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Basic Guide to the Mechanisms of Antiestrogen Action

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I. Introduction

In 1958, Lerner and coworkers published their landmark paper on the pharmacological properties of the first nonsteroidal antiestrogen ethamoxytriphetol or MER-25 (fig. 1). Lerner later wrote, "the compound was appealing not only because it completely inhibited the uterine response to estradiol (E_2)[b] but also because it was devoid of uterine stimulatory properties. This was an added bonus. Here was a possible tool for the study of oestrogen requirements and involvement in bodily functions. Was the inhibition of estrogenic activity competitive or noncompetitive? Various doses of MER-25 were studied against a single dose of oestradiol benzoate, and various doses of the oestrogen were studied against a single dose of the antagonist. The results of these studies demonstrated dose response relationships with competitive antagonism" (1981).

However, in the 1950s, the main roadblock to further progress was that no one knew how estrogen produced its effects. The target site-specific actions of estrogen on the reproductive system had been well known since the work of Allen and Doisy (1923), who identified and assayed ovarian "estrus-stimulating" hormones. But why did one tissue like the uterus and vagina respond to estrogen whereas another like muscle did not? Early studies with ^{14}C-labeled hormones could not detect any target site localization (Twombly and Shoenewaldt, 1951; Hanahan *et al.*, 1953). It subsequently would be discovered that the specific activity was too low, and tritium-labeled compounds, with high specific activity, would be necessary for success.

In 1959, in Vergennes, Vermont, Gregory Pincus and Erwin Vollmer organized a conference sponsored by the Cancer Chemotherapy National Service Center of the National Cancer Institute, entitled "Biological Activities of Steroids in Relation to Cancer." Jensen and Jacobson, from the Ben May Laboratory for Cancer Research at the University of Chicago, had synthesized [6,7-^{3}H]E_2 and for the first time illustrated the target tissue specificity of a natural hormone. They injected [^{3}H]E_2 into immature female rats and noted that the radioactivity, which they proved was E_2, was bound in and retained by estrogen target tissues (uterus, vagina, and anterior pituitary) but was not retained in nontarget tissues (muscle, kidney, and liver). These pivotal studies (Jensen and Jacobson, 1960) opened the door for the subsequent identification and study of steroid receptors. At the meeting, Dr. Gerald Mueller, then Lasker Professor of Cancer Research at the McArdle Memorial Laboratory at the University of Wisconsin, commented, "Dr. Jensen, you certainly have filled a tremendous gap in the information that we have wanted for a long time; that is, the state of hormones in the tissue during response to hormone. This beautiful work is an example of experimentation executed with good command of organic chemistry and good knowledge of the biological picture" (Mueller, 1960).

The study of estrogen and antiestrogen action converged when Pincus, the father of oral contraceptives and then Director of the Worcester Foundation for Experimental Biology (now the Worcester Foundation for Biomedical Research) in Shrewsbury, Massachusetts, invited Jensen and Jacobsen to present their findings at a Laurentian Hormone Conference in Mont Tremblant in 1961 (Jensen and Jacobson, 1962). The talk was entitled "Basic Guide to the Mechanism of Estrogen Action." Again, the authors elegantly described the target tissue specificity of estrogen, but additionally, Jensen (1962) described the first studies that demonstrated that the antiuterotropic activity of MER-25 depends, at least in part, on its ability to prevent the incorporation and

[b] Abbreviations: AF, activation function; CBP, CREB-binding protein; cDNA, complementary DNA; CNS, central nervous system; CREB, cAMP response element binding protein; DBD, DNA binding domain; DMBA, dimethylbenzanthracene; E_2, estradiol; ER, estrogen receptor; ERAP160, 160 kDa ER-associated protein; ERE, estrogen response element; ERF-1, ER factor 1; FDA, Food and Drug Administration; HBD, hormone binding domain; IGF, insulin-like growth factor; LBD, ligand binding domain; LDL, low-density lipoprotein; MER 25, ethamoxytriphetol; Met E, metabolite E; N-CoR, nuclear receptor corepressor; NLS, nuclear localization signal; NSABP, National Surgical Adjuvant Breast and Bowel Project; 4-OHT, 4-hydroxytamoxifen; PKA, protein kinase A; PKC, protein kinase C; PR, progesterone receptor; RAR, retinoic acid receptor; RRE, raloxifene response element; SMRT, silencing mediator for retinoic and thyroid receptors; TGFβR, transforming growth factor β receptor; TGF, transforming growth factor; TR, thyroid hormone receptor.

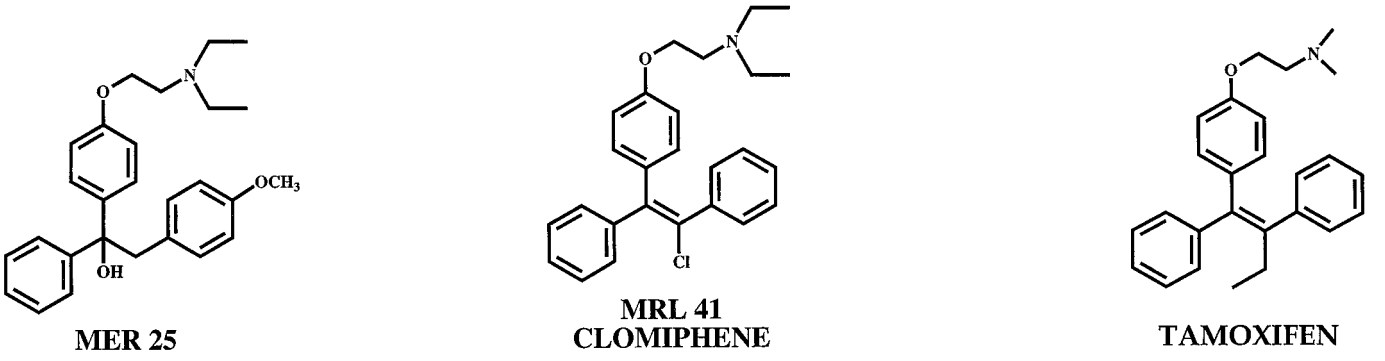


FIG. 1. The first nonsteroidal antiestrogen, MER 25, was never developed clinically because of high toxicity and low potency. The triphenylethylene compound, MRL-41, or clomiphene developed in 1961 is now the standard therapy for infertility. The triphenylethylene compound, tamoxifen, is the endocrine treatment of choice for the treatment of breast cancer.

retention of administered E_2 in the rat uterus. Thus, a foundation for the molecular mechanism of action of antiestrogens was established.

In 1963, Lerner reviewed progress in the development of antiestrogens at the Laurentian Hormone Conference (Lerner, 1964). MER-25 was not to become a clinically useful agent because of toxicity and low potency (Lerner, 1981); however, a triphenylethylene MRL-41 or clomiphene (fig. 1), as it became known (Holtkamp *et al*., 1960), was showing promise for the induction of ovulation in subfertile women (Greenblatt *et al*., 1962). The drug is now standard therapy for the treatment of infertility in anovulatory women.

After 1964, progress toward an understanding of antiestrogen action and the clinical utilization of antiestrogens was slow and largely ignored. However, by the late 1970s, with the successful clinical development of tamoxifen (fig. 1) for the treatment of breast cancer (Lerner and Jordan, 1990; Jordan, 1994), the prospects for new drug discovery changed dramatically.

Twenty years after Lerner completed the first review of nonsteroidal antiestrogens (1964), we reviewed the important developments that had occurred in our understanding of the receptor-mediated mechanism of action and the then state-of-the-art structure-activity relationships (Jordan, 1984). However, during the past dozen years, there have been enormous and far reaching changes in our basic knowledge and a new appreciation of the potential of antiestrogens as targeted agents to treat diseases associated with the menopause. This is because tamoxifen is an antiestrogen in the breast but has estrogen-like properties in other target tissues such as bone. Be that as it may, tamoxifen is used exclusively for the treatment of all stages of breast cancer (Jordan, 1997b), and clinical trials are testing the worth of tamoxifen as a preventive for breast cancer (Jordan, 1993, 1995b). By contrast, new and novel antiestrogens are being evaluated currently not only for breast cancer therapy but also for the prevention of osteoporosis (Gradishar and Jordan, 1997).

At a time when there is enormous interest in this topic, it is most appropriate to dedicate our review to Drs. Leonard Lerner and Elwood Jensen, whose seminal discoveries laid the foundations for all the subsequent research in this area. Our title is an adaptation of the original "Basic Guides to the Mechanism of Estrogen Action" used by Jensen and Jacobson at the Laurentian Hormone Conference in 1961 (Jensen and Jacobson, 1962).

We have organized our current review into two major parts. First, we will discuss the problems and inadequate understanding of antiestrogen action that occurred in 1984 and describe the enormous progress that has been achieved in understanding the fundamentals of estrogen action. Second, we will consider the current problems and potential of antiestrogens as valuable therapeutic agents and highlight the new knowledge that is emerging about the target site-specific mechanisms of estrogen and antiestrogen action. We recommend that readers refer to earlier articles for the history of the development of antiestrogens (Jordan, 1997a,b) and for a broad review of structure-activity relationships (Jordan, 1984; Lerner and Jordan, 1990).

II. Unresolved Issues in 1984

In 1984, we concluded our article in *Pharmacological Reviews* with the statement that several key issues concerning the pharmacology and mode of action of antiestrogens remained unresolved (Jordan, 1984). We wrote:

> A unifying theory of antiestrogen action is, however, impractical because there are several unexplained observations with antiestrogens that require further study. (*a*) The species differences in the pharmacology of antiestrogens is perplexing. Although it is possible that the triphenylethylene type I antiestrogens (tamoxifen) are metabolized to estrogens in rodents, no convincing evidence has been presented to show metabolic differences between chickens and rodents. (*b*) Most antiestrogens exhibit agonist or partial agonist actions in vivo but in vitro, the compounds usually have zero intrinsic efficacy. The reason for this is unknown. (*c*) Tamoxifen binds to the so called "antiestrogen binding site" with precise structural specificity

and high affinity. The binding site requires definition biochemically and its physiological role needs to be established.

As an introduction to our current review, we will briefly consider progress in addressing our previously "unresolved" issues. This retrospective illustrates the impressive progress that has been made.

A. Species Differences

There is still no satisfactory explanation for the different pharmacology of tamoxifen in the mouse, for example, where the drug is an estrogen in short-term tests, but in the chicken, it is an antiestrogen. Nevertheless, new facts have emerged to demonstrate that perhaps the answer lays more in tissue specificity than in species specificity. Long-term tamoxifen treatment of ovariectomized inbred (Jordan *et al.*, 1990) or athymic mice (Gottardis and Jordan, 1988) results in an early estrogen-like effect in the uterus, but eventually the tissue response changes so the uterus is refractory to estrogen action. Tamoxifen is a preventive for the development of mammary tumors in mice (Jordan *et al.*, 1991b); similarly, human breast tumor cell lines, which grow in response to estrogen in athymic mice, will not grow initially in response to tamoxifen (Gottardis *et al.*, 1988a). After many months, however, tamoxifen-stimulated breast tumors will grow (Gottardis and Jordan, 1988), but interestingly enough, the uterus becomes refractory to estrogen in the same animal. As a result of these findings, one could ask whether tamoxifen-stimulated tumor growth is species-specific. The answer is "no," because the tamoxifen-stimulated human tumors derived from the athymic mouse model also will grow in response to tamoxifen in the athymic rat (Gottardis *et al.*, 1989a). This excludes the possibility of species-specific metabolism.

Thus, an elucidation of the complexities of the target site-specific actions of antiestrogens may hold the most promise for resolving the unusual species differences. A combined effort to exploit the emerging molecular biology of receptor function and an understanding of the pharmacology of novel agents will prove instructive for future progress.

B. Differences Between Antiestrogens In Vivo and In Vitro

This issue has been resolved, for the most part, with the discovery that culture media contains estrogens (Berthois *et al.*, 1986). We describe this fundamental discovery in detail in Section VI.A. There are now reasonable parallels with the partial agonist actions of compounds in vivo and in vitro.

C. Antiestrogen Binding Sites

Tamoxifen and the other triphenylethylene antiestrogens bind with high affinity to microsomal sites in tissues throughout the body. We previously reviewed progress in this area (Jordan and Murphy, 1990), but no one has succeeded yet in identifying a function for the binding protein itself. In parallel studies, Lubahn and colleagues (1993) have addressed the issue indirectly by showing that an estrogen receptor (ER) knock-out transgenic mouse does not elicit a uterotropic response to 4-hydroxytamoxifen (4-OHT). Thus, if the antiestrogen binding site plays a role in the pharmacology of antiestrogens, it does not seem to be as pivotal as the ER. Conceptually, this becomes a key issue. In the earlier review, we wrote, "Finally, it is perhaps naive to believe that a clear view of the mechanism of action of antiestrogens can be described when the molecular mechanism of estrogen-controlled protein synthesis and cell division is as yet unknown (Jordan, 1984)."

At that time, before the precise structure of the ER was known, crude models of the interaction of estrogens and antiestrogens were proposed to describe the agonist, partial agonist, and antagonist actions of various ligands. These models were summarized in our earlier article (Jordan, 1984), but the proposal was based on experimental studies with ER antibodies and radiolabeled E_2 and 4-OHT conducted in collaboration with Elwood Jensen (Tate *et al.*, 1984) and in an extensive series of structure-activity relationship studies that started with a collaboration with Jack Gorski (Liebermann *et al.*, 1983a,b; Jordan *et al.*, 1984). Essentially, each study supported a model of ligand binding sites that would anchor estrogen but then be locked by a conformational folding of ER like the closing of the jaws of a crocodile. By contrast, an antiestrogen-like tamoxifen could be wedged into the ligand binding site, but the protein could not close around it correctly. The antiestrogenic molecule would be like a stick jammed into the jaws of a crocodile.

Progress to understand estrogen and antiestrogen action has been dramatic with the cloning and sequencing of the ER. The realization that the ER is a nuclear transcription factor, and just one of a superfamily of transcription factors, with many as yet unknown functions, has had a profound effect on scientific thinking during the past decade. Indeed, the conventional ER is now referred to as ERα because a second receptor ERβ has been discovered recently (Kuiper *et al.*, 1996).

Currently, evidence that our simple models of estrogen and antiestrogen action (Jordan, 1984) were close to the true state of affairs is developing. The ER has recently been crystallized with estrogens and antiestrogens revealing a similar locking of the estrogenic ligand by the mobile protein tail of the ER (Brzozowski *et al.*, 1997). Nevertheless, the overall consequences of ligand binding are now known to be far more complex. Various levels of intrinsic efficacy are related to a range of conformations (McDonnell *et al.*, 1995), and there is now knowledge of the essential role of associated proteins, or

coactivators, to construct a transcriptional unit (Katzenellenbogen *et al.,* 1996).

In our review, we will first describe the progress that has been made in the understanding of the molecular biology of estrogen action and use this as a basic foundation to consider the multifaceted actions of antiestrogens and their potential clinical applications. Finally, we will summarize the proposed molecular mechanism of action of the antiestrogen raloxifene (see Section XVII.) and suggest future studies that are necessary for a complete understanding of the multifaceted actions of a spectrum of drugs.

III. The Estrogen Receptor

The first evidence for a connection between estrogen and breast cancer growth was presented in 1896 when Beatson, a British physician, discovered that by removing the ovaries of premenopausal women, he could cause a regression of advanced breast tumors. Shortly thereafter, Stanley Boyd (1900) reported a study that established that one-third of all patients with breast cancer who had an oophorectomy would see a regression of their disease. However, the mechanism by which this occurred in these patients, and who would respond, were not to be discovered until 60 years later. The ER first was described in the uterus of rats (Jensen and Jacobson, 1962; Toft and Gorski, 1966; Jensen *et al.*, 1968), and extensive early literature on the basic biochemistry of the ER quickly developed (Jensen and DeSombre, 1973; Gorski *et al.*, 1968; Williams, 1974). Jensen and colleagues (1971) translated the basic science into clinical utility by proposing a predictive test, the ER assay, to determine which patients would respond to endocrine ablation, i.e., oophorectomy in premenopausal patients and adrenalectomy in postmenopausal patients. It was then established that patients with ER-rich tumors respond to endocrine therapy, whereas patients with ER-negative tumors are unlikely to respond (McGuire *et al.*, 1975). These pivotal observations provide an excellent example of basic research that translated to the treatment of human disease.

Nuclear hormone receptors are a family of hormone-activated transcription factors that can initiate or enhance the transcription of genes containing specific hormone response elements. The human ER, which belongs to this family, was cloned and sequenced from MCF-7 human breast cancer cells (Green *et al.*, 1986, Greene *et al.*, 1986). The ER protein consists of 595 amino acids with a molecular weight of 66 kDa (Green *et al.*, 1986) that has been separated into six different functional domains (fig. 2) (Kumar *et al.*, 1986, 1987). Two of these functional domains are highly conserved in the primary sequence of members of the nuclear hormone receptor superfamily. One of the domains, the DNA binding domain (DBD), contains two zinc fingers that mediate receptor binding to hormone response elements in the promoters of hormone-responsive genes. In the C-terminal region, the hormone binding domain (HBD) contains two regions of sequence homology with other hormone receptors and bestows hormone specificity and selectivity (Carson-Jurica *et al.*, 1990; Krust *et al.*, 1986; Kumar *et al.*, 1987; Kumar and Chambon, 1988; Orti *et al.*, 1992). The human ER is located on chromosome 6q sub band 25.1 (Menasce *et al.*, 1993), and the mouse ER is located on chromosome 10 (Sluyser *et al.*, 1988).

IV. A Second Receptor

Recently, a novel member of the nuclear hormone receptor superfamily was cloned from a rat prostate complementary DNA (cDNA) library (Kuiper *et al.*, 1996; Katzenellenbogen and Korach, 1997). This novel sequence encodes a protein of 485 amino acid residues, and the molecular weight has been calculated to be 54.2 kDa (fig. 3). ERβ bears substantial homology to ERα especially in the DBD (95%) and the HBD (55%), and these proteins are functionally homologous in that ERβ binds estrogen with high affinity as shown by saturation ligand-binding analysis. The functional homology of ERα and ERβ has been determined by measuring transcriptional activity of ERβ in a system designed to test the functionality of ERα. It has been determined by the activation of transcription of a vitellogenin A2 estrogen response element (ERE)-containing reporter plasmid in the presence and absence of estrogen that ERβ is functionally homologous (Kuiper *et al.*, 1996).

Recently, the mouse homolog of the rat ERβ was cloned and mapped to chromosome 12 (Tremblay *et al.*, 1997). The ERβ gene has been designated Estrb and is expressed in several transcripts. The corresponding cDNA has been shown to encode a 485-amino-acid protein and has 97% identity to the DBD of mouse (m)ERα and 60% identity to the LBD of mERα. The most interesting question after the identification of this novel ER is whether it has the same pharmacological properties as ERα. Tremblay and colleagues (1997) have shown that mERβ binds to the vitellogenin A2 ERE although with a lower affinity than that of mERα. More importantly, mERβ can transactivate reporter genes contain-

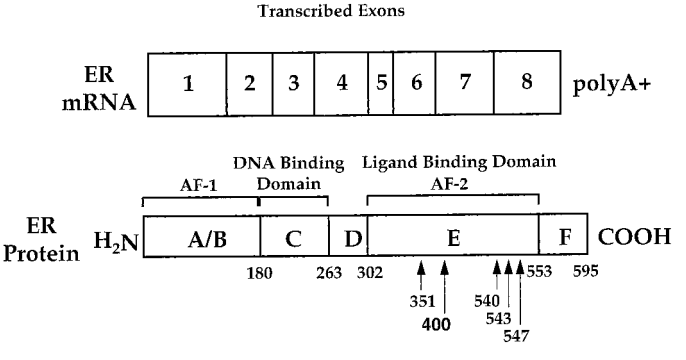


FIG. 2. The ER consists of six functional domains (AF) transcribed by eight exons. The functional domains are labeled accordingly and the relevant mutations, illustrated in the text, are highlighted with arrows.

ing EREs in transient transfection experiments with the same efficiency as mERα in HeLa and Cos-1 cell lines.

As would be expected, similarities and differences exist between the mERα and mERβ such as different aspects of regulation. For example, it is possible that mERβ can be activated via phosphorylation through the mitogen-activated protein kinase pathway as shown for ERα (Kato *et al*., 1995; Bunone *et al*., 1996). This would be predicted because of the conservation of serine 60 which could be phosphorylated in the mouse, rat and human ERβ sequences. A few differences surfaced in the pharmacology of ERβ when 4-OHT was tested in transient transfection reporter assays. The partial agonism that 4-OHT expresses in cells with ERα is not present when cells are transfected with ERβ (Tremblay *et al*., 1997). One possible explanation is the lack of homology in the amino-terminal domains of these proteins where the activation function-1 (AF-1) resides (see Section V.A.). The AF-1 is thought to be responsible for the partial agonist activity of tamoxifen in cells that express ERα (McInerney and Katzenellenbogen, 1996).

Clearly, the most important question is the distribution of ERβ in tissues and the relative importance of ERα and ERβ for the pharmacological action of antiestrogens. In addition to the presence of ERβ in the rat prostate and the mouse ovary, in situ hybridization studies have determined that the granulosa cells of the rat ovary also express ERβ (Kuiper *et al*., 1996). Previous studies tested an ERα knock-out mouse that does not express functional ERα for its ability to respond to estrogen (Lubahn *et al*., 1993). The female knock-out mice that were ERα negative were infertile and did not develop normal uteri and ovaries. Thus, if ERβ was expressed in the ovaries of these ERα knock-out mice, it was not functioning to compensate for the loss of ERα. Alternatively, ERα could regulate the expression of ERβ so that in the absence of ERα, ERβ is down-regulated. However, recent studies by Korach and colleagues (personal communication) suggest this is not highly probable. Further studies with ERα knock-out mice show residual estrogen binding of approximately 5 to 10% of the ERα level (Lubahn *et al*., 1993; Couse *et al*., 1995; Korach *et al*., 1996). What is particularly interesting is the fact that there are very high circulating levels of E_2 in the ERα knock-out mice that could be interacting with ERβ to produce the pathological states observed in the mice.

The presence of two different ERs could explain the mechanism of the target site specificity seen with antiestrogens or differential transcriptional AFs on estrogen-responsive genes (Kuiper *et al*., 1997). Even though the evolutionarily conserved regions of these two ERs are homologous, various nonconserved regions exist which probably account for the differences seen between ERα and ERβ. We will discuss the issue again in Complexity of Antiestrogen Action (Section XII.).

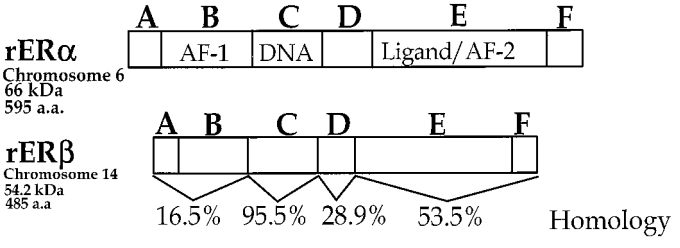


FIG. 3. Comparison of the rat (r) ERα and rERβ proteins and percent amino acid homology in the functional regions.

V. Estrogen Receptor

A. Receptor Functions

The model for estrogen action via the ERα (henceforth referred to as ER) has evolved considerably during the past 40 years. The first realistic conceptual model was proposed by Mueller and colleagues (1957) to explain the initiation of metabolic events in the rat uterus by estrogen. Since then, several models have evolved that address the mechanism of how the ER functions in the nucleus and how it activates the transcription of estrogen-responsive genes in the presence of estrogens (Gorski *et al*., 1984, 1993), an effect differentially blocked by antiestrogens. We will describe the emerging data about the functional domains of the ER to lay the foundation for our discussion of receptor regulation and antiestrogen action.

The six structural domains of the ER are regions that have been defined based on the putative functions that are contained in each area. The A/B domain contains one of the two transcriptional AFs present in the ER (fig. 2). AF-1 and AF-2 activate transcription in a cell and promoter context specific manner (Gronemeyer, 1991) and AF-1 and AF-2 are autonomous in that they are located at the N- and C-termini, respectively. In early studies, the existence of AF-1 initially was not discovered because ER deletion mutants in the A/B region retained the ability to activate the transcription of vit-tk-CAT reporter genes (Kumar *et al*., 1987). Unlike AF-2, which is induced upon hormone binding to the receptor (Kumar *et al*., 1987; Webster *et al*., 1988, 1989; Lees *et al*., 1989; Tora *et al*., 1989), we now know that AF-1 is constitutively active.

AF-1 acts in a cell type-specific fashion as shown in experiments using chimeric receptors. When the A/B region of the ER was expressed with the DBD of the yeast transcriptional activator Gal-4, this chimera was able to activate transcription of Gal-4-responsive promoters in chicken embryo fibroblasts but not in HeLa cells, thus demonstrating a cell type-specific function (Berry *et al*., 1990; Tora *et al*., 1989). The AF-2, which is located in the E region containing the HBD, when associated with Gal-4 showed activation of Gal-4-responsive promoters in both HeLa and chicken embryo fibroblasts (Webster *et al*., 1988). Thus, it is thought that AF-1 is

responsible for the promoter-specific transcriptional activation independent of the presence of ligand and that AF-2 provides ligand-specific activation (Berry *et al*., 1990; Webster *et al*., 1988).

The C region contains the DBD and a dimerization domain. The DBD is the most highly conserved region in the nuclear hormone receptor superfamily. The DBD consists of two zinc fingers that fold into two helical domains upon the coordination of one zinc to four cysteines and a third helix that extends from the zinc fingers (Schwabe *et al*., 1993). These zinc fingers are essential components of the ER because when the ER lacks the DBD, it cannot bind DNA in vitro or in vivo (Kumar and Chambon, 1988; Kumar *et al*., 1987). However, the C region alone is not sufficient to bind an ERE. As stated above, the A/B region can be deleted without compromising the DNA binding ability but deletion of the basic amino acids (amino acids 256 to 270) located downstream of the zinc fingers does impair the ability of the receptor to bind EREs (Kumar and Chambon, 1988; Chambraud *et al*., 1990).

There are many similarities in the zinc finger regions among different steroid hormones receptors, but there are precise differences that account for the specificity of each receptor. It is believed that the specificity of a certain receptor is afforded by the first of the two zinc fingers. These conclusions are based on mutagenesis in the region of the first zinc finger. The results prove that the receptor binds to specific nucleic acid residues in the major groove of the DNA helix. The second zinc finger is responsible for stabilizing this interaction through ionic bonds with the phosphate groups in the DNA backbone (Umesono and Evans, 1989; O'Malley, 1990; Parker and Bakker, 1991). In addition to these mutational studies, domain-swapping experiments in which the ER DBD was exchanged with the DBD of the glucocorticoid receptor showed that the chimeric protein activates glucocorticoid responsive genes in the presence of estrogen (Green and Chambon, 1991).

In addition to the basic requirement for DBD activity, the C region may bind to heat shock protein 90 (Chambraud *et al*., 1990) and also be responsible for nuclear localization of the receptor. The C region contains three lysine- and arginine-rich proto-nuclear localization signals (NLSs) that are ligand-independent. Several NLSs have been identified in the ER, one in the DBD and three others in the HBD (within amino acids 256 to 303) (Ylikomi *et al*., 1992). One NLS in the HBD has been shown to be ligand inducible, and the other NLSs are ligand independent. The inducible and constitutive NLSs cooperate in the presence of estrogen (Ylikomi *et al*., 1992).

The E region, the HBD, contains the AF-2 (ligand-dependent and promoter-specific), heat shock protein 90 binding function, a NLS (ligand-dependent), and a dimerization domain. The HBD is found in the C-terminus and is responsible for specific ligand recognition because it allows the ER to be transcriptionally active in a specific and selective manner. The HBD is thought to coordinate with the DBD and upon ligand binding, the coordination is lost and the receptor protein changes conformation, releases the DBD, and becomes transcriptionally active (reviewed in Gronemeyer, 1991; Parker *et al*., 1993).

B. Estrogen Action

Estrogen diffuses through the plasma membrane of cells where it binds to the ER (Rao, 1981). For many years, it generally was thought that estrogen bound to the ER in the cytoplasm and translocated into the nucleus, but it is known now that the ER is a nuclear transcription factor that initially interacts with estrogen in the nucleus (King and Greene, 1984; Welshons *et al*., 1984). Once estrogen binds to the ER, heat shock proteins dissociate and a change in conformation and homodimerization occurs (fig. 4).

Although phosphorylation of steroid hormone receptors enables them to become transcriptionally active, until recently, the role of phosphorylation of the ER was still in question (Orti *et al*., 1992). Phosphorylation of the ER from MCF-7 and calf uterus is estrogen-dependent and, in addition, increases the receptor's affinity for specific DNA sequences (Denton *et al*., 1992). The basal level of ER phosphorylation increases three- to four-fold upon treatment with estrogen and antiestrogens (Le Goff *et al*., 1994). However, the key to elucidating the mechanism of estrogen action is the identification of the selective sites for phosphorylation. Several serines in the amino-terminal portion of the human ER may play a role in hormone-regulated phosphorylation. However, when phosphopeptide maps of wild-type and mutant ERs treated with estrogen or antiestrogens are compared, the results are similar indicating that differential phosphorylation between these receptors cannot account for any differences in function (Lahooti *et al*., 1994). An alternate approach might be the identification of enzymes responsible for phosphorylation. There are several protein kinases thought to be involved in phosphorylation of the ER (ER kinase, DNA-dependent kinase, Ser-Pro kinases, protein kinase C, protein kinase A, and casein kinase II) (reviewed in Kuiper and Brinkman, 1994). Recently, a mitogen-activated protein kinase also was implicated in phosphorylation of the ER on Ser 118 resulting in the activation of ER AF-1 (Kato *et al*., 1995). Interestingly, another consequence of phosphorylation of the ER is the regulation of homodimerization through phosphorylation of tyrosine 537 (Arnold *et al*., 1995).

Although phosphorylation may play a part in receptor activation, exciting progress has been made in understanding how the receptor cooperates with other proteins to assemble a transcription unit for gene activation. The receptor can be viewed as a skeleton to assemble the unit as a prelude to DNA unwinding and the transcription of selected mRNAs. To achieve this,

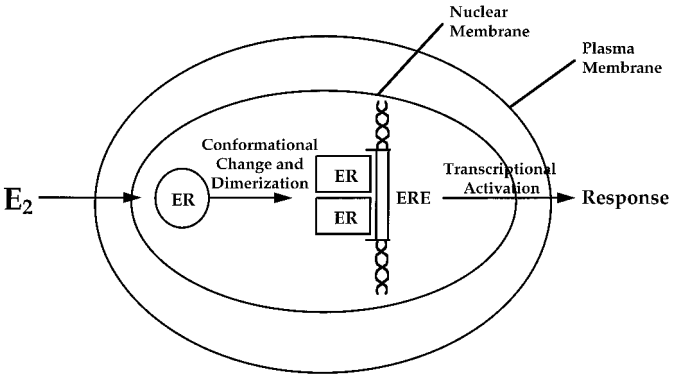


FIG. 4. Estradiol (E_2) action is transduced through ER located in the nucleus. On estrogen binding, the ER homodimerizes and interacts with EREs located in the promotor region of estrogen-activated genes. These events trigger an estrogenic response in the cell.

the receptor eventually must interact with other proteins as well as bind to one or several EREs. We will dissect the process by describing the areas needed for receptor activation, ligand binding, DNA binding, and protein-protein interactions.

The ER contains two areas called AFs: AF-1 is located in the amino-terminal region of the ER, and AF-2 is located in the carboxyl-terminal region in the ligand binding domain (LBD) of the ER; these are synergistic when the ER is activated by estrogen. Katzenellenbogen and colleagues (1995) used mammalian cells to show that the AF-1 and AF-2 regions, when expressed as separate polypeptides, functionally interact in response to estrogen and antiestrogens. The authors found that this interaction could activate transcription in response to estrogen. In addition, when mutations were made in AF-1 or AF-2 that abrogated the functional activity of these domains, no transcriptional activity was seen. Additionally, when mutations were made in the LBD that eliminated estrogen binding, no transcriptional activity could be detected. These experiments suggest that estrogen binding to the ER facilitates a conformational change that brings AF-1 and AF-2 in direct association with one another leading to synergy that results in transcriptional activation. These elegant experiments provide a mechanistic explanation for the role of the two AFs in mediating hormone-regulated transcription.

In addition to understanding the mechanism through which the ER becomes transcriptionally active, many of the amino acids important in the binding of ligand to the ER have been identified. Harlow and coworkers (1989) showed a covalent attachment between Cys530 and both an estrogen agonist and an antagonist. This work also suggested that other cysteine residues present in the LBD may be important for ligand-mediated transcriptional activation. Further mutant ERs have been constructed with mutations at the other cysteine residues present in the LBD. Each of these mutants showed an affinity similar to that of the wild-type ER (Reese and Katzenellenbogen, 1992). When these mutants were tested in reporter assays, the mutants C530A and C530S showed unaltered binding to estrogens and antiestrogens, but the transactivation response to both estrogens and antiestrogens had changed. After showing that the C530 is involved in discriminating between ligands, Pakdel and Katzenellenbogen (1992) examined the role of amino acids adjacent to the other cysteines in the LBD of the ER. The results showed that the amino-terminal domain of the LBD was important in differential transcriptional activation but not in binding affinity. When the carboxyl-terminal region of the LBD is mutated, this renders the protein transcriptionally inactive although it can still bind ligand, making this a very powerful dominant negative ER. Thus, there is a distinction between the hormone binding and the transactivation functions.

Once the ER has bound estrogen and dimerized, it binds to EREs present in the promoter region of genes. These EREs are 13 base pair palindromic sequences located upstream from the transcriptional start site. The EREs function by enhancing the transcriptional potential of a gene. EREs have been identified and defined using reporter systems to test the enhancer ability when exposed to different compounds (Gronemeyer, 1991). Also, deletional analysis has allowed the definition of the sequence of EREs. Optimally, it consists of two inverted repeats separated by any three base pairs. The exact sequence of EREs varies between species and genes (Klein-Hitpass *et al.*, 1986).

Some models of estrogen action predict that when the dimerized hormone-receptor complex binds to the palindromic ERE that it forms a looped structure allowing the ER to interact with the transcriptional apparatus at the RNA initiation site. It is thought that the hormone-receptor complex can recruit components of the transcriptional complex and serves as a nucleation site. Previous studies focus on the interaction of the ER with EREs, but more recently, there has been a shift toward the study of ER receptor interactions with ancillary proteins in the nucleus.

For example, Gorski and colleagues (1993) have suggested that the ER binds DNA in a heterodimer structure involving a variety of other proteins such as transcription factors or other DNA binding proteins. It also has been shown that estrogen is not essential for ER binding to DNA (Murdoch *et al.*, 1990) but that this does increase the ER's affinity for nuclear components. Another aspect of this study suggests that in the traditional reporter assays generally used to study these mechanisms lack the complexity that exists in the nucleus and in the nucleosome-chromatin structure.

Currently, there is intense interest in the identification of possible coactivators that can enhance ER-dependent transcription. The first candidate for a transcriptional coactivator, SPT6, was isolated from *Saccharomyces cerevisiae* and was shown to be capable of modulating ER-mediated transcription in yeast and mammalian cells and to

interact specifically with the carboxyl-terminal portion of the ER (Baniahmad *et al.*, 1995). Another steroid receptor coactivator, SRC-1, was sequenced and characterized using the yeast two-hybrid system (Oñate *et al.*, 1995). SRC-1 has been shown to interact specifically with the progesterone receptor (PR) and enhance its transcriptional activity. When SRC-1 was tested with the thyroid hormone receptor (TR), retinoic acid receptor (RAR), ER, and glucocorticoid receptor, it enhanced the transcriptional activity of each of these steroid hormone receptors. In fact, SRC-1 may have a complex role to play in steroid receptor regulation. For example, the ER can interfere with transcriptional activation by PR but SRC-1 will inhibit the effects of the ER.

Another recent discovery is that the transcription factor cAMP response element-binding protein (CREB) has an associated protein termed the CREB-binding protein (CBP) (Smith *et al.*, 1996). CBP has been shown to interact specifically with RNA polymerase II (Kee *et al.*, 1996), TFIIB (Kwok *et al.,* 1994), and with CREB in its phosphorylated form (Chrivia *et al.*, 1993). It has been postulated that the ability of CBP to stimulate transcription is through the targeted recruitment of RNA polymerase II to the promoters of genes. In addition to the above-described proteins, CBP can interact specifically with members of the steroid hormone nuclear receptor family and is able to enhance transcriptional activity in some instances (Kamei *et al.*, 1996). Thus, CBP can function as a coactivator for a rapidly growing number of transcription factors.

Ectopic expression of CBP can enhance estrogen-dependent ER transcriptional activity approximately tenfold compared with the ectopic expression of SRC-1 (Smith *et al.*, 1996). Again, CBP is partially able to reverse the transcriptional interference that activated ER has on PR-mediated transcriptional activity. Most importantly, these data suggest that CBP may be present in limited quantities in particular cells and may be able to modulate the activity of the steroid receptors. When SRC-1 and CBP are coexpressed ectopically, ER- and PR-mediated transcriptional activity is enhanced in a synergistic manner, which suggests that these two proteins are not functionally homologous.

In addition to coactivators, another category of molecules that are able to repress basal transcription induced by hormone receptors has been identified. Two corepressors termed the silencing mediator for retinoic and thyroid hormone receptors (SMRT) (Chen and Evans, 1995) and nuclear receptor corepressor (N-CoR) (Hörlein *et al.*, 1995; Kurokawa *et al.*, 1993) have been cloned using a yeast two-hybrid system. Both SMRT and N-CoR can interact with TR and RAR through specific homologous domains that have been shown to bear some homology to each other (Perlmann and Vennstrom, 1995). This finding suggests that a family of evolutionally conserved corepressors may exist that interact with other steroid hormone receptors. Corepressors that act on the ER have not yet been identified, but there is every reason to believe that they could exist.

Both SMRT and N-CoR associate with specific unliganded receptors but are released once the ligand has bound (Chen and Evans, 1995; Hörlein *et al.*, 1995). This is consistent with present dogma because when hormone receptors are unliganded, their ability to activate transcription presumably is compromised, but when ligand binds, thereby activating the receptors, the repression is alleviated leading to either an active receptor or possibly one that is open to activation by coactivators. Further evidence that these corepressors can silence receptor activity has been shown in mutational studies. The hinge region of TR and RAR which connects the DBD and the HBD has been shown to be important for a receptor's susceptibility to a repressor. When mutations are introduced into the hinge regions of the TR and the RAR, interaction with the corepressor is ablated and basal transcription levels are repressed (Chen and Evans, 1995; Hörlein *et al.*, 1995; Kurokawa *et al.*, 1993). The characterization of these corepressors could offer new insights into the molecular basis of nuclear hormone receptor modulation of transcription.

Overall, there has been enormous progress in understanding the growing levels of complexity involved in estrogen action. The key to understanding antiestrogen action is the ER, so we will now review progress in the regulation of the protein as it pertains to issues in breast cancer and antiestrogen responsiveness.

VI. Estrogen Receptor Regulation

The discovery of the ER and the fundamental role it plays in estrogen and antiestrogen action naturally has focused interest on the regulation of this nuclear transcription factor. However, progress in elucidating regulatory pathways between 1970 and 1986 had been slow partly because of the misinterpretation of data derived from the available laboratory models. In this section, we will review the change that has occurred in our basic understanding of estrogen action in cell culture.

A. Estrogen Withdrawal

Estrogen withdrawal is one of the principal treatment strategies for breast cancer (reviewed in Santen *et al.*, 1990; Jordan and Murphy, 1990). Nevertheless, throughout the 1970s and early 1980s, the direct effects of estrogen on breast cancer cell growth in culture were extremely difficult to demonstrate and results were hard to interpret. The discovery that the standard laboratory cell culture model was flawed is an important lesson that has multiple ramifications in science. Lippman and Bolan (1975) first showed that the ER-positive MCF-7 breast cancer cell line was growth inhibited by the antiestrogen tamoxifen, but this effect could be reversed by the addition of E_2. The action of E_2 alone, compared with controls, was not particularly dramatic. The inability of the research community to provoke breast cancer cell

growth reproducibly in cell culture was the subject of an intense debate for approximately a decade (1975 to 1986) and there were even suggestions that because estrogen could only cause MCF-7 cells to grow into tumors in estrogen-treated athymic animals (Shafie, 1980) but estrogen could not cause growth in vitro, then a second hormonal messenger was necessary in vivo to support growth. At the time, this was not unreasonable because both estrogen and prolactin were required for the growth of dimethylbenzanthracene-induced rat mammary tumors (Welsch, 1985).

Despite the inability to demonstrate a direct effect of estrogen-stimulated growth in all laboratories, Lippman's group did show that ZR-75 cells would respond to estrogen in a defined medium (Allegra and Lippman, 1978), and a reproducible model of estrogen-stimulated prolactin synthesis in primary cultures of cells from primary tumors also was established (Lieberman *et al.*, 1978). This latter model was used to define the structure-activity relationships of numerous antiestrogens (Lieberman *et al.*, 1983a,b; Jordan *et al.*, 1984, 1986, 1988a; Jordan and Lieberman, 1984). However, there was no adequate explanation for the finding that antiestrogens always depressed control values despite vigorous removal of all known estrogen from the culture system through serum stripping with charcoal.

The breakthrough came with the discovery that the pH indicator, phenol red, was present in micromolar concentrations in cell culture media (Berthois *et al.*, 1986). The structure of phenol red is reminiscent of the estrogens originally synthesized (fig. 5) by Sir Charles Dodds in the 1930s (Dodds and Lawson, 1936). Removal of phenol red indicator from culture media dramatically altered the cellular response to exogenous estrogen. Now, control values were not depressed by antiestrogens but E_2 did cause a huge increase in the growth response of ER-positive breast cancer cell lines in culture. As predicted, antiestrogens competitively inhibited estrogen-stimulated growth and exhibited partial agonist actions (Berthois *et al.*, 1986).

Clearly, breast cancer cells were grown unintentionally in a fully estrogenized medium, so studies of exogenous estrogen action and estrogen withdrawal were impossible. Estrogen was always present. To place this in perspective, we now know that the growth response to estrogen is so exquisitely sensitive that less than 10^{-10} M will produce maximal effects. The concentration-response curve that extends between 10^{-12} and 10^{-10} M is within the lower range of circulating levels of estrogen in postmenopausal women but often beyond the range of routine radioimmunoassays. In contrast to the profound sensitivity of replication to estrogen stimulation, the action of estrogen to induce differentiation functions of progesterone receptor or prolactin synthesis requires ten times more estrogen.

Interestingly enough, phenol red was not the actual estrogenic stimulus. Different lots of phenol red from different manufacturers had different levels of estrogenicity (Welshons *et al.*, 1988), but John and Benita Katzenellenbogen demonstrated that the phenol red alone could not account for the estrogenicity seen (Bindal *et al.*, 1988). They isolated a contaminant, produced during manufacture, that was a potent estrogen (Bindal and Katzenellenbogen, 1988). The compound is a dimerization product of components used in the synthesis of phenol red (fig. 5).

The discovery of an estrogenic contaminant in phenol red indicator is analogous to a research problem encountered in the 1930s during the first synthetic attempts to define the minimal structure of an estrogen. Anol, a simple phenol derived from anethole (fig. 5), was reported to possess extremely potent estrogenic activity with lng inducing estrus in all rats (Dodds and Lawson, 1937a). These results were not confirmed using different preparations of anol (Dodds and Lawson, 1937b; Zondek and Bergman, 1938), but it was discovered that dimerization of anol to dianol can occurr during drug synthesis and this impurity, which was know to have estrogenic

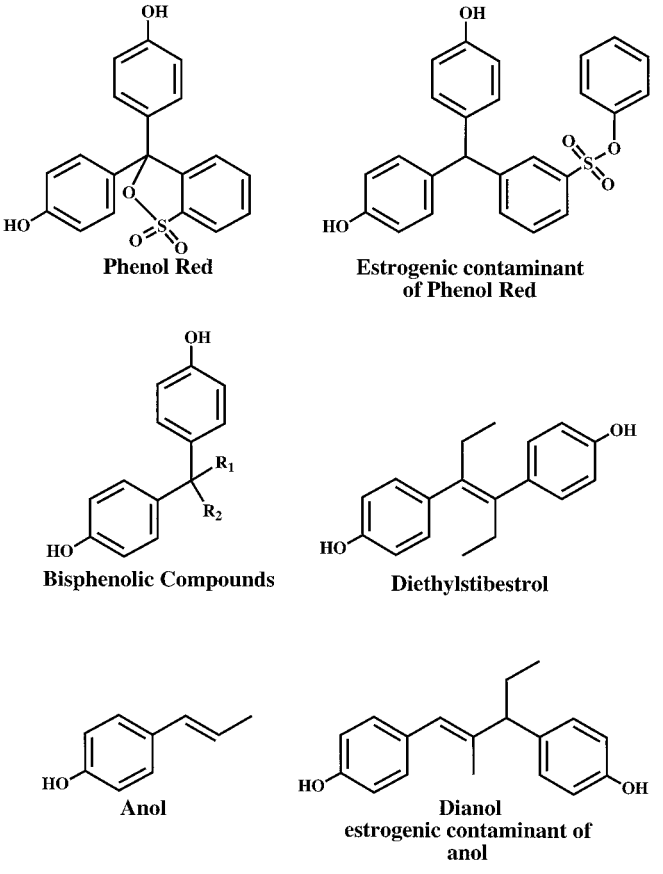


FIG. 5. The structure of the estrogenic contaminant of phenol red indicator present in culture media (Katzenellenbogen *et al.*, 1996; Bindal and Katzenellenbogen, 1988). The contaminant is a bisphenolic compound reminiscent of the nonsteroidal estrogens first reported in the 1930s (Dodds and Lawson, 1936). At that time anol was believed to be the minimal structure with estrogenic activity (Dodds and Lawson, 1937a,b), but a dimerization product was found to contaminate some samples (Campbell *et al.*, 1938a,b). The structure is very similar to the potent synthetic estrogen, diethylstilbestrol (Dodds *et al.*, 1938a).

properties (Campbell *et al*., 1938b), was responsible for the anomalous results (Campbell *et al*., 1938a). At approximately the same time, Dodd's group discovered that the diethyl substitution at the ethylenic bond of stilbesterol produces the potent estrogen diethylstilbesterol (Dodds *et al*., 1938a,b). This discovery was to revolutionize therapeutics with estrogen, and high-dose diethylstilbesterol therapy became the standard endocrine treatment for breast and prostate cancer before the discovery of antiestrogens (Haddow *et al*., 1944).

B. Receptor Regulation

With the discovery (1986) that phenol red was an estrogenic principle in cell culture, it was now possible to address the issue of ER regulation in breast cancer cells. Short-term growth in phenol red-free media can be used to determine the effects of exogenous estrogens and antiestrogens on receptor dynamics.

The regulation of ER expression in human breast cancer cells is a complex and multifaceted process that varies between different cell types and is also differentially regulated by estrogen and antiestrogens. The understanding of how different estrogens and antiestrogens affect the expression of ER in different cell types may be important in optimizing the development of new antiestrogen therapies that do not promote progression to hormone nonresponsive phenotypes. Currently, two models of ER regulation have been proposed (Pink and Jordan, 1996) that begin to elucidate how estrogens and antiestrogens direct the expression of the ER in T47D and MCF-7 cells.

In the MCF-7 cell culture system, Model I regulation dictates the response of the cell to treatment with estrogen or antiestrogens. This model is defined by down-regulation of ER expression at both the mRNA and the protein level with estrogen treatment. However, the partial antiestrogen 4-OHT (see Section VII.A.) has no effect on the mRNA levels but causes a net accumulation of ER protein by stabilization. The pure antiestrogen, ICI 182,780 (see Section VII.B.), causes a marked reduction in ER protein levels but has no effect on the mRNA levels. Thus, each of these compounds has a dramatically different effect on the expression of the ER both at the mRNA and at the protein level.

The T47D human breast cancer cell line exhibits Model II regulation. This is defined by an increase mRNA expression and a maintenance of ER protein levels with estrogen treatment. Upon treatment with 4-OHT, there is little effect on the steady-state ER mRNA levels. On the other hand, ICI 182,780 causes a marked reduction in ER protein levels and lowers levels of ER mRNA. These examples illustrate two very different mechanisms of estrogen and antiestrogen effects on ER expression in two ER-positive human breast cancer cell lines. These short-term studies could explain the response of these breast cancer cells to long-term estrogen deprivation (see Section VI.C.).

The transcriptional regulation of the ER in breast cancers seems very complicated; however, there have been recent advances in elucidating a mechanism. The control of ER expression allows the cell to increase or decrease the levels of ER in the cell according to the requirements for survival. The regulation of ER expression also plays an important role in the ER status of a cell during tumor progression. Clearly, discovery of the mechanisms for receptor regulation or re-activation hold the promise of being a valuable therapeutic target to maintain antiestrogen sensitivity.

Transcription of the ER can be initiated at two separate promoters, P_0 or P_1 (Keaveney *et al*., 1991), although the principal transcriptional start site is P_1 (Green *et al*., 1986). deConinick and colleagues (1995) found that there is an important transcriptional regulatory element in the 5′-untranslated leader sequence in the ER gene. They showed that this sequence contains two binding sites for a *trans*-acting DNA-binding protein called ER factor 1 (ERF-1). ERF-1 is expressed in higher levels in ER-positive and endometrial carcinomas and in lower amounts in normal human microvascular endothelial cells. This suggests that a correlation exists between the expression of ERF-1 and the amount of ER expressed in a given cell. The challenge is to discover whether the expression of ERF-1 is tightly regulated or whether it is susceptible to subtle changes in the cellular environment.

Recently, McPherson and colleagues (1997) cloned the gene for the ERF-1 transcription factor and also showed that ERF-1 is a member of the developmentally regulated AP-2 transcription factor family. Using a 30 base pair imperfect palindromic sequence that has been defined as a high-affinity binding site for ERF-1, they showed that ERF-1 bound specifically so they used this concept to affinity purify the ERF-1 protein. The ERF-1 is approximately 50 kDa and the predicted peptide sequence shares 65% identity and 83% similarity with AP2α and is the same as AP2γ. In vitro translated ERF-1 showed activity similar to native ERF-1 and an AP2 polyclonal antibody that specifically reacts with ERF-1. The mechanism for ERF-1 to activate transcription of the ER has yet to be elucidated.

Other positive regulatory elements exist in the ER gene further upstream from the transcriptional start site (−3778 to −3744) (Tang *et al*., 1997). The *cis*-acting element is of a 35 base pair element termed ER-EH0 that is active in ER-positive but not ER-negative cells. ER-EH0 contains not only an AP-1 but also flanking sequences that bind an as yet unknown factor. Both of the flanking sequences are required for enhancer activity. Tang *et al*. (1997) suggest that the ER-EH0 enhancer element is the predominant *cis*-acting factor in differential ER expression.

We believe it is important to stress that the regulation of the ER is a primary therapeutic target. Further progress can be facilitated by the description of models

for the loss of receptor regulation. However, this is an area of some controversy. Although dogma dictates that breast tumors progress from ER positive to ER negative, the principle is not demonstrated easily in cell culture.

C. Loss of the Receptor

Studies of long-term estrogen deprivation of MCF-7 breast cancer cells in culture illustrate that selection pressure occurs with an initial increase in ER content so that the cells now grow maximally in the apparent absence of estrogen (Katzenellenbogen *et al*., 1987; Welshons and Jordan, 1987). The cells, however, still respond to antiestrogens with an inhibition of growth. Either they have become hypersensitive (Masamura *et al*., 1995) to other environmental estrogens leached from laboratory plasticware or the cells have devised alternative growth pathways. To the first point, several chemicals have been identified that might be responsible for supporting the growth of breast cancer cells in an "estrogen-free" environment (fig. 6) (Krishnan *et al*., 1993; Soto *et al*., 1991; White *et al*., 1994) and it would illustrate the need for a breast cancer treatment strategy in patients that blocks the ER continuously. Highly estrogen-sensitive clones will be selected to develop and grow toward any source of weak estrogens. To the second point, numerous estrogen-unresponsive clones of MCF-7 cells have been developed from the original stocks kept in a phenol red-free environment for many months. One cell line, MCF-7/5C, is ER positive but does not respond to either estrogens or antiestrogens. The ER is not mutated but the receptor is incapable of initiating progesterone receptor synthesis in the presence of estrogen (Jiang *et al*., 1992b). The cell type is reminiscent of the clinical situation of breast cancers that are ER positive but progesterone receptor negative and are less responsive to endocrine therapy (Jordan *et al*., 1988b).

The cell line, MCF-7/2A, is another clonal estrogen-independent cell line derived from MCF-7 stocks maintained in an estrogen-free state for several years (Pink *et al*., 1995). The cells are unique because they express wild-type ER as well as an ER that has a duplication of exons 6 and 7 in the LBD (Pink *et al*., 1996b). The high molecular weight ER does not bind estrogens or antiestrogens (Pink *et al*., 1997). However, there is no evidence that this mutant receptor is responsible for estrogen-independent growth.

All of the studies of estrogen deprivation so far described have used one single cell line, MCF-7, and the results from different groups demonstrate that numerous clones develop to survive the loss of the primary growth stimulus, estrogen (Cho *et al*., 1991; Clark *et al*., 1989b). However, it is now apparent that different cell types respond differently to estrogen withdrawal than MCF-7 cells. ER levels seem to be regulated in different ways (Pink and Jordan, 1996).

Murphy *et al*. (1989, 1990b) first illustrated the progression of an ER-positive T47D breast cancer cell line to an ER-negative state after prolonged estrogen deprivation. Pink *et al*. (1996a) subsequently demonstrated that the loss of the ER at the mRNA and protein level in this T47D cell line was irreversible The resulting cell line (T47D: C4:2) is resistant to antiestrogens and grows maximally in estrogen-free media. This raised the questions that if the receptor is lost, how is it lost and can it be reactivated?

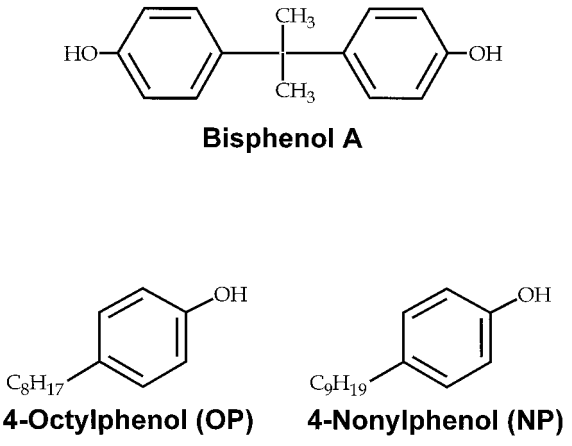


FIG. 6. The structure of environmental laboratory estrogens that can support the growth of breast cancer cells in an "estrogen-free" environment (Soto *et al*., 1991; White *et al*., 1994; Steinmetz *et al*., 1997).

One area of intense investigation is the hypermethylation of CpG islands in the 5′-promoter region of the ER gene that could silence ER synthesis. ER-negative human breast cancer cells grown in culture have an enhanced ability to methylate DNA which may explain the silencing of ER expression. Additionally, using the ER-negative MDA-MB-231 breast cancer cell line, treatment with DNA methylation inhibitors actually caused the re-expression of the ER at the protein level (Ferguson *et al*., 1995). This re-expressed ER is functional because it can activate the transcription of estrogen-responsive genes. However, this is not a universal cellular phenomenon, so further studies need to be undertaken. We have noted in our T47D cell lines that the CpG islands are not hypermethylated when the ER is lost (Chen *et al*., 1997).

The finding that ER can be retained in some cell lines in response to estrogen deprivation but not in others has clinical relevance. The levels of expression of the ER in clinical tumors as they progress to a hormone-independent state has become controversial. A recent review proposes that the actual loss of ER expression in ER-positive tumors does not occur (Robertson, 1996). However, the primary endocrine therapy today is tamoxifen and this has estrogen-like properties and may, as a result, preserve ER status. This is consistent with the observations in both cell and tumor models of antiestrogen resistance (Mullick and Chambon, 1990; Gottardis and Jordan, 1988; Katzenellenbogen *et al*., 1995). The receptor is not lost. However, the loss of the ER may occur in tumors that become resistant to the pure an-

tiestrogens (see Section VII.B.). In part, the difference in the biological response may be a result of the different mechanisms of action for tamoxifen and pure antiestrogens on the ER signal transduction pathway (see Section VIII.). This is the focus of current clinical investigations.

VII. Antiestrogen Classification

Antiestrogens can be classified into two major groups: analogs of tamoxifen or its metabolites (type I) which have mixed estrogenic/antiestrogenic actions in laboratory assays and pure antiestrogens (type II) that have no estrogen-like properties in laboratory assays. There is emerging information to suggest that the classification may also be based on different mechanisms of action (see Section VIII.).

A. Type I

The triphenylethylene structure of tamoxifen has provided the basis for several new analogs that are being investigated in the clinic. The finding that tamoxifen is metabolized to 4-OHT, a potent antiestrogen (Jordan *et al.*, 1977), also has provided a central theme for drug development (fig. 7).

The principal tamoxifen analogs currently under investigation are illustrated in figure 7. Toremifene, or chlorotamoxifen, has been investigated thoroughly as an antiestrogen and antitumor agent in the laboratory (Kangas *et al.*, 1986; Kangas, 1990) and currently is being used for the treatment of advanced breast cancer and tested as an adjuvant therapy. The compound is of interest because it does not produce DNA adducts in rat liver and, as a result, is not a potent carcinogen in rat liver (Hard *et al.*, 1993; Hirsimaki *et al.*, 1993) (see Section XIII.).

Idoxifene is a metabolically stable analog of tamoxifen synthesized to avoid toxicity reported with tamoxifen in the rat liver (fig. 7) (McCague *et al.*, 1989, 1990). Substitution of halogens in the 4-position of tamoxifen is known to reduce antiestrogen potency by preventing conversion to 4-OHT (Allen *et al.*, 1980) and it was argued that reduced demethylation of the side chain also would avoid the formation of formaldehyde in the liver (McCague *et al.*, 1989, 1990). Idoxifene is a 4-iodopyrrolidino derivative of tamoxifen that has antiestrogenic and antitumor properties in laboratory rats (Chander *et al.*, 1991).

Droloxifene, or 3-hydroxytamoxifen, has been studied extensively as an antiestrogen and an antitumor agent in the laboratory (fig. 7) (Hasman *et al.*, 1994). This drug does not form DNA adducts under laboratory conditions (White *et al.*, 1992) or produce liver tumors in rats (Hasman *et al.*, 1994). Extensive clinical testing has shown activity in the treatment of advanced breast cancer in postmenopausal patients (Rausching and Pritchard, 1994).

TAT-59 is a prodrug that is being developed for the treatment of advanced breast cancer (fig. 7). TAT-59 has

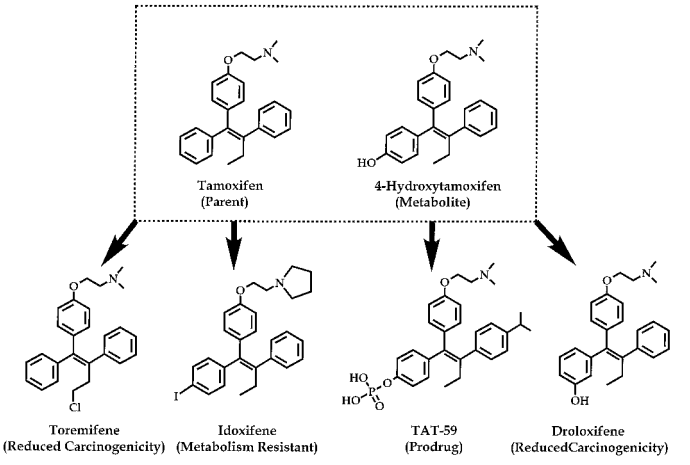


FIG. 7. The principal tamoxifen analogs currently under investigation are toremifene, idoxifene, droloxifene, and TAT-59. Each triphenylethylene is a derivative of tamoxifen, the parent drug, or its metabolite 4-OHT.

been shown to inhibit the growth of ER-positive, DMBA-induced rat mammary carcinomas (Toko *et al.*, 1990). The drug inhibits the growth of estrogen-stimulated, ER-positive breast cancer cells transplanted into athymic mice (Koh *et al.*, 1992; Iino *et al.*, 1994). The drug is activated metabolically to a dephosphorylated form (Toko *et al.*, 1990) that binds with high affinity to the ER (Toko *et al.*, 1992). Clinical studies using TAT-59 for the treatment of advanced breast cancer have not been published.

Additionally, compounds are being investigated that do not resemble triphenylethylenes but do exploit the known structural requirements for high binding affinity for the ER (Jordan *et al.*, 1978) (fig. 8). The compounds LY117018 and raloxifene have high binding affinity for the ER but a lower estrogenic activity than tamoxifen when using rodent uterine assays (Black and Goode, 1980, 1981; Black *et al.*, 1983; Jones *et al.*, 1984; Jordan and Gosden, 1983a,b). They are competitive antagonists of estrogen action but also can block the estrogen-like effects of tamoxifen in the uterus (Jordan and Gosden, 1983b). This demonstrates a single mechanism of action for this class of drugs through the ER.

B. Type II

The pure antiestrogens were discovered by Wakeling and colleagues (Wakeling and Bowler, 1987). The lead compound, ICI 164,384, is a 7α-substituted derivative of E_2 that has no detectable estrogen-like properties in vivo or in vitro (Wakeling, 1994). The structure-activity relationships are well established: 7β substitution is ineffective at producing antiestrogenic activity and the length of the carbon chain determines optimal activity (Bowler *et al.*, 1989) (fig. 9). The compound ICI 182,780 is more potent than ICI 164,384 (Wakeling *et al.*, 1991) and is being evaluated as a clinically useful agent after failure of tamoxifen (see Section XI.B.).

The discovery of ICI 164,384 and ICI 182,780 has stimulated others to improve on bioavailability and the

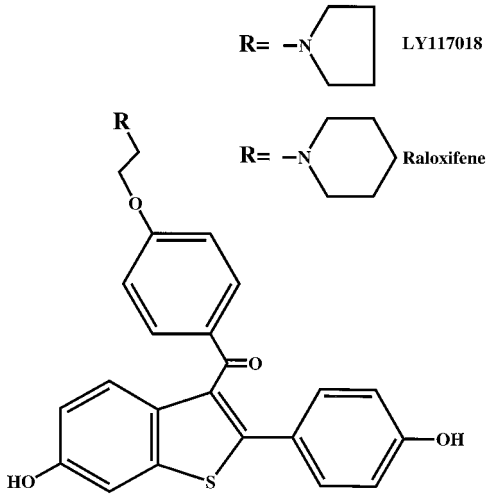
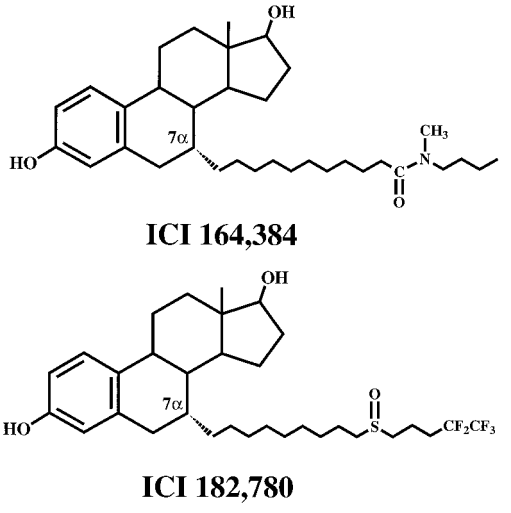


FIG. 8. LY117018 and raloxifene exploit structural characteristics necessary for high-affinity binding to the ER but have a lower estrogenicity when tested in rodent uterine assays.



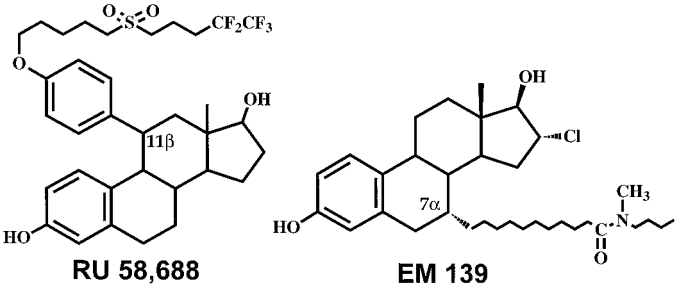


FIG. 9. The pure antiestrogens, ICI 164,384 and ICI 182,780, are derivatives of estradiol but have no estrogenic properties. ICI 182,780 is more potent than ICI 164,384 and is currently undergoing clinical investigation. The type II antiestrogens are RU 58,668, which acts as a pure antiestrogen, and EM-139, which is both an inhibitor of 17-hydroxysteroid dehydrogenase and a pure antiestrogen.

biological profile of activities. Both ICI 164,384 and ICI 182,780 are poorly soluble and have low oral activity (Wakeling *et al*., 1991) and have forced consideration of depot injections for clinical applications.

The compound RU 58,668 is substituted in the 11β-position with a long hydrophobic side chain (fig. 9) (Van de Velde *et al*., 1994, 1996). This produces the same spatial arrangement for a side chain as the 7α substitution in relation to the plane of steroid nucleus. Studies in vivo and in vitro have demonstrated that RU 58,668 has the properties of a pure antiestrogen (Van de Velde *et al*., 1994, 1996).

The compound EM-139 is both an inhibitor of 17-hydroxysteroid dehydrogenase and an antiestrogen (Li *et al*., 1995) (fig. 9). The goal is not only to block the receptor but also to reduce the conversion of estrone to the more potent estrogen, E_2, in the postmenopausal patient. As yet, this intriguing idea has only theoretical merit because an increase in E_2 production in postmenopausal women has not been described as a general method for drug resistance to an antiestrogen. On the one hand, the concept is of interest because it could produce a more efficient block of the ER. On the other hand, a more profound blockade may be produced by a combined antiestrogen and aromatase inhibitor.

VIII. Mechanisms of Antiestrogen Action

The current molecular model of estrogen action provides several potential points of weakness that can be exploited by antiestrogens (fig. 10). As described previously in Section VII., antiestrogens can be divided into two major categories based on their mechanism of action. Type I antiestrogens are the analogs of tamoxifen or structural derivatives of the triphenylethylene type of drug. Type II are the pure antiestrogens. All compounds are competitive inhibitors of the binding of E_2 to the ER but there the similarity ends. Type I antiestrogens seem to form a receptor complex that is converted incompletely to the fully activated form (Tate *et al*., 1984; Martin *et al*., 1988; Pham *et al*., 1991; Tzukerman *et al*., 1994; McDonnell *et al*., 1995; Allan *et al*., 1992). As a result of the imperfect changes in the tertiary structure of the protein, the complex is only partially active in initiating the programmed series of events necessary to orchestrate gene activation (Metzger *et al*., 1988; Jordan, 1984).

Studies in vitro demonstrate that very low concentration of triphenylethylene-type antiestrogens can cause a single round of replication in breast cancer cells, but high concentrations of these antiestrogens are completely inhibitory (Berthois *et al*., 1986). It is possible that the modest partial estrogen-like action at low concentrations causes the tamoxifen flare that sometimes is observed when therapy is started in patients with bony metastases (Reddel and Sutherland, 1984). Once steady-state levels of the drug have been achieved (approximated 4 to 8 weeks with 20 mg/day), symptoms will have disappeared and the patient will experience a response to therapy (Furr and Jordan, 1984). It is important

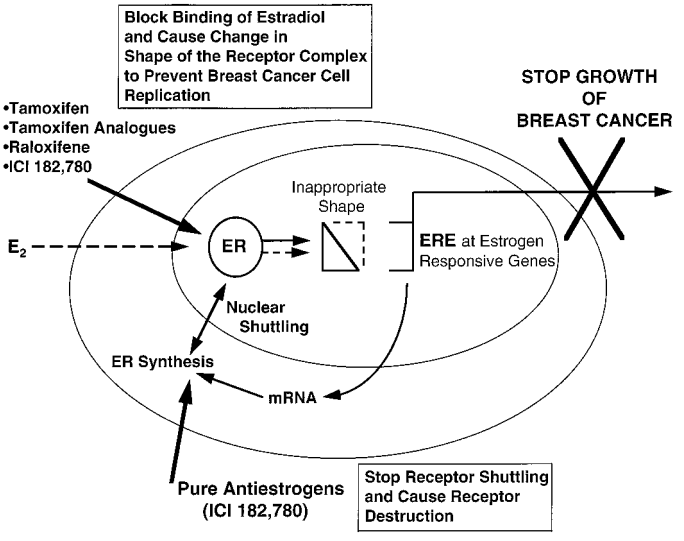


FIG. 10. The current molecular model of antiestrogen action. Different antiestrogens act at several points of weakness in the ER signal-transduction pathway.

therefore, to be able to identify tumor flare and not prematurely terminate a beneficial therapy. Nevertheless, a recent report (Vogel *et al.*, 1995) has demonstrated that clinicians often prematurely terminate antiestrogen treatment based on changes in bone scintigraphy misinterpreted as progressive disease. Because there are clear toxicological advantages in disease control with antiestrogens, a premature change to chemotherapy may be inappropriate.

Several type II antiestrogens are available for study in the laboratory (Wakeling, 1994; Van de Velde *et al.*, 1994; Dukes *et al.*, 1994; Von Angerer *et al.*, 1990) but only ICI 182,780 is being developed clinically (Wakeling *et al.*, 1991). Initially, it was believed that pure antiestrogens prevent the dimerization of receptor complexes thereby preventing binding to EREs (Fawell *et al.*, 1990). Clearly, if receptor complexes do not bind to any EREs then no genes can be activated and the compound would be a "pure" antiestrogen. However, numerous reports (Pink and Jordan, 1996; Pham *et al.*, 1991; Sabbah *et al.*, 1991) now demonstrate that pure antiestrogen-ER complexes can bind to EREs but the transcriptional unit is inactive. What is unique about the type II antiestrogens is the observation that they provoke the destruction of the ER in breast cancer cells in culture (Dauvois *et al.*, 1992), mouse uterus (Gibson *et al.*, 1991), and breast tumors in situ (DeFriend *et al.*, 1994). The ER is synthesized in the cytoplasm and transported to the nucleus where it functions as a transcription factor. A pure antiestrogen binds to the newly synthesized receptor in the cytoplasm and prevents transport to the nucleus (Davois *et al.*, 1993). The paralyzed receptor complex then is destroyed rapidly (Davois *et al.*, 1993). The complete destruction of available ER will prevent any estrogen-regulated events from occurring. Normal cells will become quiescent, whereas hormone-dependent tumors will regress rapidly because senescent tumor cells cannot be replaced by replication.

A. Receptor Mutation and Antiestrogens

Mutations in the mouse ER at residues 525 and 521/522 can abolish the ability of the ER to bind estrogen, thus prohibiting transactivation in response to this hormone. The mutant receptors retain their partial agonist response to tamoxifen similar to that of the wild-type ER in the presence of tamoxifen (Danielian *et al.*, 1993). Mutations in the ER have been used further to study the pharmacology of estrogen agonist and antagonists. As stated in Section V.A., the ER contains an AF-1 and an AF-2 region. The AF-2 activity depends on the presence of a putative amphipathic α-helix made up by the residues 538 to 552, and when hydrophobic residues (543, 544, 547, 548) of this region are mutated, estrogen-induced transactivation is reduced whereas the ligand- and DNA-binding function are not affected substantially (Mahfoudi *et al.*, 1995). The pharmacology of antiestrogens is affected dramatically. For example, tamoxifen and ICI 164,384 act as agonists in ER-negative cells transfected with the mutant ERs. Although these mutations have been suggested to promote drug resistance to tamoxifen, no clinical or laboratory evidence supports this conjecture (see Section XIV.B.).

The promoter context also can affect the transcriptional activity of both the AF-1 and the AF-2 of the ER. This has been demonstrated using a series human ER mutants (Tzuckerman *et al.*, 1994). It has been shown that both AF-1 and the AF-2 functions are required in certain promoter contexts, whereas only one of these activators is required in other promoter contexts. Using the ERs mutated at amino acids 538, 542, or 545, it has been shown that the antagonist activity of tamoxifen is a result of its insufficient ability activate the AF-2 function. However, in certain situations, tamoxifen can act as an agonist and efficiently activate transcription. It follows that if a promoter only requires the AF-1 function to activate gene transcription, tamoxifen binding may be sufficient.

The conformational changes induced by agonists and antagonists have been shown to be distinct through the use of protease digestion assays (McDonnell *et al.*, 1995). However, these studies are unable to differentiate between different types of antagonists like pure and partial antiestrogens. McDonnell and colleagues (1995) showed that functional differences between different antiestrogens depends on the cell type and promotor context. The differential ability of ER antagonists to modulate transcriptional activity is illustrated further using a mutant ER in which the AF-2 has been inactivated. 4-OHT, raloxifene, and ICI 164,384 all had different transcriptional activation profiles. When an ER mutant retaining the AF-2 region alone or neither AF sites was tested, no activity was seen (McDonnell *et al.*, 1995).

B. Interactions with Estrogen Response Elements

The interactions of the ER with EREs also depends on the nature of the ligand to which it has bound. When the effects of binding of estrogenic and different antiestrogenic ligands to an ERE are quantitated, it was found that E_2-ER and 4-OHT-ER bound a singlet ERE with similar affinity whereas ICI 164,384-ER did not bind (Klinge *et al*., 1992). However, at saturation, 4-OHT-ER binds 50% the level of E_2-ER binding. When the tandem copies of EREs were tested, E_2-ER exhibited cooperative binding whereas 4-OHT-ER and ICI 164,384-ER displayed little or no cooperativity. Therefore, specific ligand binding can alter binding affinity of the ER to DNA and the amount of receptor that is saturated presumably by inducing different conformations in the ER protein. Further studies of mechanism through which antiestrogens antagonize the transcription of estrogen-responsive genes through differential binding to EREs show that the flanking sequences and stereoalignment of EREs are important (Anolik *et al*., 1996).

A further investigation of antiestrogenic ligands demonstrated that when 4-OHT-ER binds to DNA one molecule of 4-OHT dissociates from the ER dimer (Klinge *et al*., 1996). Under the same conditions, tamoxifen aziridine, which covalently attaches to the ER, show a binding stoichiometry identical with that of E_2-ER, which is one dimeric receptor per ERE compared with one monomer of 4-OHT-ER per ERE. When DNA footprinting was used to determine ER-ligand binding to adjacent EREs, identical high-affinity binding was observed for unliganded dimeric ER or ER bound to E_2, 4-OHT, and tamoxifen aziridine (Driscoll *et al*., 1996). These results suggest that ligand-induced conformation changes primarily affect how the ER interacts with the components of the transcription initiation complex thereby mediating transcriptional activation.

IX. Antiestrogens and the Cell Cycle

The molecular description of the signal transduction pathway for estrogen and its modulation by antiestrogens now raises the question of how the protein complexes switch cell replication on and off. During the past twelve years, there have been important advances in the regulation of the cell cycle via growth factors that perform either autocrine or paracrine functions. Although precise information of immediate early genes that could link receptor/DNA interactions with cell growth is currently lacking, a database is being developed on the cell cycle and growth factor modulation. Clearly, cell lines that can be programmed to replicate provide a powerful model to discover the triggers for replication. Additionally, the antiestrogens are proving to be effective molecular tools to check the progression of the cell cycle.

The first experiments that illustrated the connection between antiestrogens and the cell cycle showed that thymidine incorporation in ER-positive cells was reduced on treatment with antiestrogens and that no effect was seen in ER-negative cells (Lippman and Bolan, 1975; Lippman *et al*., 1976). Subsequent studies determined that the arrest of cells occurred in the G1 phase of the cell cycle resulting in a lower proportion of cell in S phase (Sutherland *et al*., 1983a,b; Taylor *et al*., 1983; Osborne *et al*., 1983, 1984; Wakeling *et al*., 1989; Musgrove *et al*., 1989). Studies using synchronized cells demonstrated that antiestrogens could only inhibit the growth of cells that were in early to mid G1 phase of the cell cycle (Taylor *et al*., 1983; Musgrove *et al*., 1989). Additionally, Lykkesfeldt and colleagues (1984) studied the effects of tamoxifen treatment on the cell cycle kinetics of MCF-7 cells. They showed that after tamoxifen treatment cells not only arrested in G1 but also in G2 phase of the cell cycle. In another study using newborn calf serum as a growth inhibitory agent, it was shown that MCF-7 cells treated with newborn calf serum had an elongated G1 transit time whereas estrogen treatment shortened G1 transit time (Lykkesfeldt *et al*., 1986).

MCF-7 cells have been used to identify cell cycle regulatory genes that could be potential targets for antiestrogen action (Watts *et al*., 1994). Cells treated with steroidal and nonsteroidal antiestrogens had a significant decrease in cyclin D1 mRNA, which suggests that the G1 cyclins may be a target of antiestrogens to block entry into the S phase. Further studies using the pure antiestrogen ICI 182,780 demonstrated a reduction of the proportion of cells in the S phase and an increased proportion of hypophosphorylated Rb (Watts *et al*., 1995). Cyclin D1 message and protein were down-regulated significantly by the pure antiestrogen, but cdk protein levels remained unaffected. Nevertheless, a decrease in the kinase activity occurred after longer periods of treatment. The effects of ICI 182,780 on cdk inhibitors also were assessed showing an increase in the expression of p27KIP1 and p21WAF1/CIP1 after longer treatments (Watts *et al*., 1995).

Similar cell cycle effects are seen using T47D human breast cancer cells. Antiestrogens reduced the expression of cyclin D1 activity and cell cycle arrest occurred in the G1 phase (Musgrove *et al*., 1993; Wilken *et al*., 1996). The mRNA and protein levels of cyclin D3 or E, cdk2, and cdk4 are not affected. Finally, the treatment with ICI 164,384 resulted in a reduction in the amount of hyperphosphorylated Rb. All of the these effects of the antiestrogen were reversed by E_2 treatment.

These studies in two breast cancer cell lines provide a valuable insight into the consequences of estrogen and antiestrogen action. However, an important issue is not addressed. The critical question is whether the ER controls replication directly by nuclear interactions or via growth factor-mediated mechanisms.

X. Antiestrogens and Growth Factors

During the past 20 years, there has been considerable focus on the mechanisms whereby cells modulate the growth stimulus or stop growing when the task of replication is complete. The identification of families of stimulatory or inhibitory growth factors that affect the same cell (autocrine factors) or adjacent cells (paracrine factors) has revolutionized the concepts of hormonal regulation. The ideas have been translated during the past decade from general physiology to be applied to cancer control. We will illustrate briefly the studies that are relevant to our current understanding of antiestrogen action; however, the reader is referred to a recent review by Dickson and Lippman (1995) for an in-depth treatise on growth factors.

We will describe the effects of antiestrogens on the regulation of three different growth factor systems: transforming growth factor (TGF)α, TGFβ, and the insulin-like growth factor (IGF) system. Both TGFα and the IGFs are growth stimulatory and are modulated by estrogen. By contrast, TGFβ consists of a family of three separate proteins that are growth inhibitors.

A. Transforming Growth Factor α

Estrogen is believed to increase the production of TGFα and, through autocrine activation of the epidermal growth factor receptor, encourage replication. However, TGFα alone cannot substitute for estrogen. MCF-7 cells transfected with the cDNA for TGFα are not tumorigenic in athymic mice (Clark *et al*., 1989a).

Studies by Wakeling and colleagues (1989) compared the ability of the pure antiestrogen, ICI 164,384, and the partial antiestrogen tamoxifen or its active metabolite, 4-OHT, to attenuate the stimulatory effects of TGFα on MCF-7 cells. They showed that when MCF-7 cells are treated with TGFα, both antiestrogens partially block the stimulatory effect in the absence of E_2, but the ICI 164,380 is more effective. In contrast, studies using EGF instead of TGFα showed that antiestrogens could not block the actions of the growth factor (Cormier and Jordan, 1989) and also that antiestrogens could not block the paracrine influence of ER-negative cells from stimulating MCF-7 cells in vitro (Robinson and Jordan, 1989b). In addition, it is known that estrogens can induce the expression of TGFα in estrogen-responsive breast cancer cell lines, whereas antiestrogens generally decrease TGFα expression in vitro (Salomon *et al*., 1989) and in vivo (Gregory *et al*., 1989).

TGFα apparently is essential for estrogen-stimulated, anchorage-independent growth. TGFα or epidermal growth factor receptor antibodies can negate the E_2-stimulated, anchorage-independent growth of MCF-7 cells on soft agar (Manni *et al*., 1991). Progesterone or prolactin were not affected by the antibodies.

The effects of antiestrogens on TGFα expression in vivo have not been studied extensively; however, one study shows that tamoxifen is capable of down-regulating tumor TGFα expression in postmenopausal women with ER- and PR-positive disease but not in women with ER- and PR-negative disease (Noguchi *et al*., 1993).

The regulation of TGFα remains unclear. A few putative half-site EREs have been identified in the promotor region of the TGFα gene, but other sites in the promotor region are required for gene activation (Saeki *et al*., 1991). Constructs of the EREs alone do not appear to respond to estrogen action unless the cells are supertransfected with ER (El-Ashry *et al*., 1996). By contrast, ER-negative cells that are stably transfected with ER (Jiang and Jordan, 1992; Catherino *et al*., 1995) will induce TGFα mRNA in response to estrogen (Jeng *et al*., 1994).

Perhaps, most interesting is the effect of antiestrogens. Raloxifene acquires the ability to initiate TGFα synthesis when ER-negative cells are stably transfected with a 351-mutant ER (Levenson *et al*., 1997). However, in ER-negative transfectants containing wild-type ER, raloxifene is a complete antiestrogen. 4-OHT acts as an estrogen (induction of TGFα) in both wild-type and mutant ER stable transfectants (Levenson *et al*., 1998). The pure antiestrogen ICI 182,780 acts as an antiestrogen in all transfectants. Because antiestrogens produce different effects in transfectants expressing wild-type or mutant ER, and because 4-OHT and estrogen can both initiate TGFα mRNA transcription equally, this provides a unique model to determine which proteins associate with the antiestrogen-ERE complex to make it so promiscuous. We will consider this aspect of antiestrogen pharmacology in Section XII. and unite the concepts of receptor conformation and efficacy in Section XVII.

B. Transforming Growth Factor β

The TGFβ family of inhibitory polypeptides consists of three or more 25 kDa members which are able to homo- or heterodimerize to form complexes that interact with the TGFβ receptor (TGFβR). These peptides are implicated in breast cancer and have been found to be overexpressed and correlate with tumor progression (Gorsch, 1992). TGFβ binds to any of the different characterized TGFβRs. The receptor consists of a heterodimeric complex, one part of which is a binding protein that is unable to signal and another part that is believed to transduce signals to the cell through serine-threonine kinase activity (Bützow *et al*., 1993; Ohtsuki and Massague, 1992; Shibanuma *et al*., 1991; Massague, 1992; Ebner *et al*., 1993; Attisamo *et al*., 1993). The type II receptor is responsible for the binding of TGFβ and its ligand affinity. The type II receptor may also determine whether the effects of TGFβ binding result in growth regulation or differentiation. The ability of TGFβ to promote tumor progression is counterintuitive because TGFβ usually produces either growth inhibition or differentiation, both of which are not involved in tumor progression. Further study clearly is needed in vivo to determine

what cooperating factors dictate the effects of TGFβ on different cell types, because the results may be critical to understanding the success or failure of antiestrogen therapy.

The effect of tamoxifen on the production of TGFβ is an area of great interest. Elucidation of a mechanism could provide an explanation for the cell cycle effects of tamoxifen in ER-positive cells and also provide an explanation for the sporadic reports of the success of tamoxifen treatment in ER-negative breast cancer. Much work has been completed in cell culture but there are important translational aspects of the research that are relevant in understanding the action of tamoxifen.

Tamoxifen has a direct effect on the production of TGFβ in breast cancer cells. TGFβ expression increases in MCF-7 cells (Knabbe *et al*., 1987), and further study has shown a differential activation of members of the TGFβ family. However, the results are variable. Some studies report an increase in TGFβ-2 with tamoxifen (Jeng *et al*., 1993), whereas others demonstrate rises in TGFβ-1 (Chen *et al*., 1996; Perry *et al*., 1995). Knabbe and colleagues (1996) have shown that antiestrogen treatment causes an increase in TGFβ-1 via a nontranscriptional pathway and TGFβ-2 increases occur through transcriptional activation by TGFβ-1 (Knabbe *et al*., 1996). This observation has been translated to the clinic. Patients that respond to tamoxifen therapy show increases in TGFβ-2 and those that do not respond show no change in TGFβ-2 plasma levels. Knabbe's study suggests that the results of measuring either TGFβ-1 levels (which transcriptionally activates TGFβ-2) or TGFβ-2 (Kopp *et al*., 1995) in the plasma can be used as a predictive test for the efficacy of tamoxifen therapy.

Some support for the central role of TGFβ-2 comes from sampling tumors directly. When TGFβ mRNA levels from ER-positive breast tumors were monitored before and during tamoxifen therapy, the results were variable. Changes in TGFβ-1 and TGFβ-2 did not correlate with tamoxifen treatment, but there was a significant correlation between treatment and changes in TGFβ-2 in some tumors. The authors concluded that response to tamoxifen therapy may be mediated through an increase in the expression of a particular TGFβ isoform (MacCallum *et al*., 1996).

The effect of tamoxifen on ER-negative tumors is far more controversial. Perry and coworkers (1995) have compared and contrasted the effect of tamoxifen on the induction of TGFβ-1 in an ER-positive and an ER-negative cell line. After long-term treatment, the expression of TGFβ-1 increased, independent of ER status, but an accumulation of cells in G1/G0 and an increase in apoptosis occurred concurrently. This conclusion tends to support a model of the direct effect of tamoxifen on ER-negative cells.

By contrast, it is possible that the growth of an ER-negative cell is controlled by a paracrine mechanism. Perhaps the ER-positive cell produces TGFβ in response to tamoxifen, but the secreted growth factor stops the growth of the adjacent ER-negative cells (Knabbe *et al*., 1987). It is known that ER-negative breast cancer cells have a high density of TGFβ receptors (Artega *et al*., 1988) and the cells respond to TGFβ by growth inhibition (Jeng *et al*., 1993). The hypothesis that an ER-positive cell can control the growth of ER-negative cells during tamoxifen therapy has been demonstrated in vitro (Knabbe *et al*., 1987). However, this has not been possible to test in animal models. Different mixes of ER-positive and ER-negative cells were inoculated into athymic animals and treated with the antiestrogen toremifene (Robinson and Jordan, 1989a). Regrettably, in this model, the antiestrogen was unable to control heterogeneous tumor growth.

However, the laboratory finding that tamoxifen can induce TGFβ in fibroblasts (Colletta *et al*., 1990; Benson *et al*., 1996; van Roozendaal *et al*., 1995) has introduced a new mechanistic dimension to understand the control of ER-negative disease by tamoxifen. Clearly, if TGFβ can be induced in the supporting stromal cells of a breast cancer tumor during tamoxifen therapy, the paracrine growth inhibitor could control the proliferation of ER-negative cells. Butta and coworkers (1992) found that TGFβ production increases in stromal cells during tamoxifen therapy. Although these data illustrate that a complex cellular conversation occurs to regulate cell growth, the fact that tamoxifen is not usually successful in ER-negative disease means that the pathways are not necessarily dominant. Nevertheless, the realization that TGFβ can act both as a growth inhibitor and as a growth stimulator may ultimately make the pathways important to explain tamoxifen failure.

C. Insulin-Like Growth Factor

Many experiments have shown that IGFs are potent stimulators of the proliferation of breast cancer cells. IGFs bind to specific receptors on the cell surface and also are associated with high-affinity specific binding proteins present either in the circulation or extracellularly. Once these high-affinity specific binding proteins are secreted they are able to modulate IGF activation of their cognate receptor. These IGF-binding proteins (IGFBP) are being studied for their potential use as breast cancer therapies to inhibit the growth of breast cancer cells in vitro (Yee, 1994).

Antiestrogens decrease levels of IGF receptor in ER-positive cell lines. Conversely, antiestrogens cause a marked increase in IGFBP-1 that results in a decrease in IGF-mediated cell replication (Winston *et al*., 1994). Freiss and colleagues (1990) showed that 4-OHT can reduce the number of IGF-1 binding sites in ER-positive cells which, in turn, decreases replication. Kawamura and coworkers (1994) extended earlier findings and showed that although a 2 h pulse of droloxifene or tamoxifen can reduce the replication of MCF-7 cells, there is no decrease in the binding of IGF-1 to the cell surface.

In contrast, a 4 h incubation with droloxifene causes both growth inhibition and a decrease in IGF-1 binding.

Tamoxifen clearly can have a direct regulatory effect on the IGF-1 system, but antiestrogens also can modulate the IGF-1 system differentially in different target tissues. Estrogen induces IGF-1 in the uterus and it is believed to be responsible for the uterotropic response observed in stromal and epithelial cells. Tamoxifen also produces a uterotropic effect and doubles the expression of IGF-1. By contrast, ICI 182,780 decreases IGF-1 expression and has virtually no uterotropic effect (Huynh and Pollack, 1993).

The target tissue actions of antiestrogens to reduce IGF-1 levels could have important implications for the metastatic spread of tumor cells. IGF-1 can facilitate the growth of both ER-positive and ER-negative breast cancer cells so micrometastases may find themselves in a hostile environment without the paracrine support of tissue growth factors. Similarly, tamoxifen decreases the circulating levels of IGF-1 (Colletti *et al*., 1989; Pollack *et al*., 1990, 1992; Friedl *et al*., 1993) and causes an elevation of the circulating levels of IGFBP-1 (Lønning *et al*., 1992). Clearly, the reduction of a potent circulatory mitogen conceivably could reduce the growth rate of both ER-positive and ER-negative micrometastases.

In summary, the past decade has seen an elucidation of the role of both positive and negative growth factors in estrogen-stimulated growth. Although each effect of tamoxifen on the growth factor system may in itself be small, it is possible that the combined actions of tamoxifen are responsible for the benefits documented with tamoxifen in clinical practice.

XI. Clinical Value of Tamoxifen

The primary focus of most work with antiestrogens is related to the antitumor actions in breast cancer because it is directly relevant to the clinic. However, the clinical pharmacology of antiestrogens has been found to be complex and cannot be described simply as a blockade of estrogen action. We now will integrate the important advances that have been made in the clinical use of antiestrogens since 1984.

In 1985, tamoxifen was approved by the Food and Drug Administration (FDA) as an adjuvant therapy with chemotherapy in postmenopausal women with node-positive breast cancer, and in 1986, approval was obtained for the use of adjuvant tamoxifen alone in the same group of postmenopausal women with node-positive breast cancer.

In 1989, approval was obtained from the FDA for the use of tamoxifen in the treatment of premenopausal women with ER-positive advanced breast cancer, and in 1990, an indication as an adjuvant was approved for pre- and postmenopausal patients who had node-positive, ER-positive breast cancer.

Tamoxifen is also active in the treatment of male breast cancer. In 1993, the FDA approved the indication for the use of tamoxifen to treat advanced breast cancer in men.

Overall, tamoxifen repeatedly has been shown to increase the survival of patients with breast cancer (Early Breast Cancer Trials Collaborative Group, 1992). In 1994, the FDA approved the claim that tamoxifen prolonged the overall survival of the patient with breast cancer.

With this background of the value of tamoxifen in clinical practice, we will illustrate the target site-specific effect of tamoxifen noted in patients. We then will consider molecular mechanisms that are currently being investigated to explain target site specificity. An elucidation of these mechanisms could provide the basis for novel drug design. Nevertheless, numerous new compounds are being investigated currently, and we will discuss their strategic applications.

A. Contralateral Breast Cancer

Women with a previous diagnosis of breast cancer have a three-fold increased risk of developing a contralateral breast cancer when compared with age-matched women without breast cancer (Boring *et al*., 1994). An analysis of 11 separate trials in a total of nearly 15,000 women demonstrates that the incidence of contralateral breast tumors in a woman receiving tamoxifen therapy is reduced by 36% (table 1) (Bilimoria *et al*., 1996a). In the 6445 pre- and postmenopausal women who received adjuvant tamoxifen, there were 104 (1.6%) contralateral breast cancers, whereas in the 8033 patients randomly assigned to placebo or observation contralateral breast cancers were present in 201 (2.5%). Although the trials vary with respect to stage of disease and menopausal status, as well as duration and dose of tamoxifen therapy, the chemosuppressive and chemopreventive effects of tamoxifen were evident in nearly all of these studies.

It is also clear from the Overview Analysis (Early Breast Cancer Trials Collaborative Group, 1992, 1998) that longer durations of tamoxifen control contralateral breast cancer better than shorter durations of treatment. Women taking tamoxifen for less than 2 years have only a 26% reduction in contralateral breast cancer, whereas the reduction is 54% for women who take tamoxifen for more than 2 years.

Clearly, the antiestrogenic actions of tamoxifen produce a profound effect in controlling the growth of breast cancer. In contrast, a decade ago, it was unclear whether long-term tamoxifen treatment would have negative effects on a woman's physiology; that depends on the positive effects of estrogen. This concern prompted a broader investigation of the clinical safety of tamoxifen.

B. Endocrine Function and Tamoxifen

Tamoxifen exhibits estrogen-like effects in the postmenopausal patient causing a partial decrease in luteinizing and follicle-stimulating hormone (Jordan *et al*.,

TABLE 1
Frequency of contralateral breast cancer in patients taking adjuvant tamoxifen therapy compared with controls[a]

Clinical trial	Menopausal status	Tamoxifen-treated patients		Controls	
		Number of patients	Number of cancers	Number of patients	Number of cancers
NATO, 1985[b]	Pre and post	564	15	567	17
Stewart *et al.*, 1992	Pre and post	661	9	651	12
Rutqvist *et al.*, 1987	Post	931	18	915	32
Pritchard *et al.*, 1987	Post	198	3	202	3
Cummings *et al.*, 1986	Post	91	1	90	3
Fisher *et al.*, 1989	Pre and post	1419	23	1428	32
CRC, 1988	Pre and post	947	7	965	18
Andersson *et al.*, 1992	Post	864	10	846	8
Ryden *et al.*, 1992	Post	239	11	236	15
Mason *et al.*, 1993	Not stated	367	4	1980	57
Total		6281	101	7880	197
			1.6%		**2.5%**

[a] The incidence of contralateral breast cancer in 15,000 women receiving adjuvant tamoxifen therapy was determined by the analysis of 11 separate clinical trials (adapted from Nayfield *et al.*, 1991).
[b] NATO = Nolvadex Adjuvant Trial Organization; CRC = Cancer Research Campaign.

1987a). Consistent with this effect, tamoxifen causes an increase in sex hormone-binding globulin (Jordan *et al.*, 1987b), however, there is only a modest effect on antithrombin III. By contrast, tamoxifen causes an increase in circulating estrone and E_2 and an increase in circulating progesterone after ovulation (Jordan *et al.*, 1991a). The interactions with chemotherapy are age related. Patients younger than 40 years of age generally retain menstrual cycles after chemotherapy, whereas an increasing proportion of patients between 40 and 50 years of age stop menstruating after chemotherapy. Tamoxifen causes an increase in steroid levels in patients undergoing chemotherapy who retain menstrual function (Ravdin *et al.*, 1988), but in those patients who become menopausal, tamoxifen's effect is as a weak estrogen (Jordan *et al.*, 1987a). Although there has been some concern that the increases in estrogen caused by tamoxifen in premenopausal women will reverse the antiestrogen block in the tumor, tamoxifen has been effective in premenopausal patients with both node-negative (Fisher *et al.*, 1989) and advanced disease (Sunderland and Osborne, 1991). Laboratory studies in athymic mice have demonstrated that low circulating levels of tamoxifen cannot control the growth of extremely high levels of estrogen (Iino *et al.*, 1991). Clearly, low drug compliance from a premenopausal patient may result in the failure of treatment, but a second response could occur after oophorectomy (Sawka *et al.*, 1986).

C. Tamoxifen and Bone

Initially, it was feared that the antiestrogenic effects of tamoxifen actually would accelerate bone resorption and increase the risk of developing osteoporosis. However, studies in vitro and in vivo have demonstrated quite the opposite effect. In one study bone organ cultures pretreated with tamoxifen showed inhibition of bone absorption (Stewart and Stern, 1986). Ovariectomized rats treated with tamoxifen showed a significant decrease in bone resorption compared with controls (Jordan *et al.*, 1987c; Turner *et al.*, 1987, 1988).

The effects in patients receiving tamoxifen therapy have been equally impressive. Nine studies (Bilimoria *et al.*, 1996a) examining the effects of tamoxifen on bone resorption are summarized in table 2. Fornander *et al.* (1990) used a single photon absorptiometry technique to measure bone mineral density at the distal forearm in 75 postmenopausal patients with breast cancer, and observed no increase in bone loss in patients taking tamoxifen for 2 to 5 years. Since then several studies have used the more sensitive dual photon absorptiometry technique to study the effects of tamoxifen on bone density. Love *et al.* (1992) used this technique as part of a randomized placebo-controlled trial of 140 postmenopausal patients with breast cancer. Patients treated for 2 years with tamoxifen had a statistically significant increase in the bone mineral density of their lumbar spine when compared with patients receiving placebo. The 5 year analysis of this same study supports the conclusion that tamoxifen maintains bone density (Love *et al.*, 1994).

Seven other studies on postmenopausal patients treated with tamoxifen confirm that bone mineral density is preserved or increased with respect to controls (table 2). Three of these studies also noted preservation of trabecular bone at the femoral neck, a common site of postmenopausal osteoporotic fractures. In contrast, a recent study by Powles and coworkers (1996) shows a slight, but significant, decrease in bone density for premenopausal women taking tamoxifen. The overall result is to be expected as an expression of the antiestrogenic effects of tamoxifen, but the overall impact on the subsequent development of osteoporosis is unknown. In this context, it is interesting to note that the administration of bisphosphonates to build bone is not impaired by antiestrogen therapy in postmenopausal patients (Saarto *et al.*, 1997).

D. Tamoxifen and Lipids

When tamoxifen emerged as a proven therapy for breast cancer there were genuine concerns that treating women with an antiestrogen would affect their lipid

TABLE 2
Bone mineral content of tamoxifen-treated women versus controls[a]

Study	Duration of tamoxifen (year)	Area studied	Study design	Control	Tamoxifen-treated group	Statistical significance
Gotfredsen *et al.*, 1984	1	Distal radius	Change in BMC[b] (g/cm^2)	−2.5%	−3.2%	NS
Fornander *et al.*, 1990	2	Proximal radius	BMD (g/cm^2)	1.04	0.99	NS
	2	Distal radius	BMD (g/cm^2)	0.74	0.70	NS
	5	Proximal radius	BMD (g/cm^2)	1.05	1.06	NS
	5	Distal radius	BMD (g/cm^2)	0.74	0.78	NS
Fentiman *et al.*, 1989	0.5	Femur	gHA/cm^2	0.81	0.81	NS
	0.5	Lumbar spine	gHA/cm^2	0.95	0.94	NS
Love *et al.*, 1992	2	Lumbar spine	%/year change in BMD	−1.0%	0.6%	$P < 0.0001$
	2	Radius	%/year change in BMD	1.29%	0.88%	NS
Cuzick *et al.*, 1992	6	Lumbar spine	BMD (g/cm^2)	0.97	1.08	NS
	6	Trochanter	BMD (g/cm^2)	0.75	0.81	NS
Ward *et al.*, 1993	1	Lumbar spine	%/year change in BMD	−2.3%	0.09%	$P = 0.04$
	1	Trochanter	%/year change in BMD	−1.8%	1.4%	$P = 0.03$
Neal *et al.*, 1993	5	Lumbar spine	BMD (g/cm^2)	1.028	1.059	NS
	5	Femur	BMD (g/cm^2)	0.838	0.894	NS
Turken *et al.*, 1989	1	Lumbar spine	%/year change in BMD	−2.7%	2.4%	$P < 0.003$
Kristensen *et al.*, 1994	2	Lumbar spine	% change in BMD	−4.3%[c]	2.5%[c]	$P = 0.00074$
	2	Distal radius	% change in BMD	−6.3%[c]	−2.0%[c]	$P = 0.024$

[a] The summary of nine studies examining the effects of tamoxifen therapy on bone resorption in women (adapted) from Bilimoria *et al.*, 1996a).
[b] Abbreviations: BMC, bone mineral content; BMD, bone mineral density; gHA, grams of hydroxyapatite; NS, not significant.
[c] Percentages extrapolated from data graphs.

profile adversely and lead to an increased risk of heart disease. Since then, several studies have shown that much like the estrogenic effects of tamoxifen on bone, tamoxifen also has estrogenic effects on serum lipid profiles. Analysis of nine separate studies reveals an average decrease in total cholesterol of 13% and an average decrease in low-density lipoprotein (LDL) of 19% (table 3) (Bilimoria *et al.*, 1996a; Saarto *et al.*, 1996).

In a randomized double-blind study of tamoxifen versus placebo, Love and colleagues (1991) noted increased synthesis of very low density lipoproteins leading to increased triglyceride levels and increased apolipoprotein B receptors, which resulted in lower LDL levels. Analysis at 5 years supports the maintenance of decreased LDL and total cholesterol (Love *et al.*, 1995). Others have noted that tamoxifen and toremifene (chlorotamoxifen) interfere with cholesterol synthesis by inhibiting the conversion of Δ8-cholestenol to lathosterol (fig. 11) (Gylling *et al.*, 1995). These metabolic changes are consistent with an estrogenic effect on lipid metabolism. Interestingly enough, high-density lipoprotein levels, which are usually increased by estrogen therapy, apparently are unaffected by tamoxifen therapy.

The ability of tamoxifen to lower serum lipids translates to a significant reduction in cardiac disease. In 1991, McDonald and Stewart (1991), in a retrospective review of a randomized trial of tamoxifen versus placebo noted that 10 of 200 women in the tamoxifen-treated arm had died of myocardial infarction, whereas 25 of 251 had died of the same disease in the control group. An update of their patient data in 1995 showed that women in the tamoxifen-treated arm of the study had a rate of 14 myocardial infarctions per 1000 years at risk compared with 23 myocardial infarctions per 1000 years of risk for the control group (McDonald *et al.*, 1995). In fact, they concluded that the risk of coronary heart disease was significantly less for long-term users than short-term users of tamoxifen.

Others also have found that longer durations of tamoxifen have a greater benefit in protecting from cardiovascular disease. Rutqvist and Matteson (1993), reanalyzing the Stockholm adjuvant tamoxifen

TABLE 3
Effects of tamoxifen on serum lipids

Study	Total cholesterol (mg/dl)		LDL cholesterol (mg/dl)		HDL cholesterol (mg/dl)			TT	%change
	Control (no. of patients)	TT[b] (no. of patients)	%change	Control	TT	%change	Control		
Rossner and Wallgren, 1984	302 (10)	258 (11)	−15%	201	156	−22%	87	75	NS
Bruning et al., 1988	220 (46)	205 (46)	−7%	151	124	−18%	43	50	NS
Bertelli et al., 1988	254 (36)	213 (55)	−16%	180	127	−29%	55	56	NS
Bagdade et al., 1990	193 (8)	204 (8)	NS	122	115	−6%	45	49	NS
Love et al., 1991	216 (70)	190 (70)	−12%	138	110	−20%	57	53	NS
Ingram, 1990	259 (47)	234 (13)	−9%	193	171	−10%	66	61	NS
Cuzick et al., 1992	256 (47)	225 (14)	−12%	186	145	−22%	50	43	−14%
Dnistrian et al., 1993	244 (13)	203 (24)	−17%	169	123	−27%	55	56	NS
Thangaraju et al., 1994	224 (45)	190 (39)	−15%	149	132	−17%	54	58	7%
Average	249	214	−13%	165	134	−19%	57	52	NS

[a] The summation of nine studies examining the effects of tamoxifen therapy on serum lipid profiles in women (adapted from Bilimoria *et al.*, 1996a).
[b] TT, tamoxifen-treated group; NS, not statistically significant.

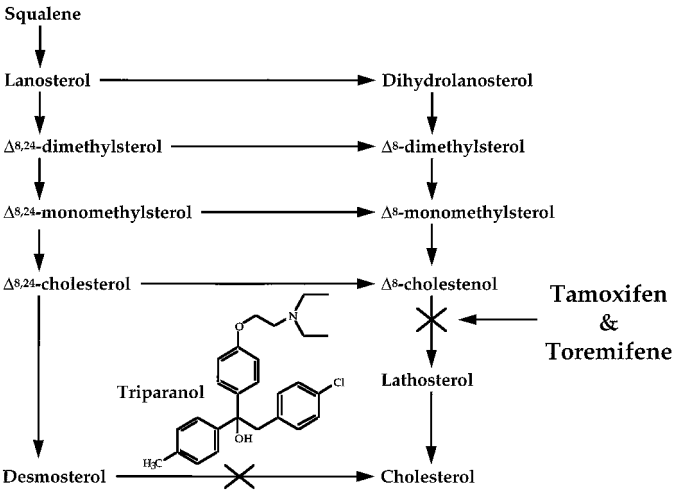


FIG. 11. The effects of toremifene and tamoxifen on cholesterol synthesis. These compounds interfere with cholesterol synthesis by inhibiting the conversion of Δ8-cholestenol to lathosterol (Gylling *et al*., 1995). Triparanol, a cholesterol-lowering drug structurally related to MER-25 (see fig. 1), blocks the conversion of desmosterol to cholesterol (Avigan *et al*., 1960; Gaylor, 1963; Frantz *et al*., 1996; Steinberg *et al*., 1961) and was associated with cataract formation (Laughlin and Carey, 1962; Kirby *et al*., 1962; von Sallmann *et al*., 1963).

randomized trial, found that hospital admissions for cardiac disease were statistically lower for women taking tamoxifen for 5 years than for women taking only 2 years of the therapy. A similar report by the National Surgical Adjuvant Breast and Bowel Project (NSABP) noted an elevation in coronary heart disease in a large population of postmenopausal women once tamoxifen has been stopped (Ganz *et al*., 1995). The group has reported that there is no significant decrease in coronary heart disease during tamoxifen therapy, but cardiac disease increases when treatment is stopped (Costantino *et al*., 1997).

The reduction in cardiovascular risk obtained from tamoxifen use apparently is mediated through the lowering of cholesterol levels mentioned earlier. It has been suggested that a 1% decrease in serum cholesterol results in a 2% decrease in the incidence of coronary heart disease (Castelli, 1988). Another possible mechanism for the cardioprotective effects of tamoxifen lies in the finding that patients treated with tamoxifen have a statistically significant reduction in serum lipoprotein(a) levels (Saarto *et al*., 1996). Several epidemiological and clinical studies have shown that increased lipoprotein(a) levels are an independent risk factor for coronary heart disease (Loscalzo, 1990; Utermann, 1990). Additionally, tamoxifen lowers homocysteine levels (Anker *et al*., 1995), and Wiseman (1995) has suggested that tamoxifen could be cardioprotective by reducing oxidation of low-density lipoproteins.

Because most women with breast cancer are postmenopausal and the number one cause of death in postmenopausal women (without a history of breast cancer) is cardiovascular disease, the lipid-lowering properties of tamoxifen become clinically significant in women with the relevant risk factors.

XII. Complexity of Antiestrogen Action

The unusual properties of nonsteroidal antiestrogens as target site-specific agents has raised the possibility that these compounds could be powerful tools to elucidate the organization of the estrogenic responses throughout the body.

Presently, there are three main theories explaining the mechanism of target site specificity. At the subcellular level, one could envision target site localization of different receptor molecules or the conversation between different cells containing different receptors in a tissue. It is possible that the new ERβ (see Section IV.) could account for the target site specificity. A recent report by Paech and colleagues (1997) suggests two potential pathways for antiestrogen action. The conventional pathway occurs via ERα where estradiol activates EREs and an antiestrogen blocks activation by occupying the steroid binding domain. The second pathway occurs when an antiestrogen-ERβ complex binds (via protein-protein interactions) to AP-1 (Fos and Jun) to activate estrogen-responsive genes at an AP-1 site.

Additionally, there are data to support two other theories to explain the target site specificity of antiestrogens. First is the idea that different cells may have different intracellular environments that determine whether an antiestrogen is perceived as an agonist or an antagonist. These differences could result from a different complement of transcription factors or other coactivator proteins (Berry *et al*., 1990). The second theory is that there could be specific EREs in the promotor region of genes that interact with the altered tamoxifen-ER complex. Also, it is possible that the sequence and the number of EREs in a particular promoter could have an effect on how an antiestrogen is perceived (Dana *et al*., 1994; Catherino and Jordan, 1995). To complement this theory, an antiestrogen response element has been identified as an alternate site for the activation of a specific gene (Yang *et al*., 1996b). In this section, we will briefly review the emerging data to support the proposed mechanisms for target site specificity.

A. Estrogen Receptor-Associated Proteins

Many ER-associated proteins have been described that may play a role in the interpretation of different ligands (see Section V.B.). Halachmi *et al*. (1993) describe a 160 kDa ER-associated protein (ERAP160) that binds to the ER in the presence of estrogen. This interaction is E_2 dependent and enables the ER to activate transcription of estrogen-responsive genes. Thus, a direct correlation between the ability of the ER to bind ERAP160 and its transcription activation potential exists providing another level of complexity to the estrogen-mediated enhancement of transcriptional activation by the ER. Antiestrogens do not promote binding of

ERAP160 to the ER and, in fact, can inhibit the estrogen-dependent interaction in a dose-dependent manner. It has been proposed recently that the ER coactivator complex, which consists of an interaction between agonist-bound ER and ERAP160, results in the recruitment of p300, which is a transcriptional coactivator (Hanstein *et al*., 1996). This provides an increasingly complicated mechanism for the ability of cells to interpret estrogen as an agonist and antiestrogens as antagonists.

There is intense interest in this area of investigation because the problem not only relates to target site specificity but also to the development of tamoxifen-stimulated tumor growth (see Section XIV.C.).

B. Antiestrogen Response Elements

When an antiestrogen binds to the HBD of the ER, this results in a subtle conformational change in the protein that distinguishes the complex from ER bound to estrogen. The antiestrogen-induced conformation then may be able to bind specifically to an antiestrogen response element and activate or inactivate transcription of that gene.

This phenomenon has been described recently for raloxifene by Yang *et al*. (1996a) in cultured bone cells. This antiestrogen shows target site specificity similar to tamoxifen regarding maintaining bone mineral density (Jordan *et al*., 1987c). Yang *et al*. (1996a) show that in cultured bone cells, raloxifene is capable of activating transcription of TGFβ3 which is involved in bone remodeling. Estrogen modestly activates transcription of this gene, but raloxifene is apparently the preferred ligand as shown by its greater enhancement of transcription. Yang and colleagues (1996a) show that the mechanism for raloxifene action is promoter mediated and ER dependent through the use of various reporter assays. The raloxifene response element (RRE) they describe was shown to be a polypurine sequence which does not require the DBD of the ER to activate transcription of the TGFβ3 gene. Thus, because raloxifene-bound ER is DBD independent, they postulate that the interaction of the raloxifene-bound ER requires an adaptor protein.

Yang *et al*. (1996b) used deletional analysis to identify the specific RRE sequence in the promotor region of TGFβ3. They showed that deletion of nucleotides -499 to -38 and $+75$ to $+110$ had no effect on reporter gene expression. However, when nucleotides $+35$ to $+75$ were deleted, reporter gene expression was ablated; thus, the RRE sequence was defined by loss of function. When this RRE was transferred to a heterologous promotor, reporter gene expression increased two-fold upon treatment with raloxifene and estrogen. When the GT repeat sequence was added raloxifene, but not estrogen, a three-fold increase in reporter gene expression was stimulated, indicating that the GT repeat sequence can act synergistically and that the RRE may not be sufficient to mediate a full raloxifene response.

Similar RREs can be found in the promoters of other genes including the urokinase-type plasminogen activator gene, the osteonectin gene, the neuron-specific growth-associated protein (GAP-43) gene and the proto-oncogene c-*myc*. All these genes are regulated by estrogen and encode proteins important in bone, the central nervous system, and the cardiovascular system. However, a note of caution has been introduced by a recent letter to the editor of Science by Yang and coworkers (1997) who now believe that the issue is much more complex.

Another ER-dependent transcriptional enhancer has been identified that consists of a new subclass of Alu DNA repeats (Norris *et al*., 1995). Alu repeats originally were thought to be functionally inert; however, in addition to conferring estrogen responsiveness. These novel elements are capable of imparting estrogen responsiveness to heterologous promoter systems in mammalian cells. These elements function as classical EREs because, in addition to responding to estrogen, transcriptional activity is attenuated by three different classes of antiestrogens. Thus, a new class of response element, consensus Alu elements, must be considered when analyzing potential estrogen-responsive genes.

XIII. Concerns with Tamoxifen

Although the target site-specific actions of tamoxifen are almost certainly responsible for the increased detection of endometrial cancer, the species-specific metabolism of tamoxifen also has introduced another dimension in the pharmacology of antiestrogens. Tamoxifen causes rat liver carcinogenesis which together with an increased uterine detection of human carcinomas has become the focus for an enormous research effort to elucidate mechanisms and describe appropriate safety guidelines.

A. Uterine Carcinogenesis

Much controversy has surrounded the associations between the use of tamoxifen and the detection of endometrial cancer. The concern resulted from observations originally made in the laboratory being extrapolated into clinical practice. The human endometrial carcinoma, EnCa 101 grows in athymic animals in response to E_2 and partially in response to tamoxifen (Satyaswaroop *et al*., 1984). However, the finding that tamoxifen exhibits target site specificity , can inhibit estrogen-stimulated breast carcinomas, but can stimulate an endometrial carcinoma transplanted in the same athymic mouse (Gottardis *et al*., 1988b) focused attention on the clinical link between tamoxifen used as an adjuvant and the risks of developing endometrial cancer in the same patient (Fornander *et al*., 1989; Fisher *et al*., 1994).

A decade later, it is now possible to provide a reasonable picture of the actual incidence of endometrial cancer and provide a balanced view of the concerns. Recent reviews (Jordan and Assikis, 1995; Assikis and Jordan,

1995; Assikis *et al*., 1996) of the literature have identified only approximately 400 cases of endometrial cancer associated with the use of tamoxifen worldwide. The disease is found predominantly in postmenopausal women and a strong association between the duration of tamoxifen use and the risks of developing endometrial carcinoma does not exist. Indeed, it is interesting to re-evaluate earlier studies that claim an association between long-term tamoxifen and endometrial cancer. Reanalysis of the Stockholm study (Fornander *et al*., 1989), which originally concluded that randomization to 2 years of adjuvant tamoxifen did not cause an increase in endometrial cancer but randomization to 5 years of adjuvant tamoxifen caused a six-fold increase in the risk of endometrial cancer, actually demonstrates that 12 of 16 recruited patients who presented with endometrial cancer actually received <2 years of the drug (Jordan and Morrow, 1994). Clearly, preexisting disease is being detected. Based on the known long genesis of cancer in humans, it would be inappropriate to suggest that early detection of endometrial cancer was caused by short courses of tamoxifen.

It is known that the uterus harbors five times the amount of occult disease than is detected clinically (Horwitz *et al*., 1981). Because tamoxifen produces symptoms like vaginal discharge, the repeated screening of these women will naturally result in increased detection rates based on detection bias. It is also important to appreciate that there is not a statistically strong increase in the incidence of endometrial cancer with a short (2 year) course of tamoxifen (Cook *et al*., 1995; Van Leeuwen *et al*., 1994). Indeed, there is little published evidence for an association between long-term tamoxifen use and an increased detection of endometrial cancer (Assikis *et al*., 1996; Assikis and Jordan, 1995).

Tamoxifen-treated patients are at a small but quantifiable risk, i.e., 2 per 1000 women per year, for the detection of endometrial cancer during or after tamoxifen treatment. However, there has been concern that the disease is aggressive. Nevertheless, the original finding by Magriples and coworkers (1993), that the use of tamoxifen is associated with poor prognosis disease has not been confirmed by any other study (Fisher *et al*., 1994; Rutqvist *et al*., 1995; Barakat *et al*., 1994). Overall the stage and grade of endometrial cancer associated with the use of tamoxifen is proportionally the same as Surveillance, Epidemiology, and End Results data (Assikis and Jordan, 1995). Therefore, it is fair to say that the overall consensus is that the benefits of tamoxifen in the treatment of breast cancer far outweigh the risks associated with a two-fold elevation in early-stage, low-grade endometrial carcinoma (Jaiyesimi *et al*., 1995; Bilimoria *et al*., 1996a; Early Breast Cancer Trialists Collaborative Group, 1992; Jordan, 1995c,d). Nevertheless, as a precaution, patients should be examined to determine whether they have preexisting gross endometrial carcinoma before starting a course of adjuvant tamoxifen therapy. Additionally, patients who present with spotting and bleeding during treatment must undergo a thorough gynecological examination. There is no justification, however, for an extensive screening program using endometrial biopsy to detect endometrial cancer in asymptomatic women taking tamoxifen (Barakat, 1997). In fact, a recent evaluation of all of these data by the International Agency for Research on Cancer concluded that no patient should stop taking tamoxifen because of concerns about endometrial cancer (http://www.iarc.fr/preleases/111e.htm). The benefits to the patient outweigh the risks.

B. Rat Liver Carcinogenesis

The concern about the association between tamoxifen and endometrial cancer in the late 1980s and the early 1990s was exacerbated by the laboratory finding that large doses of tamoxifen can produce liver tumors in rats.

Several investigators report that tamoxifen is both an initiator and a promoter of rat liver carcinogenesis (Williams *et al*., 1993; Greaves *et al*., 1993; Hard *et al*., 1993; Dragan *et al*., 1994, 1995, 1996). Tamoxifen, at high doses, causes DNA adducts in rat liver (Han and Liehr, 1992; Hard *et al*., 1993; White *et al*., 1992). However, only low adduct formation is noted in mouse liver DNA (White *et al*., 1992), a species that does not produce tumors in response to high daily doses of tamoxifen (Furr and Jordan., 1984). It also is reassuring to note that there is no increase in DNA adduct formation in the livers of patients receiving tamoxifen (Martin *et al*., 1995). As a result, it has been argued that the rat studies are not relevant to human usage (Jordan and Morrow, 1994; Jordan, 1995c,d).

Examination of the data from the rat carcinogenesis studies demonstrates that the animals receive tamoxifen (5 to 50 mg/kg daily) from puberty for more than 50% of their life (Jordan and Morrow, 1994). In contrast, the therapeutic dose of tamoxifen, as an anticancer agent in rats, is 250 μg/kg (Jordan, 1983) which is similar to the therapeutic dose in a 70 kg patient of 285 μg/kg or 20 mg of tamoxifen daily. The duration of adjuvant therapy for postmenopausal patients is usually 5 years. This would be equivalent to 8% of a woman's life. Thus the animal experiment at the lowest dose to produce tumors, 5 mg/kg, is equivalent to a teenage girl (i.e., 14 years of age) receiving 20 times the daily dose of tamoxifen until she is 40 years old. This is 40 tablets a day.

The reason that such large doses must be administered to the rat to produce drug levels comparable with the human is that the drug is cleared from the rat ten times faster than in humans (Jordan and Morrow, 1994). Thus artificially high levels of drug must be given, far outside the therapeutic range, that ultimately cause damage in the rat liver. In recent years, concerns about carcinogenesis with tamoxifen have lead to a re-

port of increases in colorectal cancer and stomach cancer (Rutqvist *et al*., 1995). These results have not been supported by either individual reports from clinical trials (Fisher *et al*., 1994) or from the current 1998 Oxford overview analysis. The International Agency for Research on Cancer also concludes that there is insufficient evidence to support human carcinogenesis at sites other than the endometrium.

The finding of liver carcinogenesis in the rat would be cause for concern with any new drug that is about to enter clinical trials. However, tamoxifen had been used extensively for 20 years before the investigation of rat liver carcinogenesis. Hepatocellular carcinoma has not increased significantly since the two initial cases reported in 1989 (Fornander *et al*., 1989). Similarly, epidemiology studies (Muhleman *et al*., 1994) have not shown a rise in hepatocellular carcinoma in breast cancer patients since tamoxifen was approved for use in the United States in 1978. In contrast, oral contraceptives cause a ten-fold increase in the risk for of hepatocellular carcinoma (Prentice, 1991), but this risk is considered to be acceptable to regulatory authorities because of the rarity of the disease.

C. Mechanism of Carcinogenesis

During the past 5 years, there has been intense interest in discovering the initiating event for tamoxifen-induced rat liver carcinogenesis and determining the relevance for humans. Han and Liehr (1992) first noted an accumulation of DNA adducts in the liver of Sprague-Dawley rats on repeated injections of 20 mg/kg (cf. human dosage of 0.3 mg/kg). This has been confirmed adequately by numerous investigators and the focus of investigation has been the identification of the actual DNA adduct. Several candidates have been proposed: an epoxide (Styles *et al*., 1994; Lim *et al*., 1994; Phillips *et al*., 1994b), 4-OHT (Randerath *et al*., 1994; Moorthy *et al*., 1996), Metabolite E (Pongracz *et al*., 1995), or α-hydroxytamoxifen (Potter *et al*., 1994; Phillips *et al*., 1994a,c). Recently, Osborne *et al*. (1996) prepared an acetoxytamoxifen that is able to react with DNA to a greater extent (1 in 50 bases) than α-hydroxytamoxifen (1 in 105 DNA bases). The products of the reaction were identical with those isolated from DNA of rat hepatocytes or the livers of rats treated with tamoxifen. The adduct of tamoxifen and DNA has been identified at the nucleoside deoxyguanosine in which the α-position of tamoxifen is linked covalently to the exocyclic amino of deoxyguanosine (fig. 12).

These important observations have provided a framework to study the metabolic activation of tamoxifen in human systems and to identify any DNA adducts in human tissues. The metabolic activation of tamoxifen and its metabolite α-hydroxytamoxifen has been compared using primary cultures of rat, mouse, and human hepatocytes (Phillips *et al*., 1996a). Although DNA adducts are identified readily in rat and mouse hepatocytes (90 and 15 adducts/108 nucleotides, respectively), DNA adducts were not detected in tamoxifen-treated human hepatocytes. Additionally, human hepatocytes also apparently produced 50-fold lower levels of α-hydroxytamoxifen from tamoxifen than rat hepatocytes. Further studies showed that if cells were treated with α-hydroxytamoxifen human hepatocytes had 300-fold lower levels of adducts than rat hepatocytes.

Studies in patients have confirmed that humans are not as susceptible as rats to DNA adduct formation with tamoxifen. The pattern of DNA adducts found in the rat liver is not found in humans treated with tamoxifen (Martin *et al*., 1995), DNA adducts are not found in lymphocytes (Phillips *et al*., 1996b), and there is a lack of genotoxicity of tamoxifen in human endometrium (Carmichael *et al*., 1996). In the latter studies DNA adducts could be produced in endometrial samples with α-hydroxytamoxifen but not with tamoxifen. The authors proved that tissue was capable of metabolizing tamoxifen to α-hydroxytamoxifen, but apparently it is incapable of producing adducts. Endometria from patients taking tamoxifen for up to 9 years were analyzed for DNA adducts. No evidence for any DNA adducts induced by tamoxifen was found in any of the patients examined. The authors concluded that the genotoxic events observed with tamoxifen in the rat may not apply to the human endometrium (Carmichael *et al*., 1996). This conclusion supports the previous suggestion that tamoxifen, or indeed any new antiestrogen that has partial agonist actions, will cause the activation and detection of preexisting disease (Jordan and Morrow, 1994). Nevertheless, recent reports using sensitive high-performance liquid chromatography techniques have isolated DNA adducts in the endometrium (Hemminki *et al*., 1996) and white blood cells (Hemminki *et al*., 1997) of patients being treated with tamoxifen; however, these have not yet been identified. There is intense debate about technol-

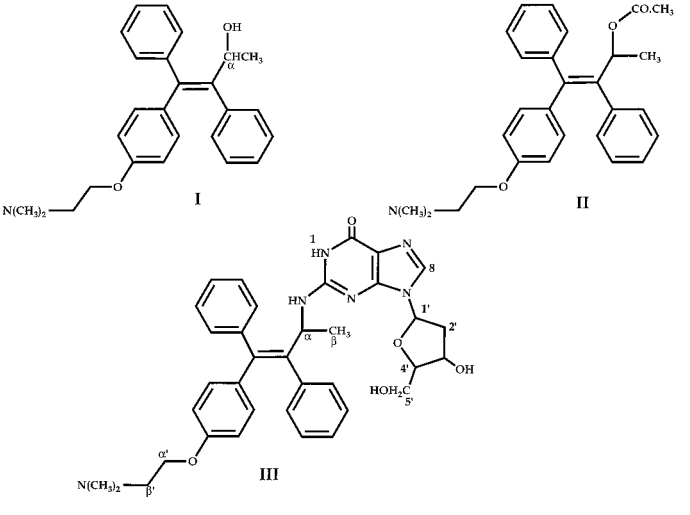


FIG. 12. A tamoxifen-DNA adduct at the nucleoside deoxyguanosine. The α-position of tamoxifen is linked covalently to DNA at the exocyclic amino of deoxyguanosine.

ogy and the actual relevance of minor high-performance liquid chromatography peaks compared with the overall known high level of adduct formation on human DNA from environmental sources (Swenberg, 1997).

D. Tamoxifen Metabolism

Extensive examination of tamoxifen has identified two principal routes of metabolism: 4-hydroxylation and the progressive degradation of the dimethyaminoethane side chain. Studies in patients reveal a stability of metabolism for many years (Langan-Fahey *et al*., 1990).

Tamoxifen is hydroxylated in the 4-position to produce 4-OHT, a minor metabolite but having a high binding affinity for the ER (Jordan *et al*., 1977). The metabolite has been noted as a minor metabolite in rats and humans, but it is a major metabolite in the mouse (Robinson *et al*., 1991). Metabolic activation seems to be a general principle for most antiestrogens based on triphenylethylene. Antiestrogens that have a methoxy group in an equivalent position, for example U 23,469 (an analog of the antiestrogen nafoxidine) or nitromifene (Katzenellenbogen *et al*., 1981; Hayes *et al*., 1981; Tatee *et al*., 1979), can be demethylated to the hydroxylated metabolite with a high binding affinity for the receptor.

In contrast, the progressive demethylation of the tamoxifen side chain first to N-desmethyltamoxifen, the principal metabolite in humans (Adam *et al*., 1979), and then didesmethyltamoxifen (Kemp *et al*., 1983) does not affect the biological actions of the triphenylethylene. However, deamination of didesmethyltamoxifen first to the glycol derivative Metabolite Y and dealkylation to Metabolite E (Met E) results in a change in pharmacology from an antiestrogen to an estrogen (Jordan *et al*., 1983). This has been proposed as an explanation for tamoxifen drug resistance and tamoxifen-stimulated growth (see Section XIV.A.).

There is much interest in understanding the mechanism of both the metabolic activation of tamoxifen to antitumor agents and the metabolic activation of the drug to a species that will form DNA adducts (see Section XIII.C.). Numerous groups (Jacolot *et al*., 1991; Mani and Kupfer, 1991; Mani *et al*., 1993a,b, 1994; White *et al*., 1993; Nuwaysir *et al*., 1995; Wiseman and Lewis, 1996) have identified P450-mediated metabolic routes for tamoxifen in rat and human liver and demonstrated the involvement of flavin-containing mono-oxygenases. It is clear from the published studies (Mani and Kupfer, 1991; Lim *et al*., 1994; Phillips *et al*., 1996a) that rat liver enzymes form tamoxifen metabolites at a much higher rate than the human and the P450s involved that have been identified. Tamoxifen N-desmethylation is catalyzed in the rat by CYP I A, CYP2C, and CYP3A enzymes and in the human by CYP3A (Jacolot *et al*., 1991; Mani *et al*., 1993a). Metabolism to tamoxifen N-oxide, a precursor of N-desmethylation, is mediated by a flavin containing mono-oxygenase (Mani *et al*., 1993b), whereas 4-hydroxylation appears to be catalyzed by constitutive P450. Kupfer's laboratory first identified the covalent binding of tamoxifen to a 52 kDa protein and they have proposed that the CYP3A enzymes activate tamoxifen to the reactive intermediate in rat and human liver microsomes (Mani and Kupfer, 1991).

The metabolic activation of tamoxifen also has been evaluated in male and female rhesus monkeys. Comoglio and colleagues (1996) found a marked induction of P450 but paradoxically, the metabolism of a test compound, 7-ethoxyresorufin, by the microsomes of treated monkeys in vitro was inhibited as was the dealkylation of two 7-alkoxyresorufin substrates. They also found that there was a significant accumulation N,N-desmethyltamoxifen, which is an inhibitor of drug metabolism. In addition, the level of DNA adduct formation was substantially lower in monkeys than in rats. When covalent binding to microsomes was assessed monkey microsomes had much lower levels than rat microsomes. It also was found that when treated with N,N-desmethyltamoxifen, microsomes from both rats and monkeys displayed significantly reduced convalent binding. Hence, the accumulation of N,N-desmethyltamoxifen in the livers of primates may inhibit P450-dependent conversion of tamoxifen into reactive metabolites, thereby protecting the animal from DNA adduct formation.

The inducibility of P450 by tamoxifen, toremifene, and droloxifene have been evaluated in the rat and mouse liver. Tamoxifen is a liver carcinogen in the rat but not the mouse (Furr and Jordan, 1984), so any differences might help to support a mechanism of carcinogenesis. The relevance of the findings would be confirmed in the rat because toremifene and droloxifene are not potent liver carcinogens (Hard *et al*., 1993; Hasman *et al*., 1994). All the antiestrogens induce CYP2BI and CYP3AI in the rat liver so these may be responsible for the promotion of carcinogenesis rather than initiation. No induction of P450s is noted in mice (White *et al*., 1993). The results with tamoxifen in rats have been confirmed (Nuwaysir *et al*., 1995) and extended with the observation that there is a striking induction of CYP2B2. Additionally, Phase 11 enzyme systems are affected by tamoxifen in the rat liver. Glutathione S-transferase (Ya1 and 2) is reduced but other isoforms are unaffected. Tamoxifen also produces a dose-related increase in rat liver UDP-glucuronosyl transferase (Nuwaysir *et al*., 1996).

XIV. Drug Resistance Mechanisms

Drug resistance to tamoxifen therapy can take many forms (Morrow and Jordan, 1993; Wolf and Jordan, 1993; Tonetti and Jordan, 1995). These are illustrated in the ER signal transduction pathway shown in figure 13. Obviously, if tumors are ER negative, there is only a small probability of a response to antiestrogen therapy. In metastatic breast cancer, approximately 10% of ER-negative and PR-negative patients respond to any form of endocrine modulation (Jordan *et al*., 1988b).Similarly

the overview analysis (Early Breast Cancer Trialists, 1992) of clinical trials suggests that postmenopausal, node-positive patients with receptor-poor disease will benefit only from adjuvant tamoxifen with a small survival advantage compared with highly receptor-positive disease. Tamoxifen and its metabolites are competitive inhibitors of E_2 binding to ER, so a large increase in E_2 possibly could reverse the antitumor action of the drug. This has been a concern in premenopausal women in whom tamoxifen increases estrogen secretion by the ovary (see Section XI.B.); however, the laboratory studies show that only very high estrogen levels and low levels of tamoxifen, i.e., that might result from a lack of compliance, would really provoke tamoxifen failure (Iino *et al*., 1991). We will consider each of the possible mechanisms of drug resistance that has been or is being evaluated.

A. Metabolic Activation

Tamoxifen undergoes metabolic conversion to two main metabolites, 4-OHT and N-desmethyltamoxifen. Although 4-OHT is a minor metabolite, it is a potent antiestrogen that binds to the human ER with an affinity similar to E_2, whereas N-desmethyltamoxifen, the major metabolite of tamoxifen, is a weak antiestrogen (Jordan *et al*., 1977; Katzenellenbogen *et al*., 1985; Murphy *et al*., 1990a). The *trans* form of tamoxifen is stable in solution; however, 4-OHT is less stable and may isomerize to the *cis* form, a less potent antiestrogen (Jordan *et al*., 1981, 1988a; Murphy *et al*., 1990a). Tamoxifen also may be metabolized to two estrogenic compounds, Met E and bisphenol (Murphy *et al*., 1990a; Jordan and Lieberman, 1984). Therefore, it has been suggested that intratumoral accumulation of tamoxifen metabolites that are either less potent antiestrogens or are estrogenic may lead to tamoxifen-resistant tumor growth (Osborne *et al*., 1991, 1992; Johnston *et al*., 1993b; Weibe *et al*., 1992).

This mechanism of tamoxifen resistance has been explored by quantitating and comparing the levels of tamoxifen and the various metabolites in tamoxifen-stimulated tumors and tamoxifen-inhibited tumors. Osborne and colleagues (1991) reported that tamoxifen-stimulated tumors have significantly reduced levels of tamoxifen compared with tamoxifen-inhibited tumors. In addition, a relative increase in the ratio of *cis*/*trans* 4-OHT was found along with accumulation of the estrogenic Met E (fig. 14) (Osborne *et al*., 1991; Johnston *et al*., 1993b; Weibe *et al*., 1992). However, Johnston *et al*. (1993a) demonstrated that ER-negative tumors only accumulate tamoxifen and its metabolites more slowly compared with ER-positive tumors. Similarly, Wolf and coworkers (1993) were unable to detect significant differences in the intratumoral concentrations of tamoxifen between tamoxifen-stimulated and tamoxifen-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 tamoxifen resistance, nonisomerizable fixed-ring analogs have been used to determine whether drug-stimulated tumor growth can occur (Osborne *et al*., 1994; Wolf *et al*., 1993). A fixed-ring tamoxifen analog incapable of forming the potent estrogenic isomer of Met E compound was found to be equally capable of supporting tamoxifen-

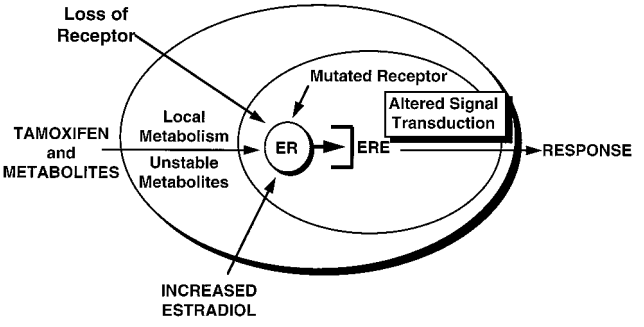


FIG. 13. The points in the ER signal-transduction pathway of breast cancer that are susceptible to modulations and can result in the acquisition of drug resistance to tamoxifen.

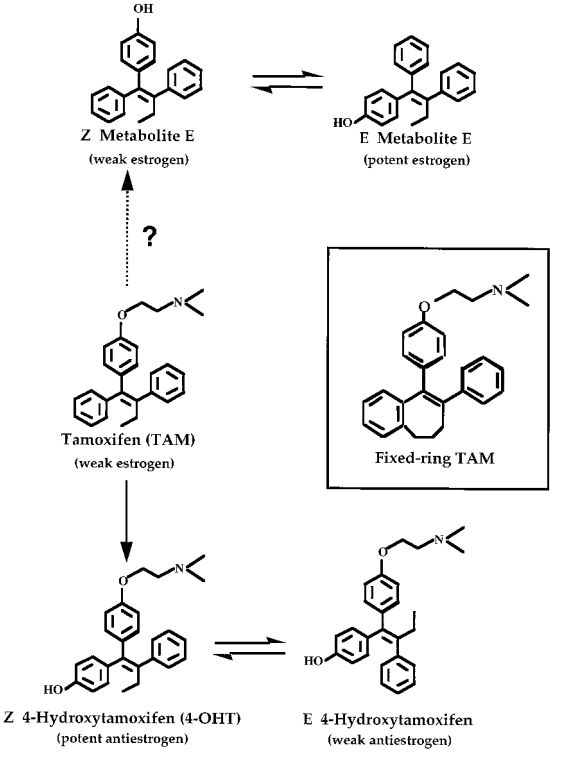


FIG. 14. The local metabolism of tamoxifen could be accomplished by two potential routes. The first is the conversion to Z-4-hydroxytamoxifen (Z-4-OHT) which acts as a potent antiestrogen. The other potential metabolite is the weakly estrogenic Z Metabolite E (Z Met E). The mechanism of tamoxifen-stimulated growth may reside in the ability of Z-4-OHT to isomerize to E-4-OHT, which is a weak antiestrogen, and Z Met E to isomerize to E Met E, which is a potent estrogen. To determine whether this is the mechanism of tamoxifen-stimulated growth, a fixed ring analog of tamoxifen which prevents isomerization of Z Met E to E Met E was synthesized, but this compound was still able to stimulate the growth of MCF-7 tamoxifen-stimulated tumors. Isomerization is unlikely to be the mechanism of tamoxifen-stimulated growth (Wolf *et al*., 1993; Osborne *et al*., 1994).

stimulated MCF-7 tumor growth as tamoxifen (Wolf *et al*., 1993). Similarly, a deoxytamoxifen analog used to eliminate the possibility of side-chain cleavage, thereby preventing the production of Met E or bisphenol, also was found to be similar to tamoxifen in stimulating tumor growth (Osborne *et al*., 1994). This evidence suggests that the isomerization of tamoxifen to estrogenic or less potent antiestrogenic metabolites is not sufficient to explain the emergence of tamoxifen-stimulated tumor growth, and therefore, alternative mechanisms must be considered.

B. Mutant Receptors

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. Therefore, the functional inactivation of the ER by a mutation that increases the efficiency of the antiestrogen-ER complex is a likely mechanism of acquired tamoxifen resistance. Site-directed mutagenesis to create specific amino acid changes in the hormone binding domain has been shown to affect the ligand binding affinity to the receptor, DNA binding, as well as transcriptional transactivation (Danielian *et al*., 1993; Reese and Katzenellenbogen, 1992; Pakdel and Katzenellenbogen, 1992; Mahfoudi *et al*., 1995). Specific mutations in the DNA and LBDs of the ER can cause an antiestrogen to transmit an agonistic rather than an antagonistic signal. Jiang and colleagues (1992a, 1993) demonstrated that a singlepoint mutation which substitutes a valine for a glycine at codon 400 in the LBD of the ER caused enhanced estrogenic activity in response to 4-OHT and other antiestrogens when stably transfected into MDA-MA-231 ER-negative human breast cancer cells (Jiang and Jordan, 1992). The ER is present in breast tumors that fail tamoxifen (Encarnation *et al*., 1993); therefore, potentially, if tamoxifen-resistant tumors have acquired such ER mutations, the mechanism of resistance should be detected easily.

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 (Graham *et al*., 1990; Fuqua *et al*., 1991; Dotzlaw *et al*., 1992; Watts *et al*., 1992; Wolf and Jordan, 1994a,b), it does not appear that mutation of the ER is the principal mechanism of acquired tamoxifen resistance. Karnik *et al*. (1994) screened eight exons of the ER cDNA from 20 tamoxifen-resistant and 20 tamoxifen-sensitive breast cancer tissue specimens using single-strand conformational polymorphism. They concluded that mutations in the ER are rare because only two mutations were found, a single base pair deletion and a 42 base pair replacement in exon 6. Similarly, Watts and colleagues (1992) analyzed 37 ER-negative and ER-positive 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.

During the past decade, there has been interest in developing in vivo laboratory models of estrogen-regulated breast cancer (Gottardis *et al*., 1988a; Shafie and Graham, 1981). Antiestrogens initially control growth (Osborne *et al*., 1985, 1987; Gottardis and Jordan, 1988; Gottardis *et al*., 1989a,b) but eventually tamoxifen-stimulated tumors develop (Wolf and Jordan, 1994a). This is analogous to the clinical situation (Canney *et al*., 1987; Howell *et al*., 1992) and provides a useful model to examine the hypothesis of mutant receptors being required for tamoxifen-stimulated growth. Based on our initial description of tamoxifen-stimulated MCF-7 tumors in athymic mice (Gottardis and Jordan, 1988), we have used single-strand conformational polymorphism to search for mutated receptors. We have characterized three tamoxifen-stimulated tumors, and one tumor contained a single-point mutation within the ER resulting in the replacement of an aspartate for a tyrosine at amino acid position 351 (Wolf and Jordan, 1994b). This mutation resulted in altered pharmacological response of an antiestrogen to an estrogen (Catherino *et al*., 1995; Levenson *et al*., 1997). This is the only report of a single base pair mutation of an ER derived from a tamoxifen-stimulated human breast tumor exhibiting altered pharmacology toward tamoxifen. However, because the remaining tamoxifen-stimulated tumors examined contained wild-type ER, this suggests that other resistance mechanisms must be available to permit growth. By contrast, Mahfoudi and coworkers (1995) suggested that specific mutations in the AF-2 region of the receptor were responsible for ER response to an tamoxifen. However, we recently have sequenced the ER in several human tumors that are stimulated to grow in response to tamoxifen and found no mutations in the AF-2 region (Bilimoria *et al*., 1996b). 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 tamoxifen-resistant or -stimulated growth. The significance of the 351 mutant ER and the mutations in the AF-2 will be considered in Section XVII.

C. Alternate Pathways

Phosphorylation of steroid hormone receptors may mediate hormone and DNA binding as well as transcriptional activation. 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 estradiol, 4-OHT, the pure antiestrogen ICI 164,384, as well as activators of protein kinase A and C (PKA and PKC) (Cho and Katzenellenbogen, 1993; Ali *et al*., 1993; Arnold *et al*., 1994; Le Goff *et al*., 1994). Various protein kinases including PKC, PKA, casein kinase, and src family kinases have been implicated in mediating phos-

phorylation (Arnold *et al.*, 1994, 1995; Le Goff *et al.*, 1994).

Tamoxifen resistance, therefore, 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. Tamoxifen is reported to be a specific inhibitor of at least one candidate protein kinase, PKC (O'Brien *et al.*, 1985, 1988). If this inhibitory activity actually occurs in vivo, tamoxifen also presumably would reduce phosphorylation of the ER and attenuate transcriptional activation, in addition to competing with estradiol for binding to the ER. If PKC acquires a mutation which prevents the inhibitory activity of tamoxifen, inappropriate activation of estrogen-responsive genes may occur. However, a recent report by Lahooti and coworkers (1994) indicates that the presence of estradiol or 4-OHT generates similar phosphopeptide maps of the ER, suggesting that tamoxifen does not inhibit ER phosphorylation. Therefore, it remains to be determined whether a defect in the phosphorylation of the ER may lead to tamoxifen resistance.

Activation of the PKA pathway has been shown to increase the agonist activity of the tamoxifen-ER complex using certain promoter-reporter constructs containing two EREs (Fujimoto and Katzenellenbogen, 1994). The transcriptional activity of the antiestrogen-ER complex was shown to increase by 20% to 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.

Another interesting alternative pathway recently has been implicated in the ability of antiestrogens to act as agonists in certain genes containing AP-1 sites (Umayahara *et al.*, 1994; Phillips *et al.*, 1993; Gaub *et al.*, 1990; Webb *et al.*, 1995). This is an ER-mediated event that seems to be cell type specific; for example, tamoxifen-stimulated AP-1 activation can be seen in cell lines of uterine origin but not of breast. Further studies of the role of the ER in transcriptional activation has lead to questions of whether or not the DBD of the ER is required suggesting that two pathways may exist. One pathway would be based on protein-DNA interactions, and the other pathway would be based on protein-protein interactions.

Many studies of antiestrogen-dependent or -stimulated cell lines have been conducted to determine the mechanism of progression to antiestrogen resistance or antiestrogen stimulation of tumor growth. The expression of jun and fos mRNA, AP-1 DNA-binding activity and transcriptional activation levels have been measured in numerous breast cancer cell lines (Chen *et al.*, 1996). These studies also showed that many growth factors and phorbol esters were able to induce the expression of jun and fos mRNA, AP-1 DNA-binding activity, and transcriptional activation levels, whereas very few of these effects were seen with estrogen treatment. Recently, an MCF-7 cell line was derived by growing tumors in nude mice in the absence of estrogen (Dumont *et al.*, 1996). This cell line became hormone independent but still grew in the presence of E_2 and tamoxifen. When the levels of AP-1 DNA-binding activity were measured in these cells, they were found to be markedly increased.

In a pivotal study, Astruc and colleagues (1995), developed unique cell lines in which the modulation of PKC is translated directly to a modulation of an AP-1 containing reporter construct. The ability of tamoxifen to affect PKC was studied in a breast cancer cell line for both short and long durations. For the short-term studies, they show that antiestrogens can inhibit phorbol ester-induced reporter activity, whereas in long-term studies, antiestrogen treatment decreased the basal AP-1 response but acted synergistically to increase the phorbol ester-induced transcriptional activation mediated by AP-1. This effect was proven to be ER mediated, mainly because treatment with E_2 abolished this effect and it did not occur in an ER-negative cell line. These studies reveal the importance of alternative pathways in the unpredictable cellular responses to long-term antiestrogen treatment and hint at the complexity of this response.

XV. Clinical Application of New Antiestrogens

Adjuvant therapy has revolutionized the prospects for survival of the patient with either node-positive or node-negative ER-positive disease (Early Breast Cancer Trialists Collaborative Group, 1988, 1992, 1998). However, these are two controversial aspects of the strategic applications of tamoxifen that are receiving increasing scrutiny. Five years of adjuvant tamoxifen provides a survival advantage for women (Breast Cancer Trials Committee, 1987; Swedish Breast Cancer Cooperative Group, 1996; Anonymous, 1996), although stopping tamoxifen at 5 years is controversial (Fisher *et al.*, 1996; Stewart *et al.*, 1996; Tormey *et al.*, 1996).

Currently, trials of extended adjuvant tamoxifen therapy are ongoing in Britain to define the optimal duration of tamoxifen for node-positive breast cancer. The trials, ATLAS (Adjuvant Tamoxifen Long Against Short) and aTTom (adjuvant Tamoxifen To offer more), will each recruit 20,000 patients who are uncertain about the value of only 5 years of tamoxifen and are electing to be assigned randomly to either stop or continue tamoxifen for a further 5 years. Overall, adjuvant tamoxifen is the cornerstone of breast cancer therapy; therefore, as much benefit must be achieved as possible and then additional new endocrine agents can be positioned to exploit drug resistance.

The second controversial strategy is the clinical trial of tamoxifen as a breast cancer preventive in high-risk women. The concept originally was based on three

known facts: the clinical safety of tamoxifen (Furr and Jordan, 1984), the ability of tamoxifen to prevent mammary cancer in rats (Jordan, 1974, 1976), and the ability of tamoxifen to prevent contralateral breast cancer (Cuzick and Baum, 1985). Pilot clinical trials originally were initiated in 1986 with 2000 women randomly assigned to receive tamoxifen or placebo for 8 years (Powles *et al*., 1989, 1990, 1994). The original vanguard study is complete and the group hopes to recruit 20,000 high-risk women in the United Kingdom and Australia. In North America, the NSABP has completed the recruitment of 13,000 very high risk women randomly assigned to receive tamoxifen or placebo for 5 years. Recent analysis shows a 45% decrease in breast cancer incidence with tamoxifen (http://www.aomc.org/News Release/BC Prevention Trial.html). In Italy, recruitment is ongoing for 20,000 normal-risk women, who have had a hysterectomy, to be randomly assigned to receive tamoxifen or placebo for 5 years.

The current clinical application of antiestrogenic strategies for breast cancer are illustrated in figure 15. After the failure of adjuvant tamoxifen, the aromatase inhibitor anastrozole is currently available (Buzdar *et al*., 1996). However, a whole range of aromatase inhibitors is currently being evaluated (Goss and Gwyn, 1994). The mechanism depends on the fact that tamoxifen-resistant disease still requires estrogen to grow, so by reducing circulating estradiol and estrone, the disease will lose its growth stimulus. Another approach would be to develop an antiestrogen that blocks ER that was not cross-resistant with tamoxifen. This new agent would then serve as a second-line endocrine therapy.

However, a broader view is being investigated currently that is aimed at the development of a preventive maintenance therapy (Jordan, 1997c). The controversy about tamoxifen and carcinogenesis has encouraged a search for a potentially safer long-term treatment that could be used in high-risk women. However, an optimal strategy to prevent breast cancer is achieved best if viewed as an overall issue of women's health. To this end, in 1987, a new approach to breast cancer prevention was proposed. Because tamoxifen and raloxifene can maintain bone density in rats, this property could be used to prevent osteoporosis in postmenopausal patients. The goal was to introduce a new hormone replacement therapy to prevent osteoporosis but to decrease the incidence of endometrial cancer and breast cancer in the general population as a beneficial side effect (Jordan *et al*., 1987b). At the same time it was shown that tamoxifen and raloxifene could prevent rat mammary carcinogenesis (Gottardis and Jordan, 1987). However, the fact that laboratory studies showed that there might be a link between tamoxifen and endometrial cancer (Gottardis *et al*., 1988b) shifted the emphasis away from tamoxifen but toward a general strategy for new drug development (Lerner and Jordan, 1990).

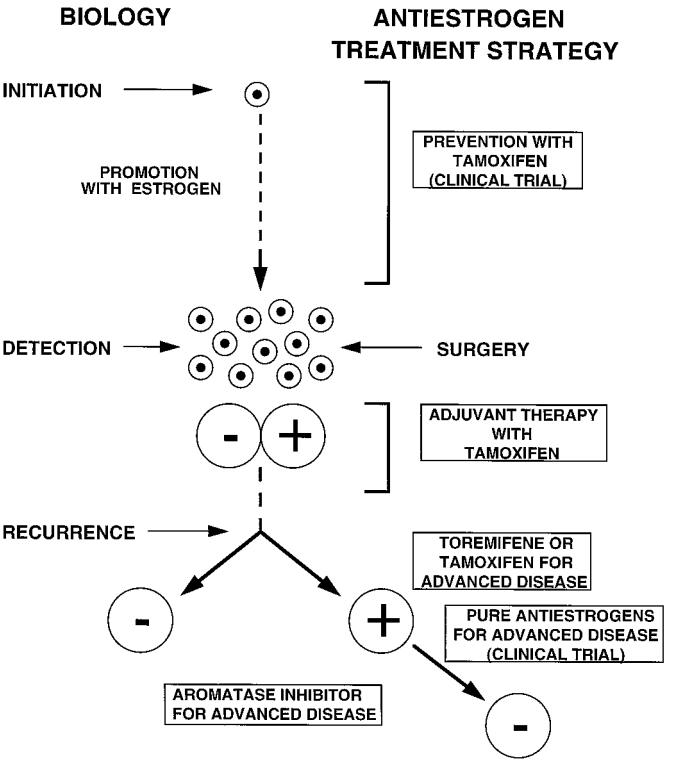


FIG. 15. The current clinical strategies to apply antiestrogens for the treatment of breast cancer. Adjuvant therapy with tamoxifen is recommended for ER-positive patients with breast cancer with or without chemotherapy. After the failure of tamoxifen, the aromatase inhibitor, anastrazole, is recommended for use. New pure antiestrogens are also in clinical trial to treat patients in whom adjuvant tamoxifen fails. In contrast, patients who present with metastatic breast cancer and who have not had adjuvant tamoxifen for early-stage disease may be treated with either tamoxifen or toremifene. Clinical trials with tamoxifen in women with a high risk of developing breast cancer will be complete early in the 21st century.

This goal has now become a reality (Jordan, 1995a; Tonetti and Jordan, 1996). In the following sections, we will describe the progress made in the clinical development of new antiestrogens during the past decade with the strategic goals of the treatment of breast cancer or the treatment of osteoporosis. It is not our aim to provide an exhaustive review of the clinical literature because this has been published recently elsewhere (Gradishar and Jordan, 1997). We will explore the laboratory rationale for current clinical testing, however, and point out the problems and potential strengths of the new agents in clinical trials.

A. Tamoxifen Analogs for Breast Cancer

Toremifene or chlorotamoxifen (Fareston®) (fig. 7) is available in the United States for the treatment of Stage IV breast cancer in postmenopausal patients.

The drug is antiestrogenic (Kangas *et al*., 1986) and has antitumor activity in carcinogen-induced rat mammary cancer (Robinson *et al*., 1988; DiSalle *et al*., 1990) but it is less potent than tamoxifen. Toremifene has approximately one third the potency of tamoxifen. This laboratory observation translates to the clinic because

60 mg daily of toremifene is recommended for the treatment of Stage IV breast cancer compared with the standard 20 mg daily dose of tamoxifen.

Originally, it was thought that toremifene would have activity in ER-negative tumors (Kangas, 1990). However, extensive studies in athymic mice demonstrate that toremifene is only active in cells that express ER and the growth of mixed ER-positive and -negative tumors cannot be controlled by toremifene (Robinson and Jordan, 1989a). Toremifene has been tested extensively in phase I to III clinical trials (Valavaara *et al*., 1988; Weibe *et al*., 1990; Hamm *et al*., 1991; Kivinen and Maenpaa, 1990; Hayes *et al*., 1995). The side effects are similar to tamoxifen's, and as with tamoxifen, responses usually are observed in the ER-positive patients. However, because most patients already have taken adjuvant tamoxifen therapy, the issue of cross-resistance is extremely important. Laboratory studies by Osborne *et al*. (1994) have demonstrated that toremifene-stimulated breast tumor growth can occurr with MCF-7 cells transplanted into athymic mice. Similarly, cross-over clinical trials demonstrate that there is little likelihood of a second response to toremifene after tamoxifen failure (Vogel *et al*., 1993; Stenbygaard *et al*., 1993). Toremifene has been studied as a treatment of Stage IV disease across a broad dose range of 40 to 240 mg daily. There is no clear dose-response relationship, and side effects increase with dose (Hayes *et al*., 1995).

The interesting aspect of the pharmacology of toremifene is the reduced liver carcinogenicity in the rat (Hirsimaki *et al*., 1993; Hard *et al*., 1993). Toremifene produces fewer DNA adducts (Hard *et al*., 1993); however, there are reports of DNA damage (Sargent *et al*., 1994, 1996; Styles *et al*., 1994). Nevertheless, toremifene has a lower carcinogenic potency than tamoxifen both as a complete carcinogen (Hard *et al*., 1993; Dragan *et al*., 1995) and a tumor promoter (Dragan *et al*., 1995). Overall, these are important observations that will aid in the understanding of the toxicology of drugs in the rat, but there is no convincing evidence that these data can be extrapolated to clinical practice (see Section XIII.B.). Additionally, there are no reports about an association between toremifene and endometrial cancer primarily because the data base is so small. The first reports about tamoxifen did not appear until 10 years after approved use in the United Kingdom and 6 years after approval in the United States. Even then, the rate is modest (see Section XIII.A.). Nevertheless, because the general pharmacology of toremifene in the uterus and endometrium is similar to tamoxifen's (Tomas *et al*., 1995), patients should be apprised of the potential risks with toremifene to encourage the growth of preexisting endometrial carcinoma.

B. Pure Antiestrogens for Breast Cancer

There is only one compound, ICI 182,780 (Faslodex®) (fig. 9), that has entered clinical trial. As yet, there are no clinical reports of RU 58,668 (see Section VII.B.) or EM-800 (see Section XVI.A.). ICI 182,780 has poor bioavailability orally but is an effective antiestrogen by depot injection (Wakeling and Bowler, 1988; Wakeling *et al*., 1991; Wakeling, 1994). Extensive studies in the laboratory all demonstrate that pure antiestrogens have virtually no estrogen-like effects and can be effective in inhibiting the growth of the tamoxifen-stimulated model of breast cancer (Gottardis *et al*., 1989a; Osborne *et al*., 1995). Furthermore, the compounds inhibit the growth of endometrial tumors (Gottardis *et al*., 1990) and extensive studies in the monkey show no agonist effects in the uterus (Dukes *et al*., 1992, 1993).

Clinical studies demonstrate that a short course of daily injections of ICI 182,780 can reduce the Ki67 index, progesterone receptors and ER in breast tumors (Defriend *et al*., 1994). A preliminary clinical trial shows good activity for ICI 182,780 as a second-line endocrine therapy after tamoxifen failure (Howell *et al*., 1995, 1996). Side effects appear to be minimal and broader studies are currently underway in the United States. Clearly, this group of agents will be valuable first- or second-line agents for the treatment of Stage IV breast cancer. Similarly, adjuvant therapy for high-risk Stage II patients with 10 plus positive lymph nodes will be a potential application. However, studies on bone density changes need to be considered before general consideration of testing in early-stage disease. Conversely, the availability and use of bisphosphonates to increase bone density may reduce long-term concerns about pure antiestrogens and decreases in bone density in elderly patients.

C. Targeted Antiestrogens for Osteoporosis

1. Raloxifene (also referred to in the literature as LY 156, 758, keoxifene, LY 139, 481-HCL, Evista®). Extensive structure-activity relationship studies have been reported (Jones *et al*., 1984; Grese *et al*., 1997) with benzothiophenes; however, two compounds, LY 117,018 and LY 156,758 (fig. 8), have been described by scientists at Lilly Laboratories to possess high binding affinity for the ER, to exhibit potent antiestrogenic activity, but to have little uterotrophic activity in rodents (Black *et al*., 1983; Black and Goode, 1980, 1981) (fig. 10). Indeed, uterotrophic activity is less than tamoxifen's in immature rats and LY 117,018 can block the uterotrophic action of both estrogen and tamoxifen (Jordan and Gosden, 1983a,b). Extensive studies in rats (Sato *et al*., 1994, 1995, 1996; Evans *et al*., 1994, 1996) have confirmed the original report that raloxifene preserves bone density in response to oophorectomy (Jordan *et al*., 1987c). Additionally, Fournier and coworkers (1996) have concluded that raloxifene exhibits estrogen-like effects in bone cells but not in uterine cells, and raloxifene has stimulated the expression of TGFβ3 in rat bone (Yang *et al*., 1996a). Overall, these results have laid the

foundation for the evaluation of raloxifene for the prevention of osteoporosis in postmenopausal women.

Additionally, raloxifene causes a decrease in circulating cholesterol in the rat (Black *et al*., 1994; Frolik *et al*., 1996) and in humans (Draper *et al*., 1996). Most importantly, this drug group displays antitumor activity against breast cancer cells in vitro (Scholl *et al*., 1983) and raloxifene prevents rat mammary carcinogenesis (Gottardis and Jordan, 1987, Anzano *et al*., 1996).

Overall, raloxifene displays the profile of a selective ER modulator that could be applied as a potential preventative for osteoporosis but with the additional benefit of preventing breast cancer and coronary heart disease.

The evaluation of raloxifene for the prevention of osteoporosis is being completed in clinical trials around the world. Eleven thousand women have been randomly assigned to receive either raloxifene or placebo. Preliminary data demonstrate that raloxifene maintains bone density (Delmas *et al*., 1997; Gunness *et al*., 1997) but has a nonestrogenic profile in the human uterus (Scheele *et al*., 1997) and breast (Jordan *et al*., 1998).

2. Droloxifene. Droloxifene or 3-hydroxytamoxifen (fig. 7) is an antiestrogen with well-documented antitumor activity in laboratory models (Wosikowski *et al*., 1993; Eppenberger *et al*., 1991; Loser *et al*., 1985). These data lead to the extensive clinical testing of droloxifene in Stage IV breast cancer (Raushning and Pritchard, 1994). As might be expected for an agent that has rapid clearance and can be conjugated rapidly by Phase II metabolic enzymes (Grill and Pollow, 1991; Lien *et al*., 1995), doses of 60 mg daily and greater are effective as breast cancer treatments.

The general pharmacology and toxicology of droloxifene has been reviewed (Hasman *et al*., 1994). Droloxifene does not produce DNA adducts or liver tumors in rats. These properties permit a broader use in therapy to exploit the activity as a bone-preserving agent (Ke *et al*., 1995a,b, Chen *et al*., 1995).

3. Idoxifene. This triphenylethylene derivative (fig. 7) was designed to be metabolically stable with the hope of less carcinogenic potential (McCague *et al*., 1989, 1990). The 4-iodo group prevents 4-hydroxylation, and the pyrrolidino group prevents side chain metabolism. The goal is to develop a drug with efficacy both in the prevention of osteoporosis and the treatment of breast cancer. However, published reports presently focus on the potential of idoxifene as an antiestrogen-anticancer agent. Idoxifene inhibits the growth of carcinogen-induced rat mammary tumors (Chander *et al*., 1991), the growth of MCF-7 breast cancer cells in vitro (McCague *et al*., 1989), and tumors inoculated into athymic mice (Johnston *et al*., 1997). Interestingly enough, idoxifene appears to develop acquired antiestrogen resistance more slowly than tamoxifen (Johnston *et al*., 1997), so the potential exists for longer durations of treatment as an adjuvant or as a second-line therapy after tamoxifen treatment fails.

Additionally, idoxifene has been evaluated as an inhibitor of calmodulin and the p-glycoprotein for multidrug resistance. Extensive studies on the structure-activity relationships of triphenylethylenes and calmodulin inhibition (McCague *et al*., 1994; Hardcastle *et al*., 1996; Edwards *et al*., 1992; Rowlands *et al*., 1990) have demonstrated that idoxifene is five times more active than tamoxifen as an inhibitor. Nevertheless, an analog with a longer side chain [$(CH_2)_8$] was 30 times more potent and a fixed ring version of tamoxifen, with equivalent antiestrogenic activity to tamoxifen, was inactive as a calmodulin inhibitor. Regrettably, the value of calmodulin inhibition as a specific drug target is unknown so these studies may be academic. However, the fact that tamoxifen has such a low toxicity profile and calmodulin is ubiquitous leads to the conclusion that the target is not relevant in the antitumor actions of antiestrogens. Conversely, focusing on calmodulin as a target may lose tumor specificity and increase serious side effects. Additionally, there has been great interest in the use of triphenylethylenes to confound multidrug resistance. At present, idoxifene has been shown to reverse multidrug resistance in cell culture (Kirk *et al*., 1994; Sharp *et al*., 1994) but no clinical studies have been reported.

Clinical studies with idoxifene as an anti-breast cancer agent are being conducted worldwide. At present, only a single report of a phase I study has appeared (Coombes *et al*., 1995). As would be expected from a metabolically stable drug, idoxifene has a very short terminal half-life of 23.3 days. Nineteen of the patients in the study previously had received tamoxifen, but four patients showed stabilization of disease for up to a year and two patients showed a partial response. Overall, idoxifene is at an early stage of clinical development for osteoporosis in postmenopausal women and as a potential treatment and preventative for breast cancer.

XVI. New Compounds and New Opportunities

We now wish to mention some intriguing reports that have appeared recently in the literature which may compliment the huge clinical efforts described in Section XV. When the first antiestrogen, MER-25, was discovered by Lerner and coworkers (1958), a primary goal for clinical investigation was as a contraceptive because the compounds were antifertility agents in the rat (Segal and Nelson, 1961). However, despite the fact that the triphenylethylenes clomiphene (Holtkamp *et al*., 1960) and tamoxifen (Harper and Walpole, 1967a,b) were both antifertility agents in rats, both induce ovulation in subfertile women. As a result, general interest in this area of investigation by the pharmaceutical industry waned in the 1960s. Remarkably, recent reports from India suggest that studies with antiestrogens as contraceptives continue. The drug centchroman (Ray *et al*., 1976) (fig. 16) has been studied extensively in the laboratory (Paliwal and Gupta, 1996; Paliwal *et al*., 1992; Singh *et*

al., 1994; Trivedi *et al*., 1995) and preliminary studies in humans have been completed (Misra *et al*., 1989; Kamboj *et al*., 1977; Lal *et al*., 1995; Gupta *et al*., 1995; Paliwal *et al*., 1989). Although contraceptive research is controversial, it would be remarkable if renewed interest in the drug group confirmed an application at the source of their origins (Kamboj *et al*., 1977).

Additionally, the drug development group in Lucknow, India has been extremely active during the past decade and described an extensive series of structure-activity relationships with centchroman. The most potent antiestrogenic compound the group described is illustrated in figure 16 (Sharma *et al*., 1990a,b; Saeed *et al*., 1990). Obviously, the potency is derived from two strategically located hydroxyls but the change in positioning of the phenyl alkylaminoethyoxy side chain is reminiscent of the location of the side chain in ICI 182,780 (fig. 9). Clearly, there is potential for further drug development but the interesting fact is that the Lucknow group did not resolve the isomers that are possible where the phenyl group joins the chromane ring. By contrast, Labrie's group in Canada recently documented an orally active pure antiestrogen (EM-800) (fig. 17) with marked similarity to the Lucknow compounds.

A. EM-800

The compound EM-800 is an orally active pure antiestrogen (Gauthier *et al*., 1997). The compound EM-800 and the de-esterified version, EM-652 (Simard *et al*., 1997b), which undoubtedly is the active compound, have stereochemistry that is reminiscent of the compound ICI 182,780 with a hydrophobic side chain at the 7α-position of E_2 (see figs. 9 and 17). Preliminary studies demonstrate that EM-800 is an orally active antitumor agent in the DMBA model (Luo *et al*., 1997c,d) and long-term studies in the mouse show clear-cut antiestrogenic activity (Luo *et al*., 1997b) with little or no estrogenic activity compared with either tamoxifen or toremifene (Simard *et al*., 1997b). The drug is extremely potent against breast cancer cells in culture (Simard *et al*., 1997a) and prevents the growth of estrogen-stimulated tumor xenografts in athymic mice (Luo *et al*., 1997a).

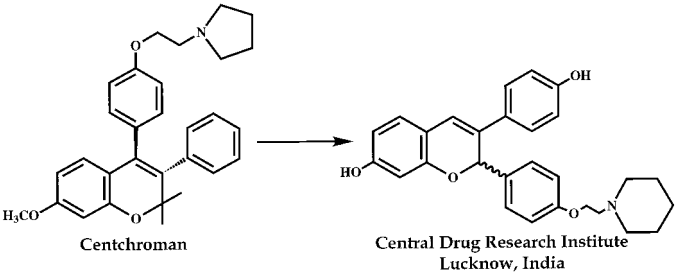


FIG. 16. A comparison of the antifertility agent, centchroman, and a structural derivative with extremely potent antiestrogenic activity discovered by the Central Drug Research Institute in Lucknow, India (Sharma *et al*., 1990a).

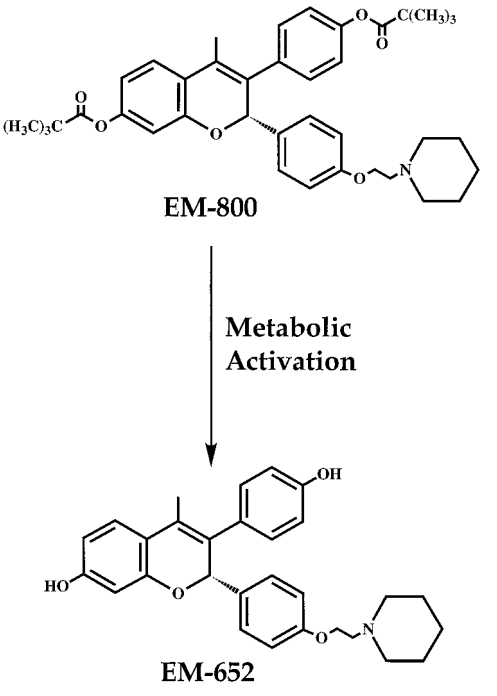


FIG. 17. The metabolic activation of the orally active pure antiestrogen EM-800 to its active metabolite, EM 652 (Simard *et al*., 1997).

Clearly, an orally active pure antiestrogen will be extremely valuable as a second-line therapy after tamoxifen treatment has failed. However, it would be prudent to evaluate the potential cross-resistance with tamoxifen to ensure a strong scientific rationale for a clinical application. Additionally, the compound does not seem to have a dramatic effect on reducing bone density in rats (Luo *et al*., 1997d), so use as an adjuvant therapy in node-positive breast cancer could be a possibility.

B. Peripheral Selectivity

In this review, we surveyed the concept of a target site-specific antiestrogen, but there are concerns that long-term treatment of postmenopausal women may not produce "estrogenic" effects in the CNS. Retrospective surveys from the epidemiological data base suggest that women who have taken postmenopausal hormone replacement therapy have a reduced incidence of Alzheimer's Syndrome (Tang *et al*., 1996). With an aging population and the proposed use of selective estrogen receptor modulator for the long-term prevention of osteoporosis, the prospect of exacerbating an already high incidence of Alzheimer's could prove to be unwise if solutions are not found.

One solution is to develop a compound that does not enter the central nervous system. This may reduce postmenopausal symptoms and the possibilities of depression. Indeed, the combination of an appropriate compound with Premarin™ could provide the benefits of estrogen in the CNS and the benefits of targeted antiestrogens in the periphery. If estrogen is proven to be beneficial in prospective trials, then the issue of exacer-

bating Alzheimer's with antiestrogens would be resolved. Two compounds presently described in the literature are of particular interest (fig. 18).

Trimethyl tamoxifen is a quaternized derivative of tamoxifen (Biegon *et al*., 1996) that has low uptake and retention in the CNS, but produces the appropriate action of tamoxifen in the periphery. Studies using athymic mice implanted with MCF-7 breast tumors show that the quaternary ammonium derivative has antitumor activity (Biegon *et al*., 1996). The fact that previous studies with quaternized compounds in vitro show poor activity probably reflects the fact that the principal route of metabolism for tamoxifen in the athymic mouse is to the 4-hydroxy derivative (Robinson *et al*., 1989c). The high potency of the 4-hydroxy derivative might allow significant biological activity at the ER even though there is low penetrance into breast tumor cells.

Nevertheless, the principal concern with the quaternary ammonium compounds is the route of administration. All the studies in vivo used injections so formulation becomes an issue. Although, it is generally believed that quaternary ammonium compounds have low oral activity, the fact that tamoxifen derivatives are extremely lipophilic suggests that absorption, in the absence of an active pump like the blood brain barrier, could be possible. Studies should compare and contrast routes of administration to confirm this hypothesis.

The compound GW 5638 (fig. 18) (Willson *et al*., 1995, 1997) is particularly interesting because it appears to fit the required criteria of a peripherally selective agent. The primary goal of targeted drug discovery is to achieve complete or pure antiestrogenic action in the breast and the uterus but to maintain full estrogenic activity in the bones and low circulating cholesterol levels (LDLs). The GW 5638 is a carboxylic acid, and as a result, there may be low penetration into the CNS (Dr. Donald McDonnell, personal communication). GW 5638 has full agonist activity to maintain bone density and lower cholesterol in ovariectomized rats but little agonist activity is detected in the uterus (Willson *et al*., 1997) and the drug possesses antitumor actions in breast cancers transplanted into athymic mice (Dr. Donald McDonnell, personal communication). Of particular interest is the observation that the compound is a complete antiestrogen in the mutant AF-2 ER assay developed in HepG2 cells using the C3 (complement) promoter system (Norris *et al*., 1996). McDonnell has classified antiestrogens based on his in vitro screen (McDonnell *et al*., 1995), but GW 5638 has a distinctly different profile (Willson *et al*., 1997) This observation creates a new class of antiestrogens, and it may be important in designing future targeted drugs without uterine activity.

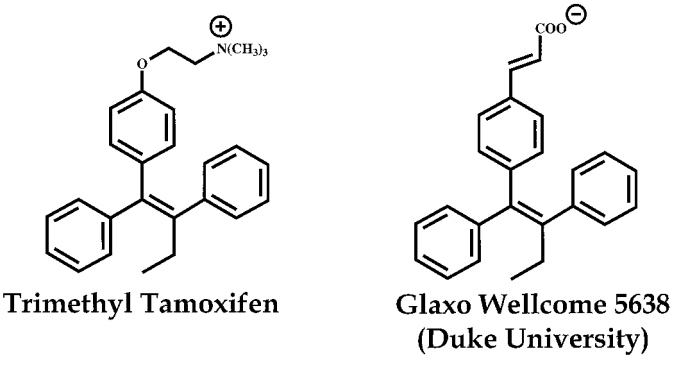


FIG. 18. The peripherally selective antiestrogens that could be used as binary therapies with estrogen.

Although it is extremely important to identify new compounds, it is also important to learn from the development of tamoxifen. Both compounds illustrated in figure 18 are derivatives of tamoxifen and both have potential for clinical testing. However, any drug introduced into general medicine today must be free from carcinogenic potential in laboratory tests. The fact that these novel agents are tamoxifen derivatives suggests that there is a high probability for complete carcinogenesis in the rat liver (see Section XIII.B. and C.). Clearly, it would be prudent to develop an alternate analog or to determine the carcinogenic potential of the new drugs at the earliest opportunity. By analogy, with tamoxifen, a simple solution would be to introduce a 3-hydroxy (or acetyoxy) that should resolve the problem immediately, if one exists.

XVII. Crystallization of the Raloxifene-Estrogen Receptor Complex

Shortly after our review was completed, the crystal structure of the LBD of the ER with estradiol or raloxifene was published (Brzozowski *et al*., 1997). This knowledge now provides an important insight into both estrogen and antiestrogen action and remarkably brings together apparently unrelated facts from the literature. We now can formulate a molecular model of the events that result in the blocking of the estrogen signal transduction pathway.

The hydroxyls of estradiol specifically bind to the amino acids 353 and 394 in the LBD (fig. 19) and this causes the large helix 12 to fold over and trap the steroid (fig. 20). Helix 12 contains three specific amino acids, 540, 543, and 547, which are critical, within the AF-2 region, for binding coactivators (Tzuckerman *et al*., 1994).

The phenolic groups of raloxifene bind to the same amino acids as estradiol (fig. 19) (Brzozowski *et al*., 1997) but the critical difference is the interaction of the alkylaminoethoxy side chain with the amino acid, aspartate, at position 351. The aminoethoxy side chain of antiestrogens is essential to block estrogen action (Jordan, 1984). Changes in the distance between the nitrogen and the oxygen (Lednicer *et al*., 1966, 1967), changes in the basicity of the nitrogen (Robertson *et al*., 1982), and the orientation of the side chain (Clark and Jordan, 1976) all results in loss of antiestrogen properties. Indeed, removal of the side chain results either in in-

creased estrogenic activity or a complete loss of activity (Jordan and Gosden, 1982).

The crystal structure of the raloxifene-ER complex demonstrates that Helix 12 becomes reoriented and cannot seal the pocket containing the ligand (fig. 20). The AF-2 region is repositioned so that coactivators cannot form a transcription complex, and signal transduction is blocked. However, the findings by Mahfoudi and colleagues (1995) and Montano and colleagues (1996) that mutations in the critical amino acids 540, 543, 544, 547, and 548 in the AF-2 region can increase estrogenicity of an antiestrogen receptor complex suggests that Helix 12 can twist so that coactivators now can bind to the AF-2 region.

It is important to appreciate that the macromolecular perturbations noted in the crystal structure are the consequences of antiestrogen binding and are not the cause or the key to antiestrogen action. Earlier models described "ligand wedging" of antiestrogens in the jaws of the LBD to prevent activation and "ligand locking" to form an "estrogenic complex" (Tate *et al*., 1984). Similarly, models devised from pharmacological assays depended on the alkylaminoethoxy side chain binding to an "antiestrogenic region" of the receptor to prevent the jaws of the LBD from closing (Lieberman *et al*., 1983a). From the crystal structure that "region" is now identified as amino acid 351 (Brzozowski *et al*., 1997).

The validity of the model comes from studies with the mutant receptor in which amino acid 351 is changed from an aspartate to a tyrosine (Wolf and Jordan, 1994b). To test the pharmacological action of the mutant receptor, both the wild-type and the 351 mutant receptor have been stably transfected into MDA-MB-231 breast cancer cells (Jiang and Jordan, 1992; Catherino *et al*., 1995). Estradiol increases TGFα mRNA in a concentration-dependent manner in both the wild-type and mutant ER transfectants. However, raloxifene is an antiestrogen in the wild-type receptor transfectants but becomes estrogenic with the 351 mutant receptor (Levenson *et al*., 1997, Levenson and Jordan, 1998). These results are illustrated in fig. 21. The pure antiestrogen ICI 182,780 is a complete antiestrogen in both transfectants; therefore, the conformation of the receptor complex must be very different from the raloxifene-ER complex.

The discovery of a 351 mutant receptor not only confirms the pharmacological importance of this amino acid as the key to the antiestrogenicity of raloxifene but also illustrates a mechanism of drug resistance to tamoxifen. The mutant receptor was isolated from a tamoxifen-stimulated breast tumor and increases the estrogenicity of tamoxifen analogs (Catherino *et al*., 1995). Obviously, the mutant receptor confers a growth advantage to breast cancer cells. This, therefore, is the first confirmed

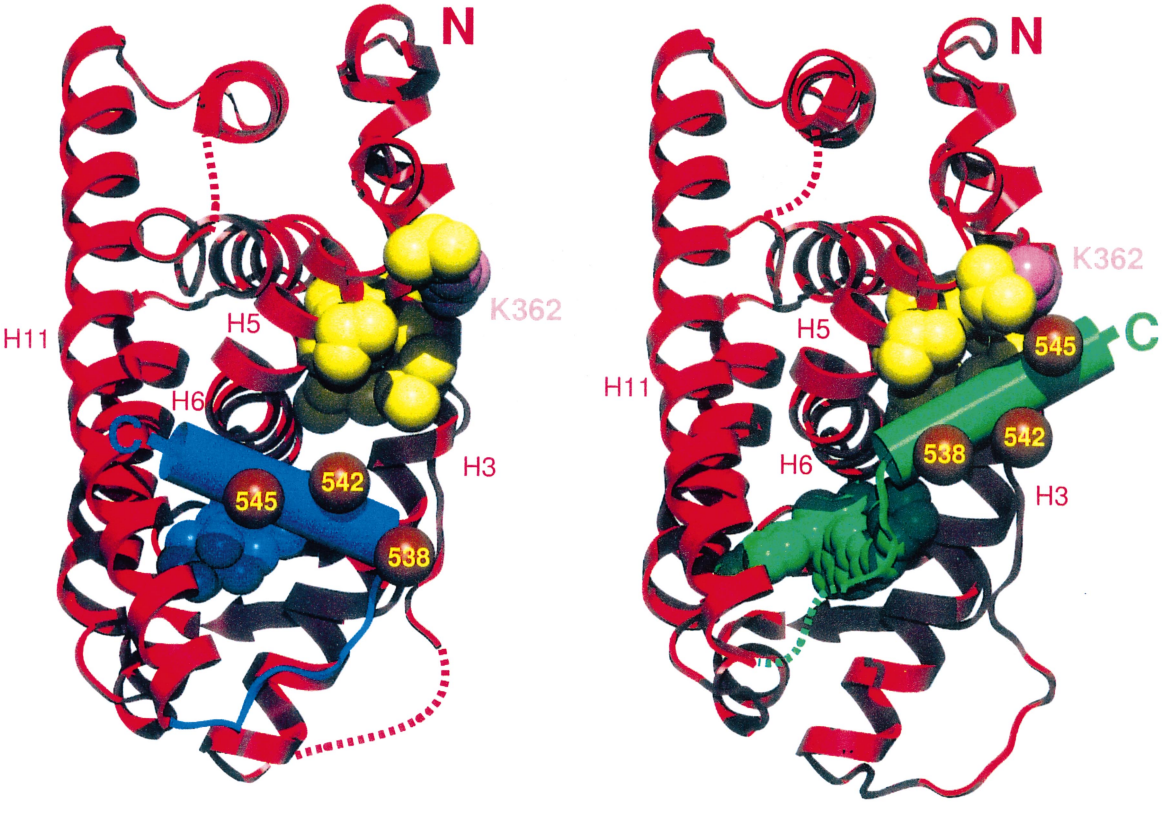


FIG. 19. The three-dimensional protein folding around estradiol to activate the receptor complex. Helix 12 must fold across the steroid and seal the ligand-binding pocket. The critical amino acids 538, 542, and 545 in the AF-2 region are exposed to allow binding to coactivators before a transcription complex can be constructed. By contrast, when raloxifene occupies the binding pocket, Helix 12 is reposited and the critical amino acids in the AF-2 region are now masked. Reproduced from Brzozowski *et al*. (1997) with permission from Nature©.

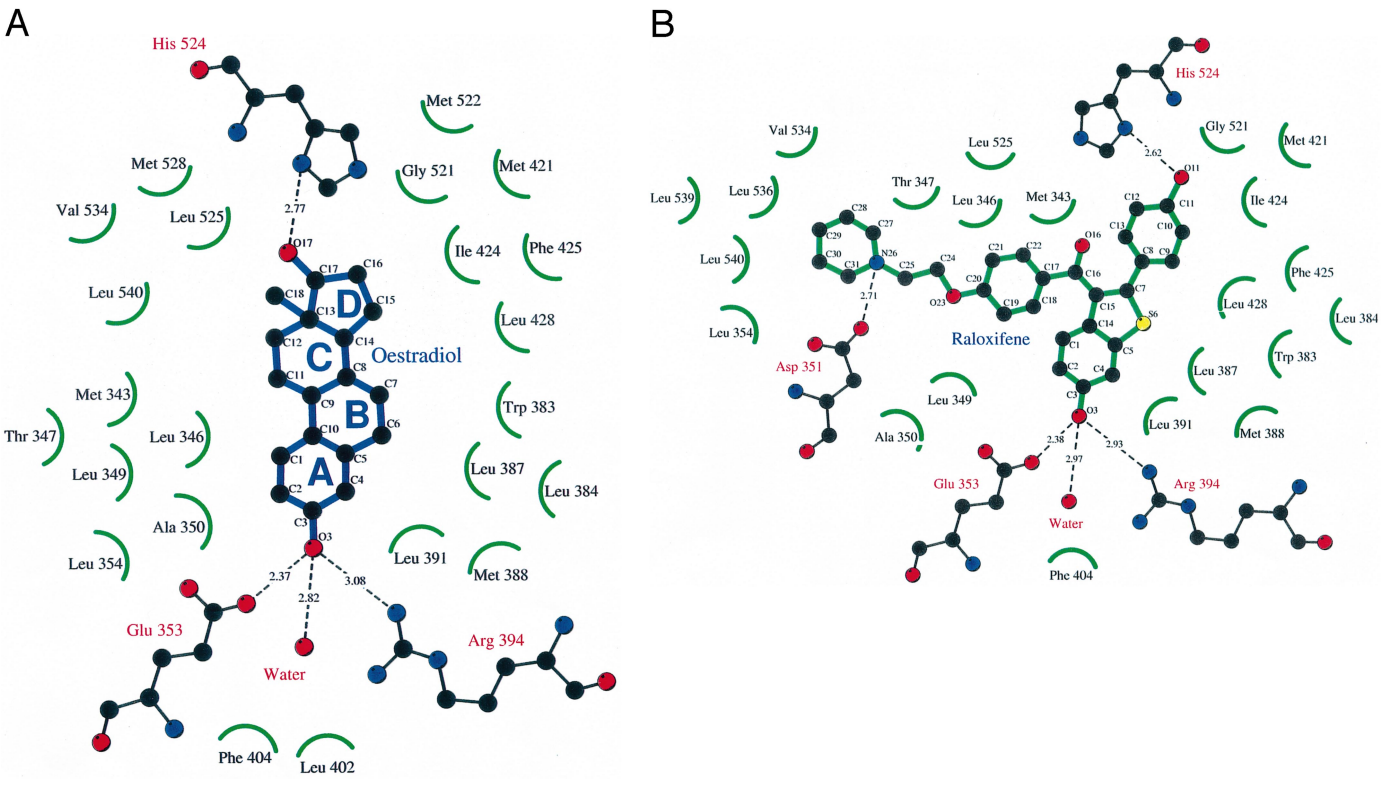


FIG. 20. The interaction of (*A*) estradiol and (*B*) raloxifene with critical amino acids in the LBD of the ER (Brzozowski *et al*., 1997).

example of a mechanism of resistance for an antiestrogen. However, because numerous mutant receptors have not been noted in either laboratory or clinical samples, other mechanisms such as an increase of coactivator molecules must be dominant or act in concert with mutant receptors.

XVIII. Summary and Conclusions

Forty years ago, Lerner and coworkers (1958) discovered the first nonsteroidal antiestrogen and Jensen (Jensen and Jacobson, 1960) identified a target for drug action, the ER. This knowledge opened the door for the clinical development of tamoxifen which we now know provides a survival advantage in both node-positive and node-negative patients with ER-positive disease (Early Breast Cancer Trialists Collaborative Group, 1992, 1998). The drug has been studied extensively, and the results have provided an invaluable insight into possible ancillary advantages of "antiestrogens", i.e., maintenance of bone density and the prevention of coronary heart disease, and possible disadvantages, i.e., rat liver carcinogenesis and an increased risk of endometrial cancer. Most importantly, the identification of the target site-specific actions of tamoxifen caused a paradigm shift in the prospective uses of antiestrogens from a direct exploitation of the antitumor properties to the broader application as a preventative for osteoporosis, but with the beneficial side effects of preventing breast and endometrial cancer.

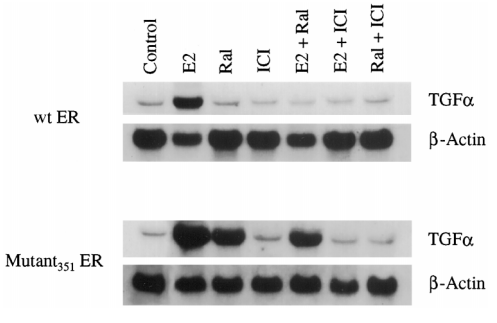


FIG. 21. The detection of TGFα mRNA by Northern blot analysis from MDA-MB-231 breast cancer cells stably transfected with cDNAs from wild-type (wt) or the 351 mutant ER. Cells were treated with either estradiol (10^{-8}M), raloxifene, or ICI 182,780 (10^{-6}M). Adapted from Levenson *et al*. (1997).

Raloxifene, a second-generation SERM, has all the properties in the laboratory that would encourage development as a safe preventative for osteoporosis (Jordan *et al*., 1997). As a result, raloxifene has been evaluated in more than 11,000 postmenopausal women and found to maintain bone density with significant decreases in breast cancer incidence and no increase in endometrial thickness. Raloxifene is now available as a preventative for osteoporosis in postmenopausal women. There is every reason to believe that a multifaceted agent like raloxifene will find widespread use, and there will be continuing interest by the pharmaceutical industry in the development of new agents with even broader applications.

The extensive clinical effort is augmented by past molecular innovations in the laboratory and the future

promise of new discoveries. The cloning and sequencing of the ER (Green *et al*., 1986; Greene *et al*., 1986) has allowed the development of an ER knock-out mouse (Lubahn *et al*., 1993) that compliments Jensen's pioneering work (Jensen and Jacobson, 1962) and describes the consequences of the loss of ERα. However, ERβ (Kuiper *et al*., 1996), the second ER, has provided an additional dimension to the description of estrogen and antiestrogen action. For the future, the development of ERβ monoclonal antibodies, the classification of target sites for the protein around the body, and the creation of ERβ and ERα,β knock-out mice will identify new therapeutic targets to modulate physiological functions. Clearly, the successful crystallization of ERα with raloxifene (Brzozowski *et al*., 1997) must act as a stimulus for the crystallization of ERβ.

The central issue for research on antiestrogen pharmacology is the discovery of the mechanism (or mechanisms) of target site-specificity for the modulation of estrogenic and antiestrogenic response. The description of a stimulatory pathway for antiestrogens through an AP-1 ERβ signal transduction pathway (Paech *et al*., 1997), although interesting, may not entirely explain the estrogenicity of antiestrogens. The model must encompass the sum of pharmacological consequences of signal transduction through ERα and ERβ with the simultaneous competition from endogenous estrogens at both sites. This is complicated because estradiol is an antagonist at ERβ through AP-1 sites (Paech *et al*., 1997), so this is clearly not the pathway for estrogen-induced bone maintenance in women. Estrogen is stimulatory through ERα, but antiestrogens are usually partial agonists and may either block or stimulate genes. However, we suggest that the ERα stimulatory pathway could be amplified through selective increases in coactivators.

The principle is illustrated with the MDA-MB-231 cells stably transfected with the cDNAs for the wild-type and the amino acid 351 mutant receptors (Jiang and Jordan, 1992; Catherino *et al*., 1995). Raloxifene has increased estrogenicity with the mutant ER transfectant compared with the transfectants containing wild-type ER where the pharmacology of raloxifene is a complete antiestrogen (fig. 21). By contrast, 4-OHT is a complete estrogen with the wild-type ER transfectants stimulating expression of the TGFα gene, and the response is amplified further in transfectants with the cDNA from the amino acid 351 mutant ER (fig. 22) (Levenson *et al*., 1998). The 4-OHT-ER complex is clearly different than the raloxifene-ER complex. This confirms the suggestions by McDonnell and colleagues (1995) that the ligand-receptor complexes can display a range of conformations. We suggest that the reason for the promiscuity of the 4-OHT-ER complex in the transfectants is an increased level of coactivator in breast cancer cells that were originally ER negative. If the coactivators can provoke transcription with the wild-type 4-OHT-ER complex, then the orientation of the H12 helix must be different than that observed with the crystal structure of raloxifene. Indeed, it is possible that there are several conformations in equilibrium so that a single crystal shape alone will not describe the spectrum of tamoxifen's actions.

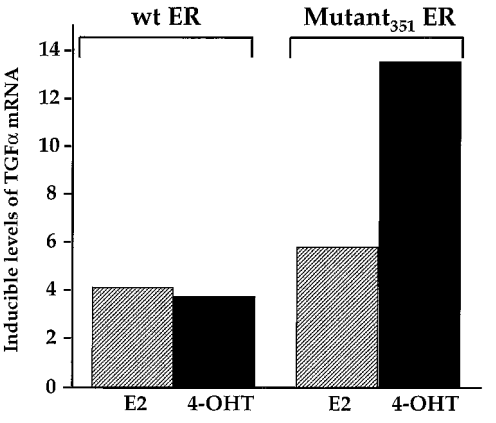


FIG. 22. Inducible levels of TGFα mRNA in wild-type (wt) and mutant (mutant$_{351}$) ER expressing from MDA-MB-231 breast cancer cells treated with estradiol (10^{-8}M) or 4-OHT (10^{-7}M). Adapted from Levenson *et al*. (1998).

This hypothesis could explain the development of tamoxifen-stimulated breast cancer. Receptor-positive cells that contain an excess of transcription factors and coactivators would be selected through a growth advantage during tamoxifen therapy. The laboratory models of tamoxifen-stimulated breast cancer are, therefore, a valuable reproducible resource to test the hypothesis. Techniques are available to identify the coactivators for the ER. However, we suggest that a solution of the molecular mechanism of antiestrogen-stimulated growth will not only solve a problem of drug resistance but also may provide an insight into the target site-specific actions of antiestrogens.

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