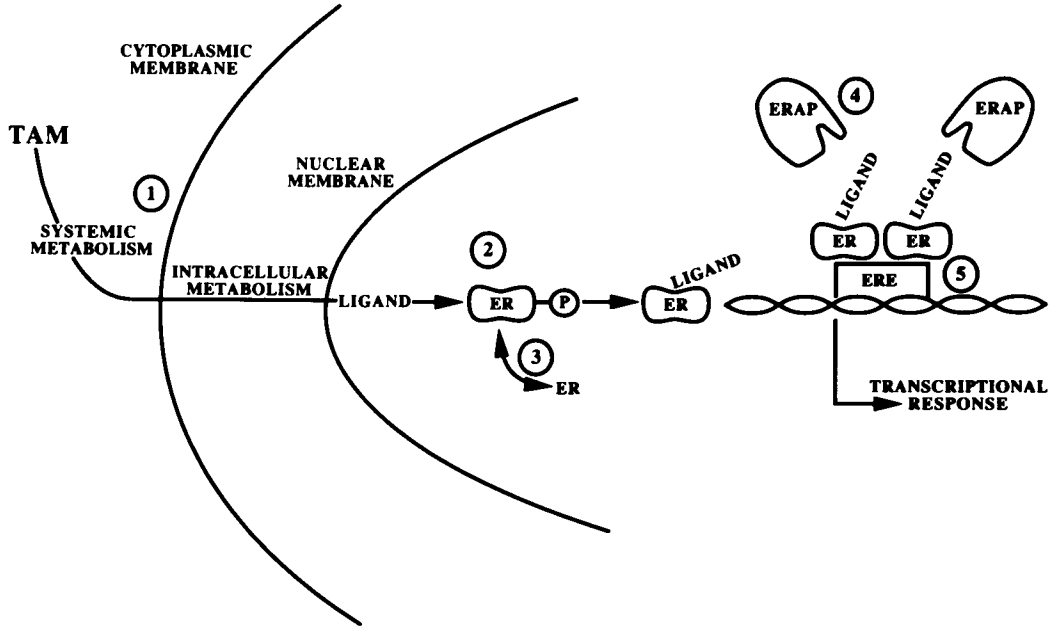

Figure 2. Potential sites of alteration of the ER-mediated signal transduction pathway resulting in TAM resistance. 1. Metabolism of TAM to estrogenic compounds. 2. Loss or mutation of the ER. 3. Aberrant post-translational modification of the ER. 4. Alteration of other transcription factors or ER associated proteins (ERAP). 5. Alteration of the ERE. The abbreviations used are the same as in Figure 1.

alteration of the ERE. We will briefly address the first two mechanisms as each of these have been summarized elsewhere.[10-13] New information regarding the phosphorylation of the ER and the identification of specific ER-associated proteins leads us to consider lesions in these steps of the pathway. In this review we wish to emphasize the ERE as a position in the signal transduction pathway with considerable potential to confer TAM resistance. This latter step in the pathway may be key to our understanding not only of TAM resistance, but may explain the tissue-specific pharmacology of TAM.

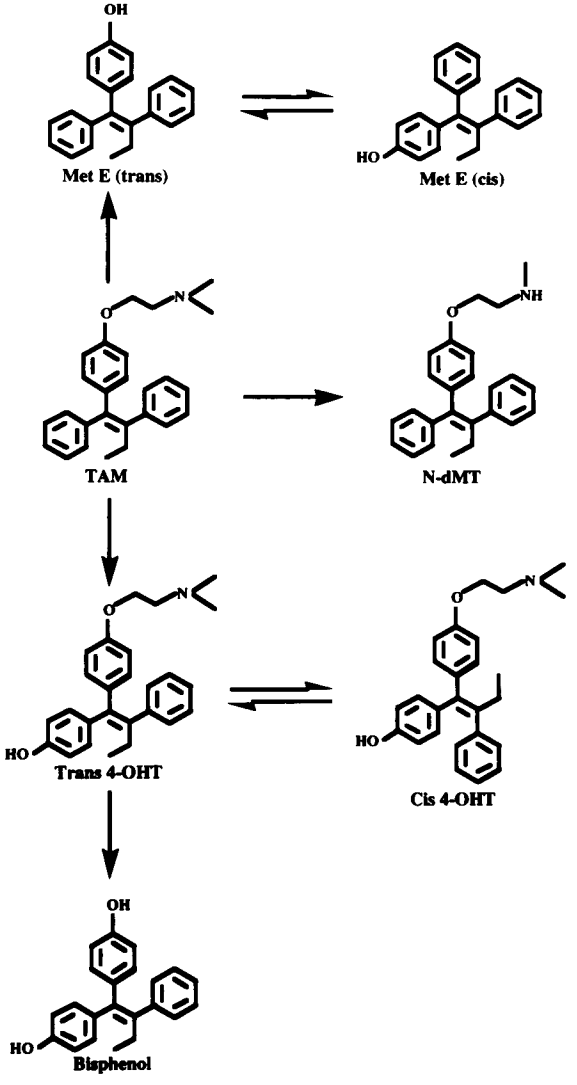

Figure 3. Potential ligands produced from TAM metabolism.

Ligand metabolism

TAM, a triphenylethylene compound, undergoes metabolic conversion to two main metabolites, 4-hydroxyTAM (4-OHT) and *N*-desmethylTAM (*N*-dMT) (Figure 3). Although 4-OHT is a minor metabolite, it is a potent antiestrogen that binds to the ER with an affinity comparable to E_2, whereas *N*-dMT, the major metabolite of TAM, is a weak antiestrogen. The *trans* form of TAM is stable in solution; however, 4-OHT is less stable and may isomerize to the *cis* form, a less potent antiestrogen.[14-16] TAM may also be metabolized to two estrogenic compounds, metabolite E (Met E) and bisphenol.[16,17] Therefore it has been suggested that intratumoral accumulation of TAM metabolites that are either less potent antiestrogens or are estrogenic may lead to TAM-resistant tumor growth.

This mechanism of TAM resistance has been explored by quantitating the levels of TAM and the various metabolites in TAM-stimulated and -inhibited tumors. Osborne *et al.*[18] report that TAM-stimulated tumors have significantly reduced levels of TAM compared with TAM-inhibited tumors. In addition, a relative increase in the ratio of *cis/trans* 4-OHT was found along with the accumulation of the estrogenic Met E.[18-20] However, Johnstone *et al.*[21] demonstrated that although ER^- tumors accumulate TAM and its metabolites much more slowly compared to ER^+ tumors, the steady-state concentration of drug achieved was similar for both ER^- and ER^+ tumors. Similarly Wolf *et al.*[22] were unable to detect significant differences in the intratumoral concentrations of TAM between TAM-stimulated and TAM-inhibited MCF-7 tumors in ovariectomized athymic mice. Nor did they find the estrogenic Met E in serum or in tumors. To address the question of whether the isomerization reaction is necessary for the development of acquired TAM resistance, non-isomerizable fixed-ring analogs were employed.[12,22] A fixed-ring TAM analog incapable of forming the potent estrogenic Met E compound was found to be equally capable of supporting TAM-stimulated MCF-7 tumor growth as TAM.[22] Similarly, a deoxyTAM analog used to eliminate the possibility of side-chain cleavage, thereby preventing the production of Met E or bisphenol, was also found to be comparable to TAM in stimulating tumor growth.[12] This evidence suggests that the isomerization of TAM to estrogenic or less potent antiestrogenic metabolites is not sufficient to explain the emergence of TAM-stimulated tumor growth and therefore alternative mechanisms must be considered.

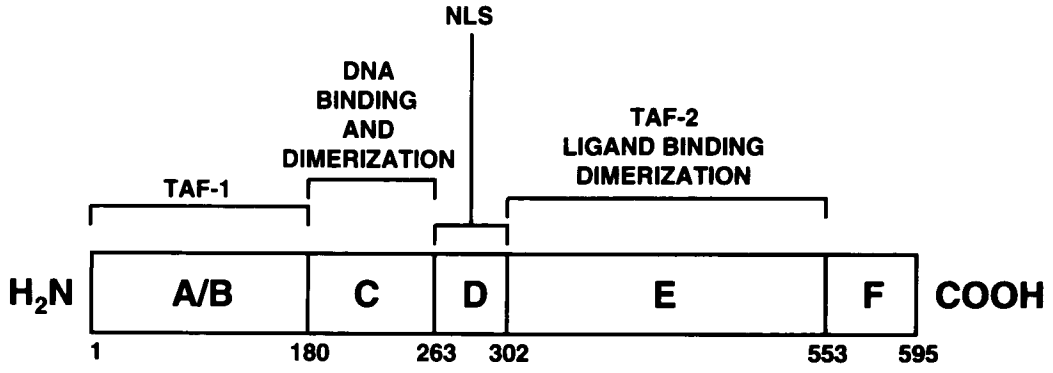

Figure 4. Structural and functional organization of the ER. TAF, transcriptional activation function; NLS, nuclear localization signal

Elimination or mutation of the ER

The mechanism of antiestrogen action is primarily by competition with E_2 for the hormone binding site of the ER. The result is the formation of a complex that is capable of interacting with the ERE, yet incapable of activating transcription.[9] Therefore, the functional inactivation of the ER by mutation is a likely mechanism of acquired TAM resistance. The ER is comprised of five functional domains, subdivided into regions A/B, C, D, E and F (Figure 4).[23] Regions C and E, which are highly conserved in human and chicken, correspond to the DNA and hormone binding domains, respectively. The A/B and E regions each contain transcriptional activation functions, TAF-1 and TAF-2. Specific ER splice variants, particularly in the hormone-binding domain, have been shown to result in dominant-positive receptors that constitutively activate transcription in the absence of a ligand. Using site-directed mutagenesis to create specific amino acid changes in the hormone binding domain has been shown to effect ligand binding affinity of the receptor, DNA binding, as well as transcriptional transactivation.[24–26] Specific mutations in the DNA- and ligand-binding domain of the ER can cause an antiestrogen to transmit an agonistic rather than an antagonistic signal. Jiang *et al.*[27,28] demonstrated that a single point mutation which substitutes a valine for a glycine at codon 400 in the hormone binding domain of the ER caused enhanced estrogenic activity in response to 4-OHT and other antiestrogens when stably transfected to MDA-MB231 ER^- human breast cancer cells. Therefore, potentially, if TAM resistant tumors have acquired such ER mutations, this mechanism of resistance should be easily detected.

Several investigators have searched for ER variants in breast cancer cell lines and breast tumor specimens, and although specific examples of ER mutations, deletions, transitions and RNA splice variants have been described in the literature,[29–33] it does not appear that mutation of the ER is the principle mechanism of acquired TAM resistance. Recently Karnik *et al.*[34] screened eight exons of the ER cDNA from 20 TAM-resistant and 20 TAM-sensitive breast cancer tissue specimens using single strand conformational polymorphism (SSCP). They concluded that mutations in the ER are rare since only two mutations were found, a single base pair deletion and a 42 base pair replacement in exon 6. Similarly, Watts *et al.*[32] analyzed 37 ER^- and ER^+ breast tumor biopsies and reported no evidence of changes affecting ER function and/or structure at the gene or mRNA level, and only one instance of gene amplification.

Based on our initial description of TAM-stimulated MCF-7 tumors in athymic mice,[35] we have developed a model to examine the possibility that TAM-resistant tumors may in fact be TAM-stimulated.[36] MCF-7 human breast cancer cells grown in athymic mice to produce solid tumors were chronically treated with TAM which resulted in the production of a TAM-stimulated phenotype. Upon characterization of three TAM-stimulated tumors, one tumor contained a single point mutation within the ER resulting in the replacement of a tyrosine for an aspartate at amino acid position 351. This mutation resulted in altered pharmacological response toward the fixed-ring 4-OHT analog from an antiestrogen to an estrogen.[37] This is the only report of a single base pair mutation of an ER derived from a TAM-stimulated human breast tumor exhibiting altered pharmacology towards TAM. However, since the remaining TAM-stimulated tumors examined contained wild-type ER, this suggests that other resistance mechanisms must be available to permit tumor growth.

Therefore, based on the inability to detect a significant frequency of ER mutations in human breast cancer tumors, other mechanisms are likely to be involved in the emergence of TAM-resistant or -stimulated growth.

Post-translational modification of the ER

It has been reported that phosphorylation of steroid hormone receptors may mediate hormone binding, DNA binding as well as transcriptional activation.[38-40] Recent evidence suggests that specific phosphorylation of at least four serine residues located in the A/B N-terminal region of the ER is induced by E_2, 4-OHT, the pure antiestrogen ICI 164,384, as well as activators of protein kinase A and C (PKA and PKC).[41–44] There appears to be cell-type-dependent phosphorylation of specific ER residues, serine 167 is the major phosphorylation site in MCF-7 cells,[43] while in COS-1 cells serines 104, 106 and 118 are phosphorylated.[44] A basal phosphorylation site located on tyrosine 537 within the hormone binding domain was recently identified in MCF-7 cells which appears to be independent of E_2 binding.[45] Various protein kinases including PKC, PKA, casein kinase and the *src* family kinases have been implicated in mediating these phosphorylation reactions.[43–45]

Therefore, TAM resistance may arise by alteration of the phosphorylation pattern required to affect appropriate transcriptional activation. The lesion may reside within the protein kinase(s) itself, resulting in aberrant phosphorylation of the ER. It is interesting to note that TAM is reported to be a specific inhibitor of at least one of the candidate protein kinases, PKC.[46,47] If this inhibitory activity actually occurs *in vivo*, TAM would in addition to competing with E_2 for binding to the ER, also presumably reduce phosphorylation of the ER and attenuate transcriptional activation. If PKC acquires a mutation which prevents TAM's inhibitory activity, inappropriate activation of estrogen responsive genes may occur. However, a recent report by Lahooti *et al.*[48] indicates that the presence of E_2 or 4-OHT generate similar phosphopeptide maps of the ER, suggesting that TAM does not inhibit ER phosphorylation. Therefore, it remains to be determined whether a defect in the phosphorylation of the ER may lead to TAM resistance.

Activation of the PKA pathway has been shown to increase the agonist activity of the TAM/ER complex using certain promoter–reporter constructs containing two EREs.[49] The transcriptional activity of the antiestrogen/ER complex was shown to increase by 20–75% that of E_2 by raising the intracellular cAMP levels or by transfection of expression vectors containing PKA catalytic subunits. This suggests that cross-talk between the cAMP and ER-dependent signal transduction pathways may exist. Therefore, increased cAMP levels may lead to the development of tamoxifen-stimulated tumor growth.

Alteration in other transcription factors

Although the precise mechanism remains unknown, the interaction of the ER/ligand complex with the ERE results in the expression or repression of the corresponding estrogen responsive genes. Upon binding of the ER/ligand complex to the ERE, which may be located 5′, 3′ or within the estrogen responsive gene, RNA polymerase II is directed to efficiently initiate transcription at the promoter. The ER contains two activation domains, TAF-1, located in the N-terminal domain can constitutively activate transcription when bound to DNA, whereas TAF-2 resides within the hormone binding domain and only activates transcription when bound by E_2 or an E_2 agonist.[50]

An abundance of information exists in the literature describing the synergistic interaction of various basal transcription factors such as Fos and Jun, TFIIB, CTF/NF-I with members of the steroid hormone receptor superfamily affecting transcriptional activation.[51–54] There are a number of recent reports which identify several proteins that specifically associate with the ER/ERE complex and influence transcriptional activation.[55–58] Therefore a possible mechanism of TAM resistance may involve alteration(s) of specific receptor-associated proteins which may be required for efficient transcriptional activation.

Halachmi *et al.*[57] reported the isolation of a 160 kDa estrogen receptor-associated protein, ERAP160, which exhibited E_2-dependent binding to the ER, whereas the pure antiestrogens ICI 164,384 and ICI 182,780 as well as 4-OHT were unable to induce the ERAP160/ER association. This protein was isolated by affinity chromatography with a fusion protein consisting of the hormone binding domain including the TAF-2 region of the ER fused to glutathione-S-transferase. This technique was also employed by Cavailes *et al.*[56] who reported the isolation of three proteins of 160, 140 and 80 kDa which also exhibited direct association with the ER in the presence of estrogen, but

not in the presence of 4-OHT or pure antiestrogens. Furthermore, mutations introduced within the hormone binding domain also abolished binding of these proteins. Cavailes *et al.*[56] suggest that hormone binding induces a conformational change resulting in the exposure of an amphipathic α-helical region located in the hormone binding domain of the ER that may mediate protein–protein interactions.

Similarly, Landel *et al.*[58] isolated four receptor-associated proteins, a 70 kDa protein identified as a heat shock protein (hsp70), a 55 kDa protein related to the protein disulfide isomerase family, and 48 and 45 kDa proteins with unknown identities. It appears that association of these proteins with the ER/ERE complex is uneffected by treatment with E_2, 4-OHT or the pure antiestrogen ICI 182,780 prior to complex isolation using DNA chromatography. However, isolation of the complexes using H222 immunoaffinity chromatography in the absence of an ERE resulted in the dissociation of hsp70 from the ER following treatment with E_2 or 4-OHT, whereas in the absence of ligand or the presence of ICI 182,780, dissociation of hsp70 did not occur. The authors suggest that the hsp70 interaction is destablized in the absence of a specific DNA target, whereas the hsp70 interaction may stabilize the ER/ERE complex similar to the stabilizing effect observed with the glucocorticoid receptor/glucocorticoid response element complex.[59] In addition to hsp70, two additional members of the heat shock protein (hsp) family, hsp90 and hsp56, have been shown to associate with steroid receptors.[60,61]

We are only beginning to appreciate the complex assembly of components that are required to effect transcriptional activation of the many estrogen responsive genes. If one considers the ER-associated proteins already identified and the proteins that have yet to be isolated, it seems that a defect in any one of these proteins may alter the signal at the ERE. The result may be the interpretation of a TAM/ER complex binding to the ERE as an estrogenic signal.

Modification of the ERE

In considering the distal events of the estrogen/antiestrogen-mediated signal transduction pathway as illustrated in Figure 2, the ERE can be considered as a position in the pathway, that if modified, may manifest the TAM-resistant phenotype. It has been well documented that modifications to the ERE such as sequence variation, placement of multiple EREs and orientation can significantly affect binding affinity of the ligand/ER complex as well as the transcriptional response to various ligands.[62–69] This evidence leads us to speculate that perhaps TAM resistance is achieved by modification of the ERE such that binding of the TAM/ER complex is interpreted as an agonistic rather than an antagonistic signal.

The ERE, a regulatory region of the DNA, may be located 5′, 3′ or within the estrogen regulated gene. The expression of the vitellogenin A2 (Vit A2) gene, a highly estrogen regulated gene of *Xenopus laevis*, is controlled via its ERE that consists of a palindromic sequence separated by a 3 bp spacer region having the sequence 5′-GGTCA CAG TGACC-3′.[70] Upon binding dimeric liganded ER, the Vit A2 ERE is capable of regulating the expression of heterologous reporter genes such as chloramphenicol acetyltransferase (CAT) in response to E_2.[71] The Vit A2 ERE is used as a molecular standard in the laboratory as a comparison of estrogen inducibility of both naturally occuring and synthetic EREs. A 13 bp consensus sequence was derived by analysis of the regulatory regions of various estrogen responsive genes. The core sequence, 5′-GGTCAnnnTGACC-3′,[71] is represented by a palindrome separated by a 3 bp spacer region, although several estrogen-inducible genes which display variations of this ERE sequence have been identified. These variations include EREs consisting of half-palindromic motifs in tandem[73–76] as well as specific base alterations to the consensus sequence.[77–82]

Dana *et al.*[68] identified 65 novel EREs using a genetic screening technique in yeast requiring the insertion of an activating sequence upstream of a selectable marker. This protocol yielded the selection of at least one putative ERE which, in response to two triphenylethylenes, TAM and nafoxidene, demonstrated a significant level of agonist activity. This finding supports the notion that the sequence of the ERE itself may affect whether a receptor/ligand complex will act as an agonist or an antagonist. Many studies have demonstrated that synergistic activation of transcription is dependent upon the spacing of tandem EREs,[62–65,67] as well as increasing transcriptional response proportional to the number of EREs in tandem.[66,67,69]

Discussion

The primary mechanism responsible for the majority of TAM-resistant tumors, either *de novo* or acquired, remains unknown. As summarized in this

review, several points of contact within the estrogen/antiestrogen signal transduction pathway have been investigated as likely candidates to mediate TAM resistance (Figure 2). It appears that the generation of estrogenic metabolites resulting from TAM metabolism and reduced intratumoral accumulation of TAM seem to be unlikely mechanisms given the findings of numerous studies in the literature.[13,22,23] Numerous mutations within the ER gene have been identified from breast cell lines and tumors that would be expected to exhibit altered ER function.[83] Considering the impact specific point mutations in genes such as *p53* and *BRCA1*[84,85] have made on protein function, it seems logical that mutations in the ER may lead to TAM resistance. However, a direct connection to TAM resistance has not been clearly demonstrated considering the low incidence of ER alterations found in surveying a number of tumors.[33,35] Recent evidence identifying the phosphorylation of specific serine residues in the ER A/B region and correlation to transcriptional activation[42-45] is an area which should be explored to determine what role ER phosphorylation plays in mediating TAM resistance. The discovery of several ER-associated proteins[56-58] is another exciting piece of the signal transduction puzzle which may add to our understanding of TAM resistance.

The mechanism which also seems to us to be quite plausible, is the possibility of multimerization and sequence alteration of the ERE which have already been shown to effect transcriptional activation. Tandem EREs are known to increase transcriptional transactivation of heterologous promoters cloned upstream of reporter genes.[65,69] It seems reasonable that such duplication events could arise within the cell as a mechanism of drug resistance, similar to the amplification of the dihydrofolate reductase gene resulting in the resistance to the antimetabolite drug, methotrexate.[86] Perhaps amplification of the regulatory ERE sequence, rather than gene amplification, is a mechanism capable of producing TAM resistance. The accumulation of known ERE sequences derived from estrogen responsive genes suggests that sequence variations affect the relative ERE strength. Similar to the hypothesis that mutation of the ER may result in altered ligand and DNA binding which may be responsible for the emergence of TAM resistance, we propose that alterations of the ERE such as those illustrated in Table 1 may contribute to the TAM-resistant phenotype.

Such variations in sequence and configurations of the ERE may also reconcile the tissue-specific differences of TAM action. As previously mentioned, TAM is an incomplete antiestrogen, acting as as antagonist in the breast and as an agonist in other tissues such as the endometrium and bone.[6-8] How does TAM achieve this dual pharmacological response? One can speculate that perhaps there is a family of ERs that are differentially expressed in a tissue-specific manner that interact with TAM differently. This scenario is difficult to imagine given that only one ER gene has been identified.[87] Another possibility is that there is a cell type-specific complement of transcription factors and/or ER associated proteins. The presence or absence of a particular factor could determine the interpretation of the ER/TAM signal at the ERE. Alternatively, it may be the configuration of the ERE itself that confers cell-type specificity. For example TAM may interact with EREs in bone that are arranged in tandem for example, resulting in agonistic activity in this tissue. Comparatively, within the breast, placement or configuration of EREs may allow TAM to produce an inhibitory signal. The systematic categorization of the effect of ERE sequence variation and multi-

Table 1. Examples of ERE alterations affecting transcriptional response

ERE configuration	Activity	Reference
Specific base changes within the half-site GGTNA CAG TGACC	Reduced ER binding Estrogenic activity in response to 4-OHT	67 68
Specific base changes within the spacer GGTCA CNG TGACC	Evidence for a 2 base preference 3′ of the half-site	68
Additions to the spacer GGTCA NCAGNNNNTGACC	A negatively regulated ERE contains a 7 base spacer region	79
Multermerization of EREs →→ →→ →→ ERE—ERE—ERE	Multimerization of EREs enhance ER binding and estrogenic activity	65, 67
Orientation and spacing of EREs →→ →→ ←← ERE—ERE–/–ERE	Different orientation and spacing of EREs yield distinct transcriptional response	62, 66

merization on estrogen/antiestrogen inducibility may allow us to predict ligand inducibility of newly identified estrogen response genes. In addition, this knowledge may aid in more specific drug design.

In summary, due to the complex nature of the ER-mediated signal transduction pathway, it appears that no one mechanism is likely to account for the emergence of TAM-resistant tumors, but probably is the result of any number of lesions. Clearly, closer examination of the ERE is warranted to determine what role this latter step in the ER-mediated signal transduction plays in the expression of the TAM-resistant phenotype.

Acknowledgments

The authors wish to thank Dr Claudia Tellez and Dr Anait Levenson for their helpful discussions and suggestions, and Mr Henry Muenzner for his computer graphics expertise in preparing the figures for this review.

References

1. Early Breast Cancer Trialists' Collaborative Group. Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. *Lancet* 1992; **330**: 1–15 and 71–85.
2. Jordan VC. The development tamoxifen for breast cancer therapy. In: Jordan, VC, ed. *Long-term tamoxifen treatment for breast cancer.* Madison: The University of Wisconsin Press 1994: 3–26.
3. Jordan VC, Wolf MF, Mirecki DM, *et al.* Hormone receptor assays: clinical usefulness in the management of carcinoma of the breast. *CRC Crit Rev Clin Lab Sci* 1988; **21**: 15–26.
4. Jaiyesimi IA, Buzdar AU, Decker DA, *et al.* Use of tamoxifen for breast cancer: twenty-eight years later. *J Clin Oncol* 1995; **13**: 513–29.
5. Walker KJ, Price-Thomas JM, Candlish W, *et al.* Influence of the antioestrogen tamoxifen on normal breast tissue. *Br J Cancer* 1991; **64**: 764–8.
6. Gottardis MM, Robinson SP, Satyaswaroop PG, *et al.* Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res* 1988; **48**: 812–5.
7. Wolf DM and Jordan VC. Gynecologic complications associated with long-term adjuvant tamoxifen therapy for breast cancer. *Gynecol Oncol* 1992; **45**: 118–28.
8. Encarnatión CA, Ciocca DR, McGuire WL, *et al.* Measurement of steroid hormone receptors in breast cancer patients on tamoxifen. *Breast Cancer Res Treat* 1993; **26**: 237–46.
9. Pham TA, Elliston JF, Nawaz Z, *et al.* Antiestrogen can establish nonproductive receptor complexes and alter chromatin structure at target enhancers. *Proc Natl Acad Sci USA* 1991; **88**: 3125–9.
10. Katzenellenbogen, BS. Antiestrogen resistance: mechanisms by which breast cancer cells undermine the effectiveness of endocrine therapy. *J Natl Cancer Inst* 1991; **83**: 1434–5.
11. Wolf DM, Jordan VC. Drug resistance to tamoxifen during breast cancer therapy. *Breast Cancer Res Treat* 1993; **27**: 27–40.
12. Osborne CK. Mechanisms for tamoxifen resistance in breast cancer: possible role of tamoxifen metabolism. *J Steroid Biochem Mol Biol* 1993; **47**: 1–6.
13. Wiebe VJ, Osborne CK, Fuqua SAW, *et al.* Tamoxifen resistance in breast cancer. *Crit Rev Oncol/Hematol* 1993; **14**: 173–88.
14. Jordan VC, Koch R, Langan S, *et al.* Ligand interaction at the estrogen receptor to program antiestrogen action: a study with non-steroidal compounds *in vitro*. *Endocrinology* 1988; **122**: 1449–54.
15. Katzenellenbogen JA, Carlson KE, Katzenellenbogen BS. Facile geometric isomerization of phenolic non-steroidal estrogens and antiestrogens: limitations to the interpretation of experiments characterizing the activity of individual isomers. *J Steroid Biochem* 1985; **22**: 589–96.
16. Murphy CS, Langan-Fahey SM, McCague R, *et al.* Structure–function relationships of hydroxylated metabolites of tamoxifen that control the proliferation of estrogen-responsive T47D breast cancer cells *in vitro*. *Mol Pharmacol* 1990; **38**: 737–43.
17. Jordan VC, Lieberman ME, Cormier E, *et al.* Structural requirements for the pharmacological activity of nonsteroidal antiestrogens *in vitro*. *Br J Pharmacol.* 1984; **26**: 272–8.
18. Osborne CK, Coronado E, Wiebe VJ, *et al.* Acquired tamoxifen resistance: correlation with reduced tumor levels of tamoxifen and isomerization of *trans*-4-hydroxytamoxifen. *J Natl Cancer Inst* 1991; **83**: 1477–82.
19. Johnstone SRD, Haynes BP, Smith IE, *et al.* Acquired tamoxifen resistance in human breast cancer and reduced intra-tumoral drug concentration. *Lancet* 1993; **342**: 1521–2.
20. Wiebe VJ, Osborne CK, McGuire WL, *et al.* Identification of estrogenic tamoxifen metabolite(s) in tamoxifen-resistant human breast tumors. *J Clin Oncol* 1992; **10**: 990–4.
21. Johnstone SRD, Haynes BP, Sacks NPM, *et al.* Effect of oestrogen receptor status and time on the intra-tumoural accumulation of tamoxifen and *N*-desmethyltamoxifen following short-term therapy in human primary breast cancer. *Breast Can Res Treat* 1993; **28**: 241–50.
22. Wolf DM, Langan-Fahey M, Parker CJ, *et al.* Investigation of the mechanism of tamoxifen-stimulated breast tumor growth with nonisomerizable analogues of tamoxifen and metabolites. *J Natl Cancer Inst* 1993; **85**: 805–12.
23. Kumar V, Green S, Stack G, *et al.* Functional domains of the human estrogen receptor. *Cell* 1987; **51**: 941–51.
24. Pakdel F, Katzenellenbogen BS. Human estrogen receptor mutants with altered estrogen and antiestrogen ligand discrimination. *J Biol Chem* 1992; **267**: 3429–37.
25. Danielian PS, White R, Hoare SA, *et al.* Identification of residues in the estrogen receptor that confer differential sensitivity to estrogen and hydroxytamoxifen. *Mol Endocrinol* 1993; **7**: 232–40.
26. Neff S, Sadawski C, Miksicek RJ. Mutational analysis of cysteine residues within the hormone-binding domain of

the human estrogen receptor identifies mutants that are defective in both DNA-binding and subcellular distribution. *Mol Endocrinol* 1994; **8**: 1215–23.
27. Jiang S-Y, Langan-Fahey SM, Stella AL, *et al.* Point mutation of estrogen receptor (ER) in the ligand-binding domain changes the pharmacology of antiestrogens in ER-negative breast cancer cells stably expressing complementary DNAs for ER. *Mol Endocrinol* 1992; **6**: 2167–74.
28. Jiang S-Y, Parker CJ, Jordan VC. A model to describe how a point mutation of the estrogen receptor alters the structure–function relationship of antiestrogens. *Breast Cancer Res Treat* 1993; **26**: 139–47.
29. Graham II ML, Krett NL, Miller LA, *et al.* $T47D_{CO}$ cells, genetically unstable and containing estrogen receptor mutations are a model for the progression of breast cancers to hormone resistance. *Cancer Res* 1990; **50**: 6208–17.
30. Fuqua SAW, Fitzgerald SD, Chamness GC, *et al.* Variant human breast tumor estrogen receptor with constitutive transcriptional activity. *Cancer Res* 1991; **51**: 105–9.
31. Dotzlaw H, Alkhalaf M, Murphy LC. Characterization of estrogen receptor variant mRNAs from human breast cancers. *Mol Endocrinol* 1992; **6**: 773–85.
32. Watts CKW, Handel ML, King RJB, *et al.* Oestrogen receptor gene structure and function in breast cancer. *J Steroid Biochem Mol Biol* 1992; **41**: 529–36.
33. Wolf DM, Jordan VC. The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. *Breast Cancer Res Treat* 1994; **31**: 129–38.
34. Karnik PS, Kulkarni S, Liu X-P, *et al.* Estrogen receptor mutations in tamoxifen-resistant breast cancer. *Cancer Res* 1994; **54**: 349–53.
35. Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 1988; **48**: 5183–7.
36. Wolf DM, Jordan VC. Characterization of tamoxifen stimulated MCF-7 tumor variants grown in athymic mice. *Breast Cancer Res Treat* 1994; **31**: 117–27.
37. Catherino WH, Jordan VC. The biological action of cDNAs from mutated estrogen receptors transfected into breast cancer cells. *Cancer Lett* 1995; *in press.*
38. Hsieh J-C, Jurutka PW, Galligan MA, *et al.* Human vitamin D receptor is selectively phosphorylated by protein kinase C on serine 51, a residue crucial to its *trans*-activation funtion. *Proc Natl Acad Sci USA* 1991; **88**: 9315–9.
39. Denton RR, Koszewki NJ, Notides AC. Estrogen receptor phosphorylation. *J Biol Chem* 1992; **267**: 7263–8.
40. Orti E, Hu L-M, Munck A. Kinetics of glucocorticoid receptor phosphorylation in intact cells. *J Biol Chem* 1993; **268**: 7779–84.
41. Cho H, Katzenellenbogen BS. Synergistic activation of estrogen receptor-mediated transcription by estradiol and protein kinase activators. *Mol Endocrinol* 1993; **7**: 441–52.
42. Ali S, Metzger D, Bornert J-M, *et al.* Modulation of transcriptional activation by ligand-dependent phosphorylation of the human oestrogen receptor A/B region. *EMBO J* 1993; **12**: 1153–60.
43. Arnold SF, Obourn JD, Jaffe H, *et al.* Serine 167 is the major estradiol-induced phosphorylation site on the human estrogen receptor. *Mol Endocrinol* 1994; **8**: 1208–14.
44. Le Goff P, Montano MM, Schodin DJ, *et al.* Phosphorylation of the human estrogen receptor. *J Biol Chem* 1994; **269**: 4458–66.
45. Arnold SF, Obourn JD, Jaffe H, *et al.* Phosphorylation of the human estrogen receptor on tyrosine 537 *in vivo* and by *src* family tyrosine kinases *in vitro*. *Mol Endocrinol* 1995; **9**: 24–33.
46. O'Brien CA, Liskamp RM, Solomon DH, *et al.* Inhibition of protein kinase C by tamoxifen. *Cancer Res* 1985; **45**: 2462–5.
47. O'Brien CA, Housey GM, Weinstein IB. Specific and direct binding of protein kinase C to an immobilized tamoxifen analogue. *Cancer Res* 1988; **48**: 3626–9.
48. Lahooti H, White R, Danielian PS, *et al.* Characterization of ligand-dependent phosphorylation of the estrogen receptor. *Mol Endocrinol* 1994; **8**: 182–8.
49. Fujimoto N, Katzenellenbogen BS. Alteration in the agonist/antagonist balance of antiestrogens by activation of protein kinase A signaling pathways in breast cancer cells: antiestrogen selectivity and promoter dependence. *Mol Endocrinol* 1994; **8**: 296–304.
50. Tora L, White J, Brou C, *et al.* The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell* 1989; **59**: 477–87.
51. Schule R, Muller M, Renkawitz R. Many transcription factors interact synergistically with steroid receptors. *Science 1988*; **242**: 1418–20.
52. Gaub M-P, Bellard M, Scheuer I, *et al.* Activation of the ovalbumim gene by the estrogen receptor involves the Fos–Jun complex. *Cell* 1990; **63**: 1267–76.
53. Martinez E, Dusserre Y, Wahli W, *et al.* Synergistic transcriptional activation receptor involves stabilized interactions with a limiting target factor. *Mol Cell Biol* 1991; **11**: 2937–45.
54. Ing NH, Beekman JM, Tsai SY, *et al.* Members of the steroid hormone receptor superfamily interact with TFIIB (S300-II). *J Biol Chem* 1992; **267**: 17617–23.
55. Smith DF, Toft DO. Steroid receptors and their associated proteins. *Mol Endocrinol* 1993; **7**: 4–11.
56. Cavailles V, Dauvois S, Danielian PS, *et al.* Interaction of proteins with transcriptionally active estrogen receptors. *Proc Natl Acad Sci,* USA 1994; **91**: 10009–13.
57. Halachmi S, Marden E, Martin G, *et al.* Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. *Science* 1994; **264**: 1455–8.
58. Landel CC, Kushner PJ, Greene GL. The interaction of human estrogen receptor with DNA is modulated by receptor-associated proteins. *Mol Endocrinol* 1994; **8**: 1407–19.
59. Srinivasan G, Patel NT, Thompson EB. Heat shock protein is tightly associated with the recombinant human glucocorticoid receptor: glucocorticoid response element complex. *Mol Endocrinol* 1994; **8**: 189–96.
60. Pratt WB, Czar MJ, Stancato LF, *et al.* The hsp56 immunophilin component of steroid receptor heterocomplexes: could this be the elusive nuclear localization signal-binding protein? *J Steroid Biochem Mol Biol* 1993; **46**: 269–79.
61. Pratt WB. Interaction of hsp90 with steroid receptors: organizing some diverse observations and presenting the newest concepts. *Mol Cell Endocrinol* 1990; **74**: C69–C76.
62. Klein-Hitpaß L, Kaling M, Ryffel GU. Synergism of

closely adjacent estrogen-responsive elements increases their regulatory potential. *J Mol Biol* 1988; **201**: 537–44.
63. Tsai SY, Tsai M-J, O'Malley BW. Cooperative binding of steroid hormone receptors contributes to transcriptional synergism at target enhancer elements. *Cell* 1989; **57**: 443–8.
64. Martinez E, Wahli W. Cooperative binding of estrogen receptor to imperfect estrogen-responsive DNA elements correlates with their synergistic hormone-dependent enhancer activity. *EMBO J* 1989; **8**: 3781–91.
65. Ponglikitmongkol M, White JH, Chambon P. Synergistic activation of transcription by the human estrogen receptor bound to tandem responsive elements. *EMBO J* 1990; **9**: 2221–31.
66. Naar AM, Boutin J-M, Lipkin SM, *et al.* The orientation and spacing of core DNA–binding motifs dictate selective transcriptional responses to three nuclear receptors. *Cell* 1991; **65**: 1267–79.
67. Klinge CM, Peale Jr FV, Hilf R, *et al.* Cooperative estrogen receptor interaction with consensus or variant estrogen responsive elements *in vitro*. *Cancer Res* 1992; **52**: 1073–81.
68. Dana SL, Hoener PA, Wheeler DA, *et al.* Novel estrogen response elements identified by genetic selection in yeast are differentially responsive to estrogens and antiestrogens in mammalian cells. *Mol Endocrinol* 1994; **8**: 1193–207.
69. Catherino WH, Jordan VC. Increasing the number of tandem estrogen response elements increases the estrogenic activity of a tamoxifen analogue. *Cancer Lett* 1995; **95**: 39–47
70. Klein-Hitpaß L, Ryffel GU, Heitlinger E, *et al.* A 13 bp palindrome is a functional estrogen responsive element and interacts specifically with estrogen receptor. *Nucleic Acids Res* 1988; **16**: 647–63.
71. Klein-Hitpaß L, Schorpp M, Wagner U, *et al.* An estrogen-responsive element derived from the 5′ flanking region of the *Xenopus* vitellogenin A2 gene functions in transfected human cells. *Cell* 1986; **46**: 1053–61.
72. Walker P, Germond J-E, Brown-Luedi M, *et al.* Sequence homologies in the region preceeding the transcription initiation site of the liver estrogen-responsive vitellogenin and apo-VLDL II genes. *Mol Endocrinol* 1984; **12**: 8611–26.
73. Seyfred MA, Gorski J. An interaction between the 5′ flanking distal and proximal regulatory domains of the rat prolactin gene is required for transcriptional activation by estrogens. *Mol Endocrinol* 1990; **4**: 1226–34.
74. Kato S, Tora L, Yamauchi J, *et al.* A far upstream estrogen response element of the ovalbumin gene contains several half-palindromic 5′-TGACC-3′ motifs acting synergistically. *Cell* 1992; **68**: 731–42.
75. May FEB, Smith DJ, Westley. The human cathepsin D-encoding gene is transcribed from an estrogen-regulated and a constitutive start point. *Gene* 1993; **134**: 277–82.
76. Vamvakopoulos NC, Chrousos GP. Evidence of direct estrogenic regulation of human corticotropin-releasing hormone gene expression. *J Clin Invest* 1993; **92**: 1896–902.
77. Weisz A, Rosales R. Identification of an estrogen response element upstream of the human c-*fos* gene that binds the estrogen receptor and the AP-1 transcription factor. *Nucleic Acids Res* 1990; **18**: 5097-106.
78. Savouret JF, Bailly A, Misrahi M, *et al.* Characterization of the hormone responsive element involved in the regulation of the progesterone receptor gene. *EMBO J* 1991; **10**: 1875–83.
79. Van Dijck P, Verhoeven G. Interaction of estrogen receptor complexes with the promoter region of genes that are negatively regulated by estrogens: the $\alpha_{2\mu}$-globulins. *Biochem Biophys Res Commun* 1992; **182**: 174–81.
80. Inoue S, Orimo A, Hosoi T, *et al.* Genomic binding-site cloning reveals an estrogen-responsive gene that encodes a RING finger protein. *Proc Natl Acad Sci* USA 1993; **90**: 11117–21.
81. Wu-Peng XS, Pugliese TE, Dickerman HW, *et al.* Delineation of sites mediating estrogen regulation of the rat creatine kinase B gene. *Mol Endocrinol* 1992; **6**: 231–40.
82. Jeung, E-B, Leung PCK, Krisnger J. The human calbindin-D_{9k} gene. Complete structure and implications on steroid hormone regulation. *J Mol Biol* 1994; **234**: 1231–8.
83. Sluyser M. Mutations in the estrogen receptor gene. *Human Mutat* 1995; in press.
84. Hollstein M, Sidransky D, Vogelstein B, *et al.* p53 mutations in human cancer. *Science* 1991; **253**: 49–53.
85. Shattuck-Eidens D, McClure M, Simard J, *et al.* A collaborative survey of 80 mutations in the BRCA1 breast and ovarian cancer susceptibility gene: implications for presymptomatic testing and screening. *J Am Med Ass* 1995; in press.
86. Curt GA, Carney DN, Cowan KH, *et al.* Unstable methotrexate resistance in human small cell carcinoma associated with double minute chromosomes. *N Eng J Med* 1983; **308**: 199–202.
87. Menasce LP, White GR, Harrison CJ, *et al.* Localization of the estrogen receptor locus (ESR) to chromosome 6q25.1 by FISH and a simple post-FISH banding technique. *Genomics* 1993; **17**: 263–5.

(Received 6 March 1995; accepted 20 April 1995)