Cellular and Molecular Pharmacology of Antiestrogen Action and Resistance

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Abstract—Antiestrogen therapy remains one of the most widely used and effective treatments for the management of endocrine responsive breast cancers. This reflects the ability of antiestrogens to compete with estrogens for binding to estrogen receptors. Whereas response rates of up to 70% are reported in patients with tumors expressing estrogen and progesterone receptors, most responsive tumors will eventually acquire resistance. The most important factor in de novo resistance is lack of expression of these receptors. However, the mechanisms driving resistance in tumors that express estrogen and/or progesterone receptors are unclear. A tamoxifen-stimulated phenotype has been described, but seems to occur only in a minority of patients. Most tumors (>80%) may become resistant through other, less well defined, resistance mechanisms. These may be multifactorial, including changes in immunity, host endocrinology, and drug pharmacokinetics. Significant changes within the tumor cells may also occur, including alterations in the ratio of the estrogen receptor αβ forms and/or other changes in estrogen receptor-driven transcription complex function. These may lead to perturbations in the gene network signaling downstream of estrogen receptors. Cells may also alter paracrine and autocrine growth factor interactions, potentially producing a ligand-independent activation of estrogen receptors by mitogen-activated protein kinases. Antiestrogens can affect the function of intracellular proteins and signaling that may, or may not, involve estrogen receptor-mediated events. These include changes in oxidative stress responses, specific protein kinase C isoform activation, calmodulin function, and cell membrane structure/function.

I. Introduction

Endocrine manipulations are among the most effective, and least toxic, of the systemic therapies currently available for the management of hormone-responsive breast cancers. Ovariectomy in premenopausal women is the oldest of these therapies (Beatson, 1896) and has long been known to produce benefit in approximately one-third of all patients (Boyd, 1900). Although ovariectomy is still an effective therapy, currently the administration of antiestrogenic drugs is the most widely applied endocrine manipulation. Antiestrogenic drugs are effective in both premenopausal and postmenopausal patients and in the metastatic, adjuvant, and chemopreventive settings. The drugs are well tolerated, the incidence of dose-limiting toxicities is low, and responses are seen in approximately 70% of patients selected on the basis of the steroid hormone receptor expression profile of their tumors (Clark and McGuire, 1988). Additional benefits associated with some antiestrogens likely include reductions in the risk and/or severity of osteoporosis. Evidence also supports a possible reduction in the risk of cardiovascular disease (McDonald et al., 1995), but this is not consistent across all studies (EBCTCG, 1998; Fisher et al., 1998). Whether the estrogenic effects of Tamoxifen (TAM2) are responsible for any reduction

2 Abbreviations: TAM, Tamoxifen; AEBS, antiestrogen binding site; AP-1, activator protein-1; CMI, cell-mediated immunity; EGF, epidermal growth factor; EGF-R, epidermal growth factor-receptor; ER, estrogen receptor; ERE, estrogen-responsive element; FOF, fibroblast growth factor; GR, glucocorticoid receptor; HRT, hormone replacement therapy; 4-hydroxyTAM, 4-hydroxytamoxifen; IC_{50}, in-
in coronary heart disease has also become somewhat controversial, since the preventive effects of estrogenic hormone replacement therapy (HRT) on coronary heart disease have been questioned (Hulley et al., 1998).

Currently, the most widely used antiestrogen is the triphenylethylene TAM (ICI 46,477), which is administered orally as the citrate salt. Cole et al. (1971) described the first clinical study demonstrating TAM’s efficacy. TAM was approved for use in advanced disease several years later. Clinical experience with this drug likely now exceeds 10 million patient years. Unfortunately, in most patients, cancers that initially respond to TAM will recur and require alternative systemic therapies. Despite extensive experience with this drug, the precise mechanisms that confer resistance remain unknown. This review will discuss evidence from recent clinical trials and experimental models that identify several possible mechanisms of resistance. Because the activity of antiestrogens is intimately involved with the role of estrogens and their receptors, a brief discussion of the role of estrogens and estrogen receptors (ERs) is included. Additional ER-independent events, which also may be important, are discussed.

A. Role of Estrogens in Affecting Breast Cancer Risk and Progression

The utility of antiestrogens as treatments and/or chemopreventives for breast cancer is closely associated with antagonizing the activity of estrogens. Estrogens have been widely implicated in affecting breast cancer risk in the postmenopause. Evidence includes the association of increased serum estrogens, or estrogen excretion, with postmenopausal breast cancer (Table 1) (see Thomas et al., 1997 for review). Prolonged HRT, which also elevates serum estrogen levels, can significantly increase breast cancer risk (CGHFBC, 1997), and the tumors arising tend to be primarily ER-positive (Lower et al., 1999). HRT is often prescribed to naturally perimenopausal or postmenopausal women, but may also be given to younger women with primary ovarian failure, or who have had their ovaries removed/irradiated.

The estrogenicity of HRTs can vary significantly, and dose is important, at least in some studies. For example, low potency oral and transdermal estrogens may not increase risk, whereas more potent estrogens significantly increase breast cancer risk (Magnusson et al., 1999). Serum estradiol concentrations can exceed 0.77 nM with some HRT regimens (Garnett et al., 1990). This concentration is almost 10-fold higher than that seen in untreated postmenopausal women and is comparable with that seen in the luteal phase of the menstrual cycle (Table 1). Recent evidence suggests that the greatest increase in breast cancer risk is associated with replacement therapies that combine estrogens and progestins (Schairer et al., 2000). Most studies observe the greatest risk in current/recent users, perhaps reflecting a promotional rather than initiating action of the estrogens.

Whereas HRT increases the risk of developing breast cancer, the resulting biology of the tumors may be different from those arising in the absence of HRT. Patients using HRT at the time of diagnosis have a reduced mortality from breast cancer (Schairer et al., 1999), perhaps reflecting a less aggressive biology (CGHFBC, 1997; Holli et al., 1997). Thus, the estrogenicity of HRT may have allowed the survival of less aggressive tumors. This is consistent with the observation that estrogen-dependent breast cancer cells selected in vivo for growth in a low estrogen environment, rather than in the presence of an adequate estrogenic stimulus, can acquire a more aggressive phenotype (Thompson et al., 1993).

Indirect evidence for a role for estrogens in affecting lifetime breast cancer risk is provided by several known risk factors. For example, breast cancer risk is increased in women who either began menstruating at a young age (<12 years) and/or ceased menstruating (menopause) at a late age (≥55 years) (Hulka and Stark, 1995). This would tend to increase the number of cycles and total lifetime exposure to ovarian estrogens. Postmenopausal obesity is also associated with increased breast cancer risk (Hulka and Stark, 1995). Peripheral adipose tissue is the primary source for the production of circulating estrogens in postmenopausal women, and serum estrogen concentrations are generally higher in obese postmenopausal women (Ingram et al., 1990; Madigan et al., 1998). There are also data implicating estrogenic exposure and risk of premenopausal breast cancer. Perhaps the most compelling evidence is the efficacy of ovariec- tomy and luteinizing hormone releasing hormone anal-ogs in inducing responses in premenopausal patients (Crump et al., 1997).

Estrogens may affect carcinogenesis by acting either as initiators (i.e., directly damage DNA) or as promoters (i.e., promoting the growth and/or survival of initiated cells). For example, administration of estrogens alone can produce tumors in some rodents (Lacassagne, 1932). This may reflect an effect mediated through mouse mammary tumor virus, and/or activities of the more chemically reactive metabolites of 17β-estradiol. Reactive estrogen semiquinone/quinone intermediates are produced by the redox cycling of the hydroxylated estrogen metabolites. These can produce DNA adducts (initiation). This has been most closely associated with the 4-hydroxy (Liehr and Ricci, 1996) and 3,4-hydroxy metabolites, with a recent study strongly implicating the
catechol estrogen-3,4-quinones as initiators (Cavalieri et al., 1997). The production of these metabolites is a function of several cytochrome P-450 isoforms that are expressed in the breast, liver, and other tissues (Zhu and Conney, 1998).

The potential role of estrogens as promoters of carcinogenesis is more firmly established. Ovariectomy—whether chemical, surgical, or radiation-induced—remains a highly effective treatment (Crump et al., 1997). Indeed, surgical ovariectomy and the suppression of gonadotropin secretion by luteinizing hormone releasing hormone analogs are as effective as TAM in managing premenopausal breast cancer (Jonat, 1998). Chemically induced mammary adenocarcinomas in rats also require functional ovaries (Russo et al., 1990), probably reflecting induction of several cytochrome P-450 isoforms that are expressed in the breast, liver, and other tissues (Zhu and Conney, 1998).

B. Antiestrogens: Partial Agonists and Antagonists

Antiestrogens primarily function through their ability to compete with available estrogens for binding to ER. However, the consequences of occupying ER with an antiestrogen appear dependent upon the cellular context, which ER is occupied (ERα and/or ERβ), and the structure of the ligand. The most important biological consequence is whether the activated receptor complex induces an estrogenic or antiestrogenic response. This has significant implications. Producing an estrogenic response in bone and an antiestrogenic response in the breast would be highly beneficial. In contrast, the reverse pattern of response could stimulate the growth of an existing breast tumor and concurrently increase the risk of debilitating fractures.

TAM provides a good illustration of several of these points. TAM is a classical partial agonist and exhibits both species and tissues specificity for inducing either an agonist or antagonist response. In the mouse, TAM is an agonist. In rats and humans, it exhibits partial agonism (Jordan and Robinson, 1987) [e.g., producing antagonist effects in the breast, but agonist effects in the vagina and endometrium (Harper and Walpole, 1967;
Ferrazzi et al., 1977). Long-term TAM use is generally associated with a reduced incidence of contralateral breast cancer (antagonist), a reduced incidence of primary breast cancer in high-risk women (antagonist), maintenance of bone density (agonist), and increased risk of endometrial carcinomas (agonist) (Fisher et al., 1998).

The ability to generate these tissue-specific effects has lead to the search for other selective ER modulators, which will have the beneficial effects seen with TAM but without the increased risk of endometrial carcinoma. Several triphenylethelene variations on TAM are already available, including Toremifene (chloro-TAM) and Droloxifene (3-OH-TAM). Both drugs seem to be approximately equivalent to TAM in terms of their antitumor activities and toxicities; both drugs are partial agonists (Roos et al., 1983; Pyrhonen et al., 1999).

The clinical utility of several of these newer antiestrogens has recently been reviewed by others (Lien and Lonning, 2000), and an exhaustive review is beyond the scope of this article. Nonetheless, several of the newer compounds are notable. Many are not triphenylethlenes [e.g., Raloxifene is a benzothiophene (previously called keoxifene; LY 156,758)]. It is now available in the U.S. as a treatment for the prevention of osteoporosis in postmenopausal women. Evidence suggests that Raloxifene may not have the same uterotropic effects as TAM (Delmas et al., 1997) and that it may regulate gene expression through novel pathways (Yang et al., 1996). In the multiple outcomes of Raloxifene randomized trial, Raloxifene significantly reduced the number of breast cancer cases, from 27/2576 to 13/5129 (Cummings et al., 1999), but did not increase the incidence of endometrial cancers (Delmas et al., 1997; Cummings et al., 1999). It also produces beneficial effects comparable with TAM on other endpoints, including lowering levels of both total and low-density lipoprotein cholesterol (Delmas et al., 1997; Walsh et al., 1998) and increasing bone mineral density (Delmas et al., 1997). However, Raloxifene increases the incidence of hot flashes (Davies et al., 1999).

Other antiestrogens that have received attention are the steroidal compounds ICI 164,384 and ICI 182,780. Both ICI 164,384 and ICI 182,780 have high affinities for ER (Wakeling and Bowler, 1988). There may also be some preference for ERβ, since ICI 164,780’s relative binding affinity for ERβ = 166%, but for ERα = 85% (Kuiper et al., 1997). Both ICI 164,384 and ICI 182,780 seem to be antagonists, being devoid of agonist activity in most experimental models. For example, ICI 164,384 does not exhibit agonist activity either in MCF-7 cells growing in the absence of estrogens (Clarke et al., 1989; Thompson et al., 1989), or in the uterus or vagina of rats and mice (Wakeling and Bowler, 1988). ICI 164,384 can inhibit the agonist effects of both estrogen and TAM (Wakeling and Bowler, 1988). The estrogenic activities of TAM induce expression of a series of estrogen-regulated genes, including the progesterone receptor (PgR) and pS2. ICI 164,384 has no notable estrogenic effects on the regulation of these genes (Wiseman et al., 1989), other than a modest induction of PgR in endometrial cells (Jamil et al., 1991). However, there is evidence that ICI 182,780 can produce an estrogen-like effect in KPL-1 breast cancer cells (Kurebayashi et al., 1998). When ICI 182,780 is administered to pregnant rats, their female offspring exhibit changes in their mammary glands similar to those seen in offspring exposed to exogenous estradiol in utero (Hilakivi-Clarke et al., 1997). This could reflect primarily ERβ-mediated events, since ERβ is the predominant form at least in some normal human and rodent mammary tissues (Speirs et al., 1999b; Saji et al., 2000). Furthermore, ICI 182,7870 is an activator of transcription at AP-1 sites (Paech et al., 1997).

The steroidal antiestrogen ICI 182,780 retains its potency in vivo as determined by its ability to inhibit MCF-7 and Br10 tumors. This compound also exhibits substantial antiuterotrophic activity in the immature rat (de Launoit et al., 1991). ICI 182,780 (trade name: Faslodex) has already completed initial phase I clinical evaluation. The first study was performed on patients who had previously demonstrated a response to TAM, but recurred. The overall reported response rate of 69% (Howell et al., 1995) is substantially higher than the 5% objective response rate reported for crossover to another triphenylethylene (Toremiphene) following TAM failure (Vogel et al., 1993) and is more in line with responses to alternative second line endocrine therapies [e.g., aromatase inhibitors (Dowsett et al., 1995)]. This observation suggests that the steroidal antiestrogens affect breast cancer cells differently than the triphenylethylenes.

The partial agonist activities of TAM and Raloxifene are thought to be responsible for their beneficial effects on bone resorption. Pure antagonists like ICI 182,780 may further exacerbate bone loss, a concern that also applies to aromatase inhibitors (Dowsett, 1997). However, when combined with alternative therapies for osteoporosis, such as bisphosphonates, these drugs may have considerable potential as first-line endocrine therapies.

C. Response Rates to Tamoxifen and Expression of Steroid Hormone Receptors

Patients with ER-positive tumors have a significantly higher response rate to antiestrogens than patients with ER-poor/ER-negative tumors. This relationship holds whether ER is measured by ligand binding or immunohistochemistry, reflecting the high concordance seen with these different techniques (Molino et al., 1997). It also holds despite the range of cut-off values used for assessing ER positivity versus ER-poor/ER negativity. TAM also seems most effective in the suppression of ER-positive tumors in the chemopreventive setting (Fisher et al., 1998).
Expression of PgR also has been implicated as a predictor of response to TAM. Several studies have reported responses in patients with ER-negative but PgR-positive tumors. However, the number of tumors is small and could reflect false negative estimations of ER expression. Concurrent expression of both ER and PgR is often associated with a higher response rate than in ER-positive, but PgR-negative, tumors. In general, approximately 70% of patients with ER-positive/PgR-positive tumors will respond to TAM, whereas response rates of 45% are seen in patients with ER-negative, but PgR-positive tumors. A 34% response rate is seen in ER-positive, but PgR-negative, tumors (Honig, 1996). The predictive power of PgR expression is likely related to the ability of estrogens to induce its expression. Thus, the presence of both ER and PgR may reflect the existence of an at least partially functional ER signaling pathway (Horwitz et al., 1975).

The Early Breast Cancer Trialists Group’s initial meta-analysis in 1992 reported both a significant reduction in recurrence or death, and a reduction in death from any cause, in patients with ER-poor tumors (Table 2). Their more recent meta-analysis found no significant reduction in recurrence rates in patients with ER-poor tumors. Indeed, a 3% (nonsignificant) increase in the risk of death from any cause was reported in women, receiving TAM, with ER-poor tumors (Table 2). These latter data do not strongly implicate ER-independent events in beneficial responses to TAM and possibly indicate an adverse effect in some women. What those adverse effects may be, whether they are real, and the extent to which they may be restricted to an undefined subset of patients, remain to be determined. It also may reflect the more aggressive biology of ER-negative tumors (Aamdal et al., 1984; Clark and McGuire, 1988). Whereas longer term TAM use (e.g., 10 yr) is less beneficial than 5 yr, it still produces an overall benefit (EBCTCG, 1992, 1998). Why the benefit should be lower with longer use is not known, but may also reflect an adverse effect in some women.

D. Overview of Antiestrogen Resistance Mechanisms

Antiestrogens clearly produce several beneficial effects in some patients, including improved disease-free survival and overall survival from breast cancer. However, most patients with initially responsive tumors will experience a recurrence, indicating acquired antiestrogen-resistant disease. There are several possible mechanisms that could influence response to antiestrogens and, when altered, contribute to resistance. These include changes in host immunity, host endocrinology, or antiestrogen pharmacokinetics. Competition with en-

<table>
<thead>
<tr>
<th>Table 2</th>
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<tbody>
<tr>
<td><strong>Treatment with TAM, its potential as a chemopreventive agent, and the potentially confounding effects of HRT on response to TAM</strong></td>
</tr>
<tr>
<td>Early Breast Cancer Trialists Collaborative Group (1992)</td>
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<tr>
<td>Endpoint</td>
</tr>
<tr>
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</tr>
<tr>
<td>Recurrence</td>
</tr>
<tr>
<td>Contralateral breast cancers</td>
</tr>
<tr>
<td>Mortality</td>
</tr>
<tr>
<td>Recurrence or Prior Death</td>
</tr>
<tr>
<td>ER+ (n = 14,972)</td>
</tr>
<tr>
<td>ER poor ((n = 5,366)</td>
</tr>
<tr>
<td>Early Breast Cancer Trialists Collaborative Group (EBCTCG, 1998)</td>
</tr>
<tr>
<td>Endpoint</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Recurrence (ER +)</td>
</tr>
<tr>
<td>Recurrence (ER poor)</td>
</tr>
<tr>
<td>Death: any cause (ER +)</td>
</tr>
<tr>
<td>Death: any cause (ER poor)</td>
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**Chemoprevention**

<table>
<thead>
<tr>
<th>Study</th>
<th>Placebo</th>
<th>TAM</th>
<th>Reduction in Risk</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>U.K. (Powles et al., 1998)</td>
<td>36</td>
<td>34</td>
<td>6%; 1.06</td>
<td><em>p = 0.8</em></td>
</tr>
<tr>
<td>(n = 2,471)*</td>
<td>(0.7, 1.7)</td>
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<tr>
<td>Italian (Veronesi et al., 1998)</td>
<td>22</td>
<td>19</td>
<td>14%</td>
<td><em>p = 0.6</em></td>
</tr>
<tr>
<td>(n = 5,408)*</td>
<td></td>
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</tr>
<tr>
<td>NSABP P-1 (Fisher et al., 1998)</td>
<td>175</td>
<td>89</td>
<td>49%; 0.51</td>
<td><em>p &lt; 0.00001</em></td>
</tr>
<tr>
<td>(n = 13,388)*</td>
<td>(0.39; 0.66)</td>
<td></td>
<td></td>
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<tr>
<td>Invasive cancers</td>
<td>69</td>
<td>35</td>
<td>50%; 0.50</td>
<td><em>p &lt; 0.002</em></td>
</tr>
<tr>
<td>(Noninvasive cancers</td>
<td></td>
<td></td>
<td>(0.35; 0.77)</td>
<td></td>
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</table>

* Data are adapted from each study. Significance estimates are from the appropriate study. In some cases, the precise *p*-values are not available. N.S. = not significant.
* ER poor is generally taken as <10 fmol/mg protein.
* 42% used HRT.
* TAM appears effective in 14% of women taking HRT (hazard ratio = 0.13; confidence interval = 0.02, 1.02).
* Subjects did not receive HRT.
Endogenous ligands for binding to an antiestrogen’s primary intracellular target(s), or altered function of its target(s), could also contribute to resistance (Fig. 1). The low rate of responses in ER-negative tumors is most consistent with antiestrogen action being primarily mediated through interactions with ER. However, antiestrogens, and TAM in particular, have been shown to bind intracellular proteins in addition to ER. It might be expected that, if these targets were critical for generating a response, many ER-negative tumors also would be responsive. Although such responses are not common, the ability of antiestrogens to influence the function of targets other than ER may still be important.

It is apparent that the cellular context (i.e., the gene/protein expression pattern in a cell) can affect how a cell responds to a specific stimulus (Clarke and Brünner, 1996). For example, ER’s transcriptional activities can be influenced by phosphorylation events regulated by signaling, which activates mitogen-activated kinase (MAPK) (Kato et al., 1995). Downstream signaling from the ER also is likely to be complex and may interact with other (ER-independent) signaling pathways. Antiestrogens could influence the activities of these other pathways (e.g., through binding to non-ER proteins) and alter cellular context (Clarke and Brünner, 1996). Whereas such events are probably not sufficient to induce an antiestrogenic effect in most ER-negative cells, they may be necessary permissive for signaling to a fully antiestrogenic effect in responsive cells. Thus, perturbations in the activity of some ER-independent effects could contribute to an acquired antiestrogen resistance. Both ER-mediated and ER-independent targets for antiestrogens are considered in this review.

II. Endogenous and Exogenous Estrogens in Antiestrogen Resistance
A. Origins of Intratumor Estrogens

In women, the biosynthesis of estrogens may arise from several sources. Ovarian production is the main source of circulating estrogens in premenopausal women, the primary estrogen being 17β-estradiol. The efficacy of ovariectomy and luteinizing hormone releasing hormone analogs in premenopausal women (Crump et al., 1997) strongly support a role for ovarian estrogen production in the breast cancers that arise in these women. Conversion of adrenal androgens in peripheral tissues is the predominant source of circulating estrogens in postmenopausal women. The primary estrogen produced in the postmenopause by the action of aromatase is the relatively weak estrone, which is generally present in serum as the inactive estrone sulfate. Breast cancer cells can release the biologically active estrone through the action of the steroid sulfatase enzyme (Pasqualini et al., 1988) and can further convert estrone to 17β-estradiol through the action of 17β-hydroxysteroid dehydrogenase type 1 (Brodie et al., 1997).

![Fig. 1. Overview of the likely targets of antiestrogen action and resistance. E2, estradiol.](image-url)
Mammary tissues accumulate serum estrogens to concentrations significantly higher than those present in serum (Masamura et al., 1997; Miller, 1997). However, breast tissues also synthesize estrogens through a pathway similar to that in peripheral adipose tissues. This biosynthesis can occur within the epithelial cells (Brodie et al., 1997), the associated breast adipose tissue (Bulun and Simpson, 1994), and in some infiltrating lymphoid cells (Mor et al., 1998).

The importance of the aromatase enzyme in generating biologically active estrogens is evidenced by the efficacy of aromatase inhibitors in inducing clinical responses in postmenopausal breast cancer patients. These drugs already are established as second-line endocrine therapies (Dowsett, 1997). Because they inhibit both peripheral and breast aromatase activities, it is often difficult to assess which site of synthesis predominates. Both peripheral and intratumor/stromal aromatase activities are likely to be important, with the relative contribution varying among tumors. Studies in experimental models suggest that local production may be more important (Santen et al., 1999). Although peripheral aromatization is reduced to comparable levels by both aminoglutethimide and testolactone in women, testolactone produces a much lower clinical response rate (Lonnning et al., 1989a). However, aminoglutethimide significantly increases estrone sulfate clearance in addition to its inhibition of aromatase activity (Lonnning et al., 1989b; Lonnning et al., 1990). These data suggest that both serum estrogens and intratumor/stromal biosynthesis may contribute to intratumor estrogen concentrations.

**B. Intratumor Estrogen Concentrations**

High intratumor estrogen concentrations could prevent antiestrogens from blocking ER action and produce a resistant phenotype. Data in Table 3 show that normal, benign, and malignant breast tissues in postmenopausal women contain concentrations of 17β-estradiol up to 10-fold higher than those seen in serum. The range among tumors is considerable, from undetectable to over 5 μM 17β-estradiol, with these levels being essentially equivalent regardless of patients’ menopausal status. The mean concentration estimated from these studies is 1.28 nM (Table 3). If this reflects the concentration in epithelial cells, and it is fully available for ER binding, there would be sufficient intratumor estradiol to produce a maximal stimulation of ER signaling. In serum, <5% of estrogens are “free” [i.e., not bound to serum proteins]. Using this as an estimate of intracellular availability within tumors, and with a $K_d$ of approximately 0.1 nM in breast cancer and other cells (Beil et al., 1996), only 25% of ER would be occupied.

Generally, biological response is proportional to receptor occupancy. However, some cells up-regulate receptor expression, these “spare” receptors producing a left shift in the dose-response relationship (Ross, 1996). If this occurred in some breast tumors, they might exhibit a greater biological response than would be predicted by the proportion of occupied receptors. Consistent with the concept of spare receptors, MCF-7 cells respond to 17β-estradiol at concentrations well below its $K_d$ for ER. Some MCF-7 cells selected in vitro for growth in the absence of estradiol further up-regulate ER expression

### TABLE 3

<table>
<thead>
<tr>
<th>Intratumor concentrations of 17β-estradiol</th>
<th>Study</th>
<th>Mean ± S.D./S.E. (nM)</th>
<th>Range (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. Intratumor Estrogen Concentrations</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bonney et al., 1983 (n = 13)</td>
<td>1.76 ± 0.3</td>
<td>0.148–1.77</td>
<td></td>
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<tr>
<td>de Jong et al., 1997 (n = 9)</td>
<td>0.84 ± 0.58</td>
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<tr>
<td>Drafa et al., 1983 (n = 41)</td>
<td>1.58 ± 0.10</td>
<td></td>
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<tr>
<td>Edery et al., 1981 (n = 78)</td>
<td>2.92 ± 0.12</td>
<td></td>
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<tr>
<td>Fishman et al., 1977 (n = 129)</td>
<td>0.33 ± 0.21</td>
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<tr>
<td>Maynard et al., 1978</td>
<td>N.D.</td>
<td></td>
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<tr>
<td>Mehta et al., 1987 (n = 65)</td>
<td>1.34 ± 0.13</td>
<td>0.7 nM–5.5 μM</td>
<td></td>
</tr>
<tr>
<td>Millington, 1975 (n = 18)</td>
<td>3.1 ± 1.19</td>
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<td>Mistry et al., 1986 (n = 16)</td>
<td>0.756 ± 0.49</td>
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<td>Pasqualini et al., 1996 (n = 34)</td>
<td>1.4 ± 0.7 (postmenopausal)</td>
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<tr>
<td>Recchione et al., 1995 (n = 34)</td>
<td>0.169 (median value)</td>
<td>0.033–0.775</td>
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<tr>
<td>van Landeghem et al., 1985 (n = 105)</td>
<td>0.62 ± 0.39</td>
<td>0.02–1.52</td>
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<td>Vermeulen et al., 1986 (n = 50)</td>
<td>1.64 ± 1.89</td>
<td>0.07–9.02</td>
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<td>Normal and benign breast tissues</td>
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<tr>
<td>Kyo et al., 1999</td>
<td>0.625 ± 0.018 (adjacent normal tissue)</td>
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<td>Szynyczak et al., 1988 (n = 30)</td>
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<td>Vermeulen et al., 1986 (n = 14)</td>
<td>1.05 ± 0.9 (glandular tissue)</td>
<td>0.15–2.76</td>
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Where values are missing, they cannot be readily identified from the publication(s). N.D. = not detected.

a All values are nM unless otherwise indicated. Numbers in parentheses are the number of subjects in the study.

b Mean estimates are provided with either the standard deviation or standard error and are based on the data presented in the studies using the following conversions:

(a) 1 g tissue weight = 1 ml; and (b) 50 mg protein = 1 g tissue weight.

c $p < 0.01$ for ER+ vs. ER– (Drafa et al., 1983).

d $p < 0.001$ for ER+ vs. ER– (Edery et al., 1981).

*e $p < 0.02$ for ER+ vs. ER– (Fishman et al., 1977).
(Jeng et al., 1998). However, MCF-7 cells, which represent the most widely used endocrine responsive experimental model (Levinson and Jordan, 1997), have ER levels of ~400 fmol/mg protein (Martin et al., 1991). This is 40 times greater than the lower limit used to determine ER positivity in tumors. Relatively few breast tumors express these very high levels of ER, nor the levels seen in an estrogen supersensitive MCF-7 variant (Masamura et al., 1995).

In the absence of spare receptors, our estimate of 25% receptor occupancy would predict that many breast tumors exist in a weak estrogenic environment. Evidence of a suboptimal estrogenic environment being present in tumors is apparent from the associations of increased serum estrogens, HRT (Table 1), and oral contraceptive use (Hulka and Stark, 1995; CGHFBC, 1997) with increased breast cancer risk in some populations. Similarly, some metastatic tumors, which develop while a patient is taking HRT, regress upon withdrawal of HRT (Dhodapkar et al., 1995). Generally, the effects of HRT are not seen in heavier women (Magnusson et al., 1999; Schairer et al., 2000), probably reflecting the ability of higher serum estrogen levels, derived from peripheral adipose tissues, to compensate for a low intratumor estrogenic environment. In lean postmenopausal women, HRT could stimulate tumors with otherwise suboptimal intratumor estrogen concentrations.

Tumors arising in women exposed to HRT tend to be ER-positive (Lower et al., 1999). In one recent study, the mitogenic effects of HRT (high S-phase fraction) were seen only in ER-positive tumors (Cobleigh et al., 1999). ER-positive tumors often proliferate more slowly than ER-negative tumors (Wenger et al., 1993), which have no obvious need of estrogens for proliferation. This may reflect a suboptimal estrogenic/mitogenic environment, and could contribute to the different biologies apparent between ER-positive and ER-negative tumors.

Some tumors with no effective estrogenic stimulation could be driven by a ligand-independent activation of the ER signaling network (Tzukerman et al., 1990; Clarke and Brünnér, 1996). Others with insufficient ligand may benefit from a concurrent ligand-independent activation of the remaining unoccupied ER. Generally, ligand independent activation is weaker than ligand activation. Both forms of activation can be blocked by antiestrogens (Clarke and Brünnér, 1996; Tzukerman et al., 1990). Thus, tumors driven exclusively or partly by ligand-independent activation of ER should still exhibit responses to several endocrine therapies.

C. Does the Pituitary-Ovarian Axis Affect Response to Tamoxifen in Premenopausal Women?

The potential contribution of serum estrogens to intratumor estrogen concentrations implies that factors influencing serum estrogen concentrations might affect response to antiestrogens. Some early studies suggested that TAM is of greater benefit when administered to postmenopausal rather than premenopausal women. However, these data are not supported in the recent Breast Cancer Trialists Cooperative Group meta-analysis, where it is clear that TAM is equally effective in both postmenopausal and premenopausal patients (EBCTCG, 1998). This does not exclude possible important mechanistic differences concerning how tumors respond in premenopausal versus postmenopausal women. For example, the presence of functional ovaries, particularly if these provide a major component of intratumor estrogenicity, could affect responsiveness.

The release of estrogens from the ovaries is regulated by the pituitary-ovarian axis. Estrogens can regulate the release of gonadotropins at two levels: the release of gonadotropin releasing hormone from the hypothalamus and the release of gonadotropins from the anterior pituitary. If TAM effectively blocks the ER in both the hypothalamus and anterior pituitary, this would disrupt the negative feedback on gonadotropin releasing hormone, ultimately producing a “hyperstimulation” of the ovaries. This might partly explain how TAM increases the circulating levels of estrogens in some premenopausal women (Ravdin et al., 1988; Szamel et al., 1994). Other studies have not reported an ability of TAM to affect circulating estrogen levels. However, ovariectomy and aromatase inhibitors can induce remissions in premenopausal women who initially responded to TAM but eventually recurred. This suggests that TAM produced an incomplete antiestrogen action, possibly as a result of increased circulating estrogens.

TAM can affect gonadotropin levels in premenopausal women, but its ability to do so in postmenopausal women is not so clear (Lien and Lonning, 2000). Small increases in serum dehydroepiandrosterone, estrone, and estradiol levels are also produced by antiestrogens in postmenopausal women (Szamel et al., 1994; Pommier et al., 1999). This probably reflects an effect mediated either through the release of adrenal androgens and/or increases in adrenal estrogen production in postmenopausal women (Pommier et al., 1999).

Where serum estrogens are increased, a consequent elevation in intratumor 17β-estradiol concentrations would be predicted, reflecting the ability of tumors to accumulate serum estrogens. Such an effect might compromise response to TAM by increasing intratumor estrogen competition for binding to ER. Whether this occurs to an extent sufficient to affect the response to TAM is unclear. Response rates to TAM are comparable in premenopausal and postmenopausal women, but serum estrogen levels are higher in premenopausal women. A clearer understanding of the role of serum estrogens in influencing TAM response will probably await data from appropriately designed clinical trials. Nonetheless, it is evident that estrogens can readily reverse the inhibitory effects of antiestrogens in experimental models in vitro and in vivo. Since the primary estrogen produced in premenopausal women in response to TAM is also the
most potent (17β-estradiol), and tumors can significantly accumulate estrogens to levels in excess of that seen in serum (Masamura et al., 1997; Miller, 1997), changes in serum estrogens could affect TAM responsiveness in some individual tumors.

D. Can Endogenous Estrogens or Hormone Replacement Therapies Produce Antiestrogen Resistance?

Antiestrogens can block both ligand-dependent and ligand-independent ER activation (Tzukerman et al., 1990; Clarke and Brüner, 1996). Thus, the precise origin of the ligand, and whether or not it is required for receptor activation, is less important than the potential of available intratumor estrogens to prevent antiestrogen action. Free intracellular estrogens could compete with antiestrogens for binding to ER, reducing their ability to block ligand dependent receptor activations.

The mean intratumor concentration (1.28 nM from Table 3) would probably not be sufficient to fully compete with TAM and its metabolites. This is consistent with evidence from experimental models suggesting that combinations of an antiestrogen and an aromatase inhibitor is no better than either drug alone (Lu et al., 1999). However, where reduced intratumor TAM accumulation also occurs (Johnston et al., 1993), the higher intratumor estradiol concentrations in some tumors might overcome TAM’s antiestrogenic activities. Very high intratumor estrogen levels (up to 5 μM) are only occasionally observed, but would provide sufficient estrogenicity to compete with the mean intratumor concentrations of triphenylethylene antiestrogens (3.4 μM; see Section III.A.). Assuming that both estrogens and antiestrogens have equivalent intracellular availability for binding ER, it is theoretically possible for some tumors to acquire sufficient intratumor estrogen concentrations to either eliminate or reduce the inhibitory effects of TAM and its major metabolites.

Although this is a reasonable hypothesis, it has been inadequately addressed in clinical trials. It is evident that approximately 30% of tumors that acquire TAM resistance will respond to a second-line aromatase inhibitor. The proportion may be higher in selected populations (Dowsett et al., 1995). This response pattern is consistent with an important role for estrogen biosynthesis in acquired TAM resistance. It implies that the responding tumors have retained both a functional ER signaling network and a dependence upon that network’s estrogenic activation/regulation for continued survival/proliferation. In some of these tumors, the levels of intratumor estrogens may reach sufficient levels to overcome any antiestrogenic activities of TAM and support an estrogen-dependent proliferation.

Currently, determining the possible contribution of HRT to antiestrogen resistance can also be done only indirectly. The National Surgical Adjuvant Breast and Bowel Project (NSABP)-P1 TAM chemoprevention trial precluded women who were receiving HRT, but found a significant reduction in the incidence of invasive breast cancers (Fisher et al., 1998). The apparent lack of a chemopreventive effect of TAM in the Italian (Veronesi et al., 1998) and United Kingdom studies (Powles et al., 1998) has been partly attributed to their inclusion of women receiving HRT. This explanation for the failure of these studies remains somewhat controversial. For example, it is not clear that many HRT’s, particularly those using low-dose/potency estrogens, would produce an environment any more estrogenic than that occurring naturally in TAM-responsive premenopausal women. Tumors in premenopausal patients have a response rate comparable with those arising in postmenopausal women (EBCTCG, 1998). Other differences in the chemoprevention trials probably account for the lack of activity in the European studies. These may include differences in the patient populations and the greater statistical power of the NSABP study (Pritchard, 2000).

The timing of TAM treatment relative to any HRT may affect clinical outcome. Initiation of HRT during TAM may have a greater inhibitory effect on TAM’s ability to affect serum lipid profiles than initiation of TAM in current HRT users (Decensi et al., 1998). Since these are agonist cardiovascular endpoints rather than antagonist cancer endpoints, extrapolation to the antiestrogenic effects of TAM in breast cancer is difficult. Nonetheless, data raise the possibility that the timing of HRT may affect TAM’s antineoplastic activity in these patients. Additional studies are required to definitively answer the possible contribution of HRT to TAM resistance. The limited information available does not provide strong evidence for an effect of HRT on TAM responsiveness, which, if it occurs, may be restricted to specific HRT formulations and/or specific populations.

III. Pharmacokinetics in Resistance to Tamoxifen

There are several pharmacologic properties of TAM that directly influence its biological activity and that, when significantly altered, could contribute to the emergence of an antiestrogen resistant phenotype. These include the classical pharmacokinetic parameters of absorption, distribution, biotransformation, and elimination. The intracellular availability of TAM will determine the concentration free to interact with ER. This could be affected by changes in TAM accumulation in tumors. There are several likely major intracellular binding compartments for TAM that could limit intracellular availability. These include binding to antiestrogen binding sites (AEBSs) and other intracellular proteins, and partition into the lipophilic domains of cellular membranes. Such interactions could effectively sequester active TAM and its metabolites to produce the resistance phenotype. Since TAM is extensively metabolized in humans, and several metabolites are agonists,
a resistance phenotype could also be conferred by a switch to the generation of predominantly estrogenic metabolites.

A. Basic Pharmacology of Tamoxifen

Steady-state serum concentrations of TAM are generally achieved after approximately 4 weeks with the conventional dosing regimen of 20 mg TAM daily (Buckley and Goa, 1989; Etienne et al., 1989). Following administration of 30 mg/day, the mean steady-state plasma concentrations of parent drug and major metabolites can be up to 1.1 μM (Etienne et al., 1989). High-dose TAM, 150 mg/m² twice daily following a loading dose of 400 mg/m², produces plasma concentrations of 4 μM TAM and 6 μM N-desmethyl TAM (Trump et al., 1992). In most studies, clinical response does not seem to correlate with TAM plasma levels (Bratherton et al., 1984; Clarke and Lippman, 1992).

Greater than 98% of TAM and its major metabolites are bound to serum proteins. Most of this appears to reflect binding to serum albumin, which can bind drugs in a ratio of 1:1 (Lien et al., 1989). The extensive degree of association with albumin (Lien et al., 1989), peripheral tissues (Daniel et al., 1981; Lien et al., 1989) and cellular membranes (Clarke et al., 1990), and its large volume of distribution (Herrlinger et al., 1992) may contribute to TAM’s long terminal elimination phase. The relatively low affinity binding to serum albumin might facilitate transport to tissues, where dissociation may occur to allow for tissue accumulation. This role for albumin as a transporter has been described for estrogens, with albumin-bound estrogens often being considered within the available component (Moore et al., 1986; Jones et al., 1987).

Despite the low free concentrations in serum, TAM concentrations of 5 to 110 ng/mg protein (25 ± 27 ng/mg protein; mean ± S.D.) have been reported in the breast tumors of women receiving 40 mg TAM/day (Daniel et al., 1981). This would approximate 0.67 to 14 μM (3.36 ± 3.63 μM; mean ± S.D.) using the conversions in the legend to Table 3. Similar intratumor concentrations have been described for brain metastases, with mean concentrations of TAM ≈ 4 μM, 4-hydroxytamoxifen (4-hydroxyTAM) = 0.13 μM, and N-desmethyl TAM = 8 μM (approximate values derived from the published data) detected in a small study of patients receiving 30 to 50 mg TAM/day (Lien et al., 1991). Thus, as with estrogens, there is clear evidence of intratumor accumulation of TAM and its major metabolites to concentrations significantly in excess of that seen in serum (MacCallum et al., 2000).

When compared with the mean intratumor 17β-estradiol concentration (=1.28 nM; Table 3), and assuming approximately equivalent intratissue availability, it is apparent that there should be sufficient TAM present to effectively compete with most concentrations of intratumor estrogens. This would be the case even if all the drug was present as either the relatively weak parent or the N-desmethyl TAM metabolite. The latter is present at concentrations of approximately 7 ± 8 μM (estimated from the values of Daniel et al., 1981). However, a significant proportion of the antiestrogenic activity will be provided by the 4-hydroxyTAM metabolite (77 ± 64 nM estimated from the values of Daniel et al., 1981), which has an affinity for ER ≈ 17β-estradiol (Kuiper et al., 1997). Although these estimates were obtained several years ago, a more recent study by MacCallum et al. (2000) obtained mean intratumor concentrations of TAM and its major metabolites (4-hydroxyTAM = 0.18 μM; N-desmethyl TAM = 0.61 μM; TAM = 0.32 μM) within the range of these prior studies.

The potentially significant intratumor excess of antiestrogenicity over estrogenicity (>10-fold for 4-hydroxyTAM) explains, in part, why TAM is an effective therapy in many patients with ER-positive tumors. This likely also contributes significantly to the apparent lack of a strong dose-related response rate in clinical trials. Many of the lower doses studied could still produce antiestrogen concentrations in excess of any intratumor estrogens.

B. Intracellular Antiestrogen Binding Sites

Several intracellular binding proteins have been identified for estradiol (Anderson et al., 1986; Takahashi and Breitman, 1989; Masamura et al., 1997), and it would be remarkable if none of these also bound TAM. Indeed, it is likely that there are several such proteins that can sequester TAM and reduce its intracellular availability. One intracellular binding component, at least for the triphenylethlenes, is the AEBS protein. AEBS seems to be predominately microsomal (Katzenellenbogen et al., 1985) and may represent a novel histamine receptor (Clemmons et al., 1999). More recent data imply a protein complex containing the microsomal epoxyde hydrolase as one of the subunits (Mésange et al., 1998). This is a type II detoxification enzyme involved in the hydrolysis of aliphatic and aromatic electrophilic epoxides. TAM-AEBS interactions could contribute to the putative mutagenicity of TAM in some species (Greaves et al., 1993; Mésange et al., 1998). Whereas TAM induces expression of the epoxyde hydrolase mRNA (Nwaisir et al., 1995), it is an inhibitor of the enzyme’s catalytic activity (Mésange et al., 1998). Such an inhibition could leave reactive epoxyde metabolites of TAM, or other electrophilic epoxides, available to induce DNA damage (Mésange et al., 1998). TAM-induced hepatocellular carcinomas have been reported in rats (Greaves et al., 1993), but the incidence of these tumors is not increased in humans (Muhlemann et al., 1994). Any role for the epoxyde hydrolase-TAM interactions may be tissue- and species-specific.

A basic alkylether side chain, as occurs in many of the nonsteroidal antiestrogens, seems important for recognition of AEBSs by triphenylethlenes (Murphy and
Sutherland, 1985). AEBSs do not bind either the natural estrogens or the steroidal antiestrogens with high affinity (Pavlik et al., 1992) and will not interfere with intratumor estrogen activation of ER. Thus, overexpression of AEBSs could contribute to TAM resistance in the presence of continued ER expression. The antiestrogen-resistant LY2 cells (Bronzert et al., 1985; Clarke et al., 1989c) overexpress AEBSs relative to ER, as do a significant proportion of human breast (Pavlik et al., 1992) and ovarian carcinomas (Batra and Iosif, 1996). The affinity of TAM for AEBSs in ovarian cells is estimated <1 nM (Batra and Iosif, 1996) significantly greater than its affinity for ER. This implies a preferential binding of TAM to AEBSs relative to ER. Where TAM inhibits the epoxide hydrolase activity of AEBSs allowing reactive metabolites to persist, this could increase the genetic instability of some tumors. One consequence could be an increased potential to induce mutations in genes required for TAM function, with a subsequent increased risk of producing mutations that produce antiestrogen resistance.

The biological potency of antiestrogens does not correlate with their affinity for AEBSs (Katzenellenbogen et al., 1985). Although it has generally been assumed that the primary function of AEBSs has been to sequester drugs, several studies imply otherwise. Lymphoid cells that express AEBSs, but not ERs, are growth inhibited by antiestrogens (Tang et al., 1989; Hoh et al., 1990; Teo et al., 1992). The compound N,N-diethyl-2-(4 phenyl-methyl)-phenoxy ethamine HCl binds AEBSs, but not ERs, and is growth inhibitory in MCF-7 cells (Brandes, 1984). A TAM-resistant MCF-7 variant (RTx6) does not express AEBSs (Faye et al., 1983) and is not inhibited by either benzylphenoxy ethanamine derivatives (Poir et al., 1990) or other selective ligands for AEBSs (Fargin et al., 1988; Teo et al., 1992). Parental MCF-7 cells are growth inhibited by these compounds.

Polyunsaturated fatty acids can block TAM binding to AEBSs (Hoh et al., 1990). Cholesterol and lipoproteins can reverse the inhibitory effects of antiestrogens in an ER-negative lymphoid cell line (Tang et al., 1989). The antiproliferative activities of oxygenated sterols may be mediated by AEBSs. Ligand binding to AEBSs also affects cholesterol metabolism. Benzofurans can inhibit de novo cholesterol metabolism in ER-negative cells that express AEBSs (Teo et al., 1992). This raises the possibility that the hypocholesterolemic effects of some antiestrogens may be related to effects mediated by binding AEBSs.

Whereas AEBSs can sequester TAM, the extent to which antiestrogen-mediated activation of any AEBS function contributes to the antiproliferative effects of antiestrogens is unclear. If sufficient alone to confer responsiveness, the response rate to antiestrogens would be expected to be high in ER-negative tumors. However, responses in ER-negative tumors are infrequent (EBCTCG, 1998). The relationship between AEBS affinity and the IC$_{50}$ for antiproliferative effects is also of concern. The affinities of the antiestrogens TAM and clomiphene for AEBSs are two to three orders of magnitude greater than their respective antiproliferative IC$_{50}$s (Lin and Hwang, 1991). Whatever the role of AEBSs, these sites cannot affect the activities of the steroidal antiestrogens because steroids do not bind AEBSs (Pavlik et al., 1992).

C. Binding to Plasma Membranes

Many lipophilic compounds are sequestered within plasma membranes and other intracellular bilipid membranes. This is probably a relatively nonspecific phenomenon, reflecting their physicochemical properties. Compounds with a high degree of lipophilicity would be expected to preferentially partition into lipophilic domains in cellular membranes. This has been widely reported for steroids (Duval et al., 1983). We have previously shown that both TAM and estradiol can affect membrane structure in breast cancer cells in vitro (Clarke et al., 1990). Sequestration of TAM in a cell’s plasma membrane, and potentially within other intracellular bilipid membranes, could significantly reduce intracellular availability for binding to ERs. Some breast tumors exhibit a marked desmoplastic response, associated with the presence of fibroblastic and myofibroblastic cells, and/or significant infiltration of lymphoreticular cells (Clarke et al., 1992b). Thus, TAM could be further sequestered within the membranes of infiltrating cells and adjacent adipose tissue.

D. Altered Drug Accumulation/Transport and P-glycoprotein (mrd1)

The precise mechanism for intracellular uptake of TAM is not known. Passive diffusion, as probably occurs for steroids, seems most likely. Although tumors can concentrate TAM relative to its levels in serum (Fromson and Sharp, 1974; Daniel et al., 1981; Lien et al., 1989), intracellular sequestration could produce a relatively low concentration of unbound TAM, favoring its diffusion from extracellular sources. Some tumors may appear to have high TAM concentrations, but respond poorly because of low intracellular drug availability.

Reduced uptake of TAM from extracellular sources could confer resistance, provided the intracellular levels of available drug/metabolites fell below those required to effectively compete with any intratumor estrogens. Lower intratumor levels of TAM have been reported in some resistant versus sensitive tumors (Osborne et al., 1991, 1992; Johnston et al., 1993) and in some cell lines (Kellen et al., 1986). However, data are inconsistent. In a recent study, tumor concentrations of TAM, 4-hydroxy-TAM, and N-desmethyl TAM did not correlate with responsiveness or resistance. Indeed, the serum concentrations of 4-hydroxyTAM and N-desmethyl TAM were significantly higher among nonresponding patients.
significant difference in accumulation of $[^3]H$TAM (Maenpaa et al., 1994). We also have not found any not seen in one TAM-stimulated MCF-7 xenograft model these could affect accumulation. Altered TAM levels are intracellular binding proteins, altered expression of these could affect accumulation. Altered TAM levels are not seen in one TAM-stimulated MCF-7 xenograft model (Maenpaa et al., 1994). We also have not found any significant difference in accumulation of $[^3]H$TAM among TAM-resistant and TAM-responsive breast cancer cells growing in vitro (unpublished results).

TAM's ability to diffuse into cells could be related to specific plasma membrane domains into which it initially partitions (Clarke et al., 1990). The structure of these domains might depend on critical membrane-associated proteins or lipids, the altered expression of which could contribute to reduced diffusion/uptake. A simple reduction in the number of such putative domains also could reduce accumulation. These comments are speculative; further studies are required to determine the extent to which TAM's association with, and diffusion through, the plasma membrane is dependent upon definable membrane domains and/or functions.

The mechanism for TAM efflux also is not known, although a passive diffusion again seems most likely. We and others (Ramu et al., 1984; Leonessa et al., 1994) have described the ability of TAM to interact with the P-glycoprotein (also known as MDR1, gp170, and PGP) efflux pump, the product of the mdr1 (multidrug resistance 1) gene. P-glycoprotein is widely expressed in human breast tumors and is associated with a worse than partial response to cytotoxic chemotherapy (Trock et al., 1997). To determine the ability of P-glycoprotein to alter response to TAM, the MDR1 gene was overexpressed in MCF-7 cells. TAM can compete with azidopine for binding to P-glycoprotein and reverse the multidrug resistance phenotype in the transfectants (Leonessa et al., 1994). However, the transfectants' response to TAM is unaffected (Clarke et al., 1992a), and TAM accumulation is equivalent to wild-type cells (Clarke and Lippman, 1996). Thus, TAM is an inhibitor but not a substrate for this efflux pump, and expression of P-glycoprotein is probably not a contributor to TAM resistance.

E. Metabolism and Resistance

TAM is subject to extensive hepatic metabolism. Not surprisingly, several of the metabolites are predominately estrogenic, rather than antiestrogenic. Differences in TAM metabolism among mice, rats, and humans probably contribute to its species-specific agonist versus partial agonist properties (Jordan and Robinson, 1987).

The most relevant metabolites will be discussed only briefly, since the metabolism of TAM has been extensively reviewed elsewhere (Buckley and Goa, 1989; Lonning et al., 1992b). Demethylation of the aminoethoxy side chain produces N-desmethyl TAM, with further N-demethylation producing the primary amine (N-didesmethyl TAM). Deamination of the primary amine produces the primary alcohol (Kemp et al., 1983). Metabolite E is generated when the aminoethane side chain is removed. Hydroxylation of the parent drug produces the two more polar metabolites 4-hydroxyTAM and 3,4-dihydroxyTAM. Loss of the aminoethane side chain and hydroxylation at position 4 produces the bisphenol. Metabolite E and the bisphenol are estrogens and exhibit a lower affinity for ER than TAM (Jordan and Robinson, 1987). The other metabolites (B, D, X, Y, and Z) are partial agonists. The relative affinities for ERs are 4-hydroxyTAM $\geq$ 17$\beta$-estradiol $>$ TAM $>$ N-desmethyl TAM $>$ metabolite Y (Jordan et al., 1983; Katzenellenbogen et al., 1984).

Increased isomerization of TAM to estrogenic metabolites is observed in some TAM-resistant breast tumors (Osborne et al., 1991, 1992). A preferential generation of estrogenic metabolites could compete with the antiestrogenic metabolites for binding to ERs, perhaps interacting additively with existing intratumor estrogens to block antiestrogen action. It also would reduce the concentrations of antiestrogenic metabolites, potentially shifting the ratio of estrogenic:antiestrogenic metabolites in an unfavorable direction.

Evidence firmly establishing altered metabolism as a clinically relevant event remains elusive. Data from one animal model of TAM-stimulated growth, a phenotype that could reflect the preferential intracellular generation of estrogenic metabolites, clearly excluded the generation of such metabolites in this phenotype (Wolf et al., 1993). A series of elegant studies were performed using nonisomerizable TAM. These could not be metabolized to estrogenic metabolites, but the tumors still exhibited a mitogenic response to these derivatives (Wolf et al., 1993). Subsequent studies implicated a mutant ER protein in conferring the phenotype (Jiang et al., 1992). In a similar model from Dr. Osborne's laboratory (Baylor College of Medicine, Houston, TX), nonisomerizable TAM analogs also produced a stimulation of tumorigenesis. These data imply that the TAM-stimulated phenotype, at least in these models, is unlikely to
be explained by the significant conversion of parent drug to estrogenic metabolites (Osborne et al., 1994).

F. Comments

Altered intracellular availability could be a key event in affecting response and may account for a proportion of those ER-positive tumors that fail to respond to TAM. Ultimately, the ability of intracellular binding sites to affect TAM’s availability will reflect both the relative affinities of each site for TAM versus ER and their intracellular localization. For example, binding proteins in the cytosol may sequester TAM such that it never reaches the nuclear ER. Clearly, it will be important to determine the relevance and relative importance of intracellular availability. Identifying additional intracellular binding proteins may provide useful intermediate biomarkers for identifying those patients with ER-positive tumors that will fail to respond to TAM.

The importance of reduced TAM accumulation also requires further study. It is unlikely that P-glycoprotein contributes to lower intratumor TAM levels. However, we have preliminary data suggesting that P-glycoprotein may confer resistance to steroidal antiestrogens (Leonessa et al., 1998). The role of other membrane transporters has not been well defined.

The extent to which metabolism of TAM to estrogenic metabolites confers resistance remains to be clearly established. TAM-stimulated growth, the predicted response to this mechanism, can arise from mutations in ER and may not require estrogenic metabolites (Jiang et al., 1992). Nonetheless, it may be premature to entirely exclude the generation of estrogenic metabolites as a possible contributing resistance mechanism in some breast tumors.

IV. Cell Culture Models of Antiestrogen Responsiveness and Resistance

The study of acquired resistance has been greatly facilitated by the generation of several series of resistant variants. Most have been obtained by in vitro selection of the MCF-7 human breast cancer cell line. Almost all of these variants retain ER expression and show various patterns of resistance and cross-resistance. Resistant variants of other estrogen-responsive cell lines also have been reported. Although not a full listing, Table 4 describes several antiestrogen-resistant models. This section will focus primarily on those models of apparent pharmacological resistance (i.e., cells that do not exhibit a growth response to specific antiestrogens). Models that are growth stimulated by TAM are discussed in Section V. The models presented are selected to reflect the most widely used models and the diversity of phenotypes.

A. R27 and LY2

These were among the first stable antiestrogen-resistant variants reported. R27 cells were obtained following anchorage-independent cloning of MCF-7 cells in the presence of TAM. The cells retain an attenuated response to estradiol and are resistant to the growth inhibitory activities of TAM (Nawata et al., 1981). The LY2 cells were generated by a stepwise selection against the benzothiophene antiestrogen LY 117,018 (Bronzert et al., 1985). While retaining some responsiveness to estrogens, LY2 cells are cross-resistant to 4-hydroxy-TAM (Bronzert et al., 1985; Clarke et al., 1989c) and ICI 164,384 (Clarke et al., 1989c). Unfortunately, LY2 cells are nontumorigenic, restricting their use to in vitro studies (Clarke et al., 1989c). The tumorigenicity of R27 cells is not reported.

B. MCF-7RR

The MCF-7RR subline was obtained by selecting MCF-7 cells for their ability to grow in medium supplemented with 2% calf serum and 1 μM TAM (Butler et al., 1986). The cells exhibit an altered chromatin structure and chromatin acceptor sites for the antiestrogen 4-(N,N-diethylaminoethoxy)-4’-methoxy-α-(p-hydroxyphenyl)α-ethylstilbene (Singh et al., 1986). Of interest is MCF-7RR cells’ retinoic acid cross-resistance (Butler and Fontana, 1992), which has not been fully studied in many other antiestrogen-resistant variants. Whereas the cross-resistance pattern among other antiestrogens is not reported for MCF-7RR, these cells provide a novel model for studying the relationships among responsive-

<table>
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<td>MCF-7</td>
<td>LY2</td>
<td>+/−</td>
<td>E2-independent; TAM and ICI 164,384 cross-resistant</td>
</tr>
<tr>
<td>MCF-7</td>
<td>R27</td>
<td>+/?</td>
<td>TAM-resistant</td>
</tr>
<tr>
<td>MCF-7</td>
<td>RR</td>
<td>+/?</td>
<td>E2-independent; TAM-resistant</td>
</tr>
<tr>
<td>MCF-7</td>
<td>MCF7/LCC1</td>
<td>+/+</td>
<td>E2-independent; antiestrogen-responsive</td>
</tr>
<tr>
<td>MCF7/LCC1</td>
<td>MCF7/LCC2</td>
<td>+/+</td>
<td>E2-independent; TAM-resistant/ICI 182,780-responsive</td>
</tr>
<tr>
<td>MCF7/LCC1</td>
<td>MCF7/LCC9</td>
<td>+/+</td>
<td>E2-independent; TAM and ICI 182,780 cross-resistant</td>
</tr>
<tr>
<td>MCF-7</td>
<td>MCF7/WE5</td>
<td>+/+</td>
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</tr>
<tr>
<td>ZR-75-1</td>
<td>ZR75/LCC3</td>
<td>−/−</td>
<td>E2-independent; TAM and ICI 182,780 cross-resistant</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>ZR-75-9a1</td>
<td>−/−</td>
<td>E2-independent; TAM and ICI 182,780 cross-resistant</td>
</tr>
<tr>
<td>T47D</td>
<td>T47Deco</td>
<td>−/+</td>
<td>E2-independent; TAM and ICI 182,780 cross-resistant</td>
</tr>
</tbody>
</table>

a ER/PgR expression in variants.

b Citations for the cells and their phenotypes can be found in the text.
ness and resistance to both antiestrogens and retinoids. Another MCF-7 variant selected against 4-hydroxyTAM (MCF/TOT) has also been shown to exhibit cross-resistance to retinoic acid (Herman and Katzenellenbogen, 1996).

C. The LCC Series

This series was established to facilitate a further evaluation of cross-resistance phenotypes and to identify underlying molecular mechanisms. LCC variants were established from an estrogen-independent variant of MCF-7 cells (MCF7/MIII), initially selected for growth in vivo in ovariectomized nude mice (Clarke et al., 1989b). Circulating estrogen concentrations in these mice are similar to those found in postmenopausal women (Seibert et al., 1983), and the parent MCF-7 cells were derived from a postmenopausal patient (Soule et al., 1973). MCF7/MIII cells form proliferating tumors in these mice, but their growth is further increased upon estrogen supplementation. The cells retain ER expression and are growth inhibited by antiestrogens (Clarke et al., 1989b). A further in vivo selection produced the MCF7/LCC1 variant (Brüner et al., 1993a). These cells are similar to the MCF7/MIII, but tend to produce tumors more rapidly in ovariectomized nude mice. MCF7/LCC1 cells also retain ER expression, are estrogen-independent for growth, and are inhibited by triphenylethylene and steroidal antiestrogens (Brüner et al., 1993a; Brüner et al., 1997).

To generate antiestrogen-resistant variants, MCF7/LCC1 cells were stepwise selected against increasing concentrations of either 4-hydroxyTAM or ICI 182,780. Cells selected against the TAM metabolite produced stable, TAM-resistant cells (MCF7/LCC2), which also retain estrogen-independent growth in vitro and in vivo (Brüner et al., 1993b; Coopman et al., 1994). However, the MCF7/LCC2 cells are not cross-resistant to ICI 182,780. This predicts that tumors that responded and then failed TAM might show a strong response to a steroidal antiestrogen (Brüner et al., 1997). This prediction has now been confirmed in the clinic. The first trial of ICI 182,780 was performed in TAM responders who subsequently recurred. Consistent with the MCF7/LCC2 phenotype, the overall response rate to ICI 182,780 (69%) was substantially higher than would be predicted if the patients had been treated with another triphenylethylene (Howell et al., 1995). Using similar approaches, others have reported a MCF-7 variant (MCF-7/TAMR1) expressing a phenotype similar to MCF7/LCC2 (Lykkesfeldt et al., 1994).

Cells resistant to ICI 182,780 (MCF7/LCC9) were generated by selecting the MCF7/LCC1 variant against ICI 182,780. The resulting phenotype is clearly ER-positive, ICI 182,780-resistant, estrogen-independent, and TAM-crossresistant. Indeed, TAM cross-resistance emerges at early passages during the selection, arising before stable ICI 182,780 resistance is apparent (Brüner et al., 1997). The cross-resistance pattern may reflect the greater potency of ICI 182,780 relative to TAM and/or the differences in its interactions with ER (Fawell et al., 1990; Dauvois et al., 1992), which may have more substantial effects on ER functioning/signaling. Others have selected MCF-7 cells against ICI 182,780, but have not seen TAM cross-resistance (Jensen et al., 1999). The clinical relevance of these diverse phenotypes remains to be established.

D. ZR-75–9a1

ZR-75–1 cells are another of the relatively few, well established, estrogen-responsive human breast cancer cell lines. They were established from an ascites that developed in a 63-yr-old woman with an infiltrating ductal breast carcinoma (Engel et al., 1978). The patient had been receiving TAM for 3 months before the time when cells were removed to establish the ZR-75–1 cell line (Engel et al., 1978). ZR-75–1 cells are ER-positive and PgR-positive (Engel et al., 1978; van den Berg et al., 1987) and are growth stimulated by estrogens and inhibited by antiestrogens in vitro (Engel et al., 1978; van den Berg et al., 1989). However, the patient did not respond to TAM (Engel et al., 1978). A stepwise selection of the ZR-75–1 cells produced a resistant variant (ZR-75–9a1) that is not growth inhibited or stimulated by TAM (van den Berg et al., 1989). Unlike the MCF-7 TAM-resistant variants, the ZR-75–9a1 variant has lost expression of both ERs and PgRs. The cells remain stably resistant and receptor negative for only 3 months in the absence of selective pressure (van den Berg et al., 1989). Thus, ZR-75–9a1 cells are a useful model for studying initial acquired receptor negativity as an antiestrogen resistance phenotype.

E. Resistance Phenotypes Implied by Cell Culture Models

Some tumors with little or no effective estrogenic stimulation could be driven by a ligand-independent activation of the ER signaling network. This type of activation has been clearly described in vitro (Clarke and Brüner, 1996). Although independent of estrogens, antiestrogens are able to inhibit, and estrogens can further increase this ER activation. Consistent with these observations, cells acquiring estrogen independence retain responsiveness to antiestrogens and are growth stimulated by estrogens in vivo (e.g., MCF-7/MIII and MCF7/LCC1 phenotypes). Thus, proliferation of some estrogen-independent cells, which continue to express ERs, may be primarily maintained by ligand-independent ER signaling. This also suggests that available intracellular estrogens may not be required for some tumors to exhibit an ER-positive, antiestrogen responsive phenotype. It is also apparent that estrogen independence and antiestrogen resistance are independent phenotypes (Clarke et al., 1989c).
Together, these observations suggest the existence of at least three ER-positive phenotypes: 1) estrogen-dependent (requires an adequate estrogenic stimulus for proliferation); 2) estrogen-independent, but responsive (does not require, but may be stimulated by, available intracellular estrogens); and 3) estrogen-independent and unresponsive (does not require, and will not respond to, available intracellular estrogenic stimuli even if estrogens are present). Phenotype (1) would be responsive to both antiestrogens and aromatase inhibitors, whereas phenotype (3) would be cross-resistant to these therapies. Phenotype (2) would be antiestrogen responsive and also might exhibit responses to aromatase inhibitors. For example, removal of the estrogenic stimulation by the aromatase inhibitors would leave the cells reliant on the less potent ligand-independent ER-activated signaling. Estrogen-independent, but responsive, cells would either grow more slowly, or undergo growth arrest but perhaps not die, in response to an effective aromatase inhibitor. TAM-stimulated growth might be seen in both phenotypes (1) and (2). Since breast tumors are highly heterogeneous, the overall clinical response would partly reflect the relative proportions of the responsive phenotypes within the tumor.

**V. Tamoxifen-Stimulated Proliferation as a Resistance Mechanism**

TAM-stimulated growth is one possible mechanism for clinical resistance, a response not unusual in some normal tissues. For example, TAM stimulation of uterine proliferation (estrogenic/agonist effect) has been known for many years (Harper and Walpole, 1967). Switching to a TAM-stimulated phenotype can arise in MCF-7 cells following in vivo selection against TAM, spontaneously in estrogen-deprived cells, and after transfection with members of the fibroblast growth factor (FGF) family of proteins. There also is limited evidence suggesting that TAM-stimulated tumor growth may occur in a minority of breast cancer patients (see Section V.E.).

**A. In Vivo Selection against Tamoxifen or ICI 182,780**

Perhaps the most consistent models of TAM-stimulated growth are generated by in vivo selection of established MCF-7 xenografts against TAM (Osborne et al., 1987; Gottardis et al., 1989). Since MCF-7 tumors require estrogens for growth in vivo, tumors are first established in the presence of estradiol, which is then replaced with TAM. Tumors initially stop proliferating or regress, but prolonged therapy produces re-emergent tumors. These appear to be TAM-stimulated because they subsequently regress upon removal of TAM (Osborne et al., 1987; Gottardis et al., 1989). The TAM-stimulated tumors are not cross-resistant to the steroidal antiestrogens (Osborne et al., 1995), consistent with the cells now selectively perceiving TAM as an agonist. MCF-7 tumors also have been selected in vivo for resistance to ICI 182,780. ICI 182,780 resistance arises, but takes longer than does the development of TAM resistance (Osborne et al., 1995), perhaps reflecting the greater potency of ICI 182,780 relative to TAM (Brüner et al., 1993b).

**B. MCF-WES and MCF/TOT**

Although most in vitro selection models have identified phenotypes that are no longer growth inhibited by antiestrogens, the MCF-WES cells are growth stimulated by TAM (Dumont et al., 1996). MCF-WES was obtained from a MCF-7 tumor growing in an ovariectomized nude mouse. The cells are estrogen-independent, but respond mitogenically to estrogens. While being growth stimulated by TAM, MCF-WES cells are cross-resistant to ICI 182,780 [i.e., treatment with the steroidal antiestrogens does not affect growth rate (Dumont et al., 1996)]. The ability of these cells to grow both in vitro and in vivo provides a novel model to study TAM-stimulated proliferation. A MCF-7 cell population that is stimulated by 4-hydroxyTAM (MCF/TOT) has also been obtained by long-term exposure to 4-hydroxyTAM in vitro (Herman and Katzenellenbogen, 1996) and may be derived from a subpopulation similar to that which produced MCF-WES cells. These cells appear to have a TAM-responsive phenotype broadly comparable with the MCF/WES cells, but the cells do not exhibit cross-resistance to ICI 164,384 (Herman and Katzenellenbogen, 1996).

**C. Fibroblast Growth Factor-Transfected MCF-7 Variants and Their Role(s) in Antiestrogen Resistance**

The expression of several growth factors have been implicated in estrogen independence and antiestrogen resistance. Several angiogenic growth factors, most notably members of the FGF family, have recently been evaluated for their ability to produce antiestrogen resistance. Overexpression of FGF-1 by transfection into MCF-7 cells produces cells that generate highly vascularized, estrogen-independent, metastatic tumors (Zhang et al., 1997). Estrogen-independent growth is not affected by 4-hydroxyTAM, indicating the ability of FGF-1 overexpression to confer TAM resistance. When FGF-4 is overexpressed, the cells become TAM-stimulated in vivo (Kurebayashi et al., 1993; Zhang et al., 1997), a response similar to that seen in the MCF-WES cells and some in vivo TAM-selected models (see above). FGF-1 and FGF-4 transfected MCF-7 cells are still growth inhibited by ICI 182,780 in vitro, but exhibit some reduction in responsiveness compared with controls (McLeskey et al., 1998). Thus, overexpression of these FGFs is sufficient to confer TAM resistance, but not full cross-resistance to ICI 182,780.

The ability of overexpression of FGFs to produce these phenotypes may reflect the induction of both mitogenic and growth inhibitory effects in breast cancer cells.
FGF-1 are important for this TAM-stimulated growth. and that the paracrine and/or angiogenic effects of This indicates that the tumors can be driven by TAM, dominant negative FGF receptor. Importantly, xeno- autocrine stimulation because of the coexpression of a importances of both autocrine and angiogenic responses. In an elegant approach, these investigators generated many reports. When combined, data suggest significant with-drawal responses (i.e., where the patient obtains a beneficial clinical response if treatment is continued. This is quite different from recurrence on TAM, where continued treatment provides little benefit. Flare probably reflects TAM’s pharmacology. Steady-state levels of TAM in patient sera are not reached for up to 4 weeks (Buckley and Goa, 1989; Etienne et al., 1989). In cell culture, low concentrations of TAM can be mitogenic (Clarke et al., 1989). Thus, the low TAM serum/ tissue concentrations at the initiation of treatment in patients may be mitogenic, producing the flare response. Once the elevated steady-state levels are reached in patients, the antagonist properties of TAM could predominate, accounting for the subsequent remissions. Another possibility is a TAM-induced increase in serum dehydroepiandrosterone (estrogen precursor), estrone, and estradiol concentrations (Pommier et al., 1999). These hormones could stimulate proliferation until the levels of TAM become sufficient to overcome this effect. It is possible that both the direct (low concentrations of TAM perceived as an estrogen) and indirect effects (increased estrogen production) contribute to TAM flare. Since we can delineate TAM flare from a TAM-stimulated resistance phenotype, it is important to estimate the frequency of the latter. The precise frequency of the TAM-stimulated phenotype is difficult to assess in patients. One approach is the measurement of clinical withdrawal responses (i.e., where the patient obtains a beneficial response upon cessation of treatment). Unfortunately, the number of TAM withdrawal cases may be underdocumented. Table 5 shows those identified using a proven literature retrieval approach (Trock et al., 1997). Despite approximately 10 million patient years of experience, only 16 cases of partial and complete responses were found in five relatively small studies. The few other reports were identified as individual case reports. When combined, data suggest significant withdrawal responses in approximately 7% of patients. When disease stabilization is included, the estimate of the incidence of putative TAM withdrawal clinical responses approaches 20%.

Nomura et al. (1990) measured the ability of TAM to increase the proliferation (≥150%) of breast tumor biop-

D. Angiogenesis and Tamoxifen Resistance

Data from the FGF transfected cell lines imply a role for angiogenesis in TAM resistance. Limited evidence from studies in humans also suggests that more angiogenic tumors have a poor response to antiestrogens. In node-positive patients, those with ER-positive and poorly vascularized tumors have the best prognosis in response to TAM therapy (Gasparini et al., 1996). Antiestrogens are antiangiogenic in some experimental models (Gagliardi and Collins, 1993). Thus, an antiangiogenic effect could contribute to good TAM responses, or conversely, highly angiogenic tumors may respond poorly to TAM.

Angiogenesis will increase tumor perfusion and might increase TAM accumulation. This could increase the number of cells to which TAM is delivered and perhaps increase the intracellular concentrations of TAM in previously poorly vascularized regions. Such an effect might be expected to increase responsiveness rather than induce resistance. However, increased angiogenesis will also increase intratumor concentrations of estradiol precursors, improve perfusion of oxygen and nutrients, and improve removal of cellular waste and dead/dying cells. These events would be expected to improve the overall “health” of tumor cells. However, the simplest explanation might be that highly angiogenic tumors may have a higher metastatic potential. This could produce an effect independent of ER expression, as seen in the study by Gasparini et al. (1996).

Signaling through receptors for angiogenic growth factors could also contribute to cellular resistance by changing the activation of cell signaling pathways within the cell. This seems most likely in some models, since the cells are resistant in vitro where the angiogenic effects are irrelevant. Zhang et al. (1998) have used a dominant negative FGF-receptor to assess the relative importance of both autocrine and angiogenic responses. In an elegant approach, these investigators generated cells that overexpress FGF-1, but cannot respond to autocrine stimulation because of the coexpression of a dominant negative FGF receptor. Importantly, xenografts from these cells require either estrogen or TAM. This indicates that the tumors can be driven by TAM, and that the paracrine and/or angiogenic effects of FGF-1 are important for this TAM-stimulated growth.

E. Tamoxifen Stimulation as a Resistance Phenotype in Patients and Tamoxifen Flare

If the TAM-stimulated phenotype arose in a patient, the tumor would be considered resistant. Thus, TAM-stimulated growth can be considered a resistance mechanism in the broadest sense. However, the tumor is clearly not resistant in the pharmacologic sense. Superficially, this resistance phenotype looks like TAM-induced tumor flare, which occurs when patients respond by a temporary worsening of their disease shortly after initiation of TAM treatment. This response is often accompanied by increased pain, hypercalcemia, and progression of metastatic disease (Plotkin et al., 1978).

Many patients who initially exhibit TAM flare obtain a beneficial clinical response if treatment is continued. This is quite different from recurrence on TAM, where continued treatment provides little benefit.

Flare probably reflects TAM’s pharmacology. Steady-state levels of TAM in patient sera are not reached for up to 4 weeks (Buckley and Goa, 1989; Etienne et al., 1989). In cell culture, low concentrations of TAM can be mitogenic (Clarke et al., 1989). Thus, the low TAM serum/tissue concentrations at the initiation of treatment in patients may be mitogenic, producing the flare response. Once the elevated steady-state levels are reached in patients, the antagonist properties of TAM could predominate, accounting for the subsequent remissions. Another possibility is a TAM-induced increase in serum dehydroepiandrosterone (estrogen precursor), estrone, and estradiol concentrations (Pommier et al., 1999). These hormones could stimulate proliferation until the levels of TAM become sufficient to overcome this effect. It is possible that both the direct (low concentrations of TAM perceived as an estrogen) and indirect effects (increased estrogen production) contribute to TAM flare. Since we can delineate TAM flare from a TAM-stimulated resistance phenotype, it is important to estimate the frequency of the latter. The precise frequency of the TAM-stimulated phenotype is difficult to assess in patients. One approach is the measurement of clinical withdrawal responses (i.e., where the patient obtains a beneficial response upon cessation of treatment). Unfortunately, the number of TAM withdrawal cases may be underdocumented. Table 5 shows those identified using a proven literature retrieval approach (Trock et al., 1997). Despite approximately 10 million patient years of experience, only 16 cases of partial and complete responses were found in five relatively small studies. The few other reports were identified as individual case reports. When combined, data suggest significant withdrawal responses in approximately 7% of patients. When disease stabilization is included, the estimate of the incidence of putative TAM withdrawal clinical responses approaches 20%.
sies in short-term culture in vitro (data adapted in Table 5). Approximately 7% of ER-positive biopsies exhibit a mitogenic response to TAM. The biopsies appear to have been collected from previously untreated patients. Thus, at the time of diagnosis, a small proportion of tumors may already contain cells that will perceive TAM as an estrogen.

Half of the TAM-stimulated tumor biopsies did not respond to estradiol (Table 5), suggesting that the true proportion perceiving TAM as an estrogen could be as low as 4% of all ER-positive tumors. This raises the possibility that some tumors might be TAM-stimulated through other mechanisms. For example, TAM can sensitize cells to the proliferative activities of IGF-1 (Wise-\textit{man et al.}, 1993b). This would still require ER expression, and is consistent with the low frequency of TAM-stimulated, ER-negative, breast biopsies in the data adapted in Table 5. Data from the TAM withdrawal responses clearly implicate TAM stimulation in about 7% of recurrences, equivalent to the estimated proportion of TAM-stimulated biopsies from naive patients (Nomura \textit{et al.}, 1990). TAM treatment would tend to select for these cells, which would be predicted to have a clear proliferative advantage over other cell populations within the tumor, ultimately producing a TAM-stimulated tumor.

Data in Table 5 are consistent with acquired TAM stimulation being one of several mechanisms that contribute to clinical resistance. However, it is not entirely clear that this phenotype exclusively reflects cells that perceive TAM as an estrogen. Since >80% of tumors probably do not use this mechanism to acquire resistance, it may not be the primary resistance mechanism in most breast tumors.

### VI. Estrogen Receptors, Mutant Receptors, Coregulators, and Gene Networks

Two ER proteins exist (ER\textalpha{}, ER\textbeta{}), each being the product of different genes on separate chromosomes. Both proteins have similar functional domains including ligand binding, DNA binding, and two transcriptional activating domains (AF-1; AF-2). These have been extensively discussed and reviewed by others (Kumar \textit{et al.}, 1987; Enmark and Gustafsson, 1998). ERs function as nuclear transcription factors and regulate the expression of a considerable number of different genes. The patterns of gene regulation probably differ across cell types and can be thought of as regulating a series of different gene networks. These networks may be independent, interdependent, and/or intersecting (Clarke and Brü\text{"u}rner, 1995, 1996; Clarke and Lippman, 1996).

ER proteins adopt various conformations when occupied by different ligands (Brzozowski \textit{et al.}, 1997; Grese \textit{et al.}, 1997) and may recruit different proteins into the transcription complexes being formed at the promoters of target genes (Shiau \textit{et al.}, 1999). The potency and direction of transcriptional regulation (induction or repression) are strongly affected by the ligand and receptor. For example, ICI 182,780 inhibits ER\textalpha{}-mediated transcription, but activates ER\textbeta{} transcriptional activities at an AP-1 site (Paech \textit{et al.}, 1997). The mix of coregulators recruited (coactivators or corepressors) (Clarke and Brü\text{"u}rner, 1996; Horwitz \textit{et al.}, 1996) and probably the phosphorylation status of the receptor (Arnold \textit{et al.}, 1995; Kato \textit{et al.}, 1995; Notides \textit{et al.}, 1997) are also important components that can affect transcription.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Worse than PR</th>
<th>PR</th>
<th>CR</th>
<th>PR + CR/Duration (Range)</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advanced disease</td>
<td>19/19</td>
<td>0/19</td>
<td>0/19</td>
<td>0%</td>
<td>Beex et al., 1981</td>
</tr>
<tr>
<td>Postmenopausal with metastatic disease</td>
<td>6/9</td>
<td>1/9</td>
<td>2/9</td>
<td>22%/10–14 months</td>
<td>Rudolph, 1986</td>
</tr>
<tr>
<td>Postmenopausal with metastatic disease</td>
<td>84/87</td>
<td>3/87</td>
<td>0/87</td>
<td>3%/9–10.3 months</td>
<td>Taylor et al., 1986</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>56/61</td>
<td>4/61</td>
<td>1/61</td>
<td>8%/3–10 months</td>
<td>Canney et al., 1989</td>
</tr>
<tr>
<td>Advanced disease</td>
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<td>5/65</td>
<td>0/65</td>
<td>8%/3–40 months</td>
<td>Howell et al., 1992</td>
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<tr>
<td>Mean (PR + CR)</td>
<td>225/241</td>
<td>13/241</td>
<td>3/241</td>
<td>6.6%</td>
<td></td>
</tr>
<tr>
<td>Overall (PR + CR + DS)</td>
<td>19.5% (47/241)</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>TAM Stimulation of Primary Breast Tumors In Vitro</th>
<th>ER Status</th>
<th>Response</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER-positive</td>
<td>TAM-stimulated</td>
<td>11/153</td>
<td>(7)</td>
</tr>
<tr>
<td>ER-negative</td>
<td>TAM-stimulated</td>
<td>1/71</td>
<td>(1.4)</td>
</tr>
<tr>
<td>ER-positive</td>
<td>Estradiol-stimulated</td>
<td>47/153</td>
<td>(31)</td>
</tr>
<tr>
<td>ER-negative</td>
<td>Estradiol-stimulated</td>
<td>10/71</td>
<td>(14)</td>
</tr>
<tr>
<td>ER-positive</td>
<td>TAM-stimulated and estradiol-stimulated</td>
<td>6/153</td>
<td>(4)</td>
</tr>
<tr>
<td>ER-negative</td>
<td>TAM-stimulated and estradiol-stimulated</td>
<td>0/71</td>
<td>(0)</td>
</tr>
</tbody>
</table>

\* PR, partial response; CR, complete response.
\* All patients were selected on the basis of having experienced a response to TAM.
\* All responses were seen in the group of 28 patients who had originally responded to TAM (18% of initial responders).
\* DS = disease stabilization.
\* Data adapted from Fig. 1 in Nomura \textit{et al.}, 1990.
Since most antiestrogen-resistant tumors retain ER expression (Johnston et al., 1995), continued signaling through ER may be required for cell proliferation. This is probably the case in those tumors that remain responsive to other antiestrogens or aromatase inhibitors, but may also apply to other phenotypes. If sufficient ERs remain occupied by antiestrogens, either the cells have eliminated the antiestrogenic signaling, changed how this signaling is perceived by the cell, and/or altered the expression of other genes that counteract any remaining antiestrogenic signals. Such effects could be produced by changes in receptor function, perhaps through the emergence of either mutant receptors, perturbations in post-translational receptor modifications (e.g., phosphorylation patterns), and/or other changes in the cellular context (e.g., coregulator expression/availability; changes in the regulation of intersecting/interdependent signaling pathways).

Membrane-associated ERs have been reported for many years (Nelson et al., 1987) and are also present on human breast cancer cells (Nelson et al., 1987; Watson et al., 1999). These membrane-associated ERs were generally considered experimental artifacts once the predominately nuclear localization was reported (Welschons et al., 1984). More recently, proteins derived from both the ERα and ERβ genes have been identified in the cell membranes of Chinese hamster ovary cells transfected with the respective cDNAs (Razandi et al., 1999). Moreover, there is an increasing body of evidence suggesting that membrane-associated ERs are functional. For example, estrogens that cannot enter cells induce critical biological events in pituitary tumor cells (Watson et al., 1999), human sperm (Luconi et al., 1999), rat hypothalamic cells (Prevot et al., 1999), and human neuroblastoma cells (Watters et al., 1997). In some (Prevot et al., 1999), but not all, instances (Watters et al., 1997), these estrogenic effects can be blocked by antiestrogens. Some investigators used high concentrations of ligands, and these can produce nonspecific effects. However, the ability of antiestrogens to block the estrogenic activities of membrane receptors implies a signaling similar to that of nuclear ERs. Clearly, additional studies on the role and function of membrane ERs are required.

A. Wild-Type and Mutant Estrogen Receptor-α and Estrogen Receptor-β

Since the ERβ gene was cloned in 1996 (Kuiper et al., 1996; Mosselman et al., 1996), and ERβ-selective reagents have only recently been reported (Sun et al., 1999), most studies have focused on the role of ERα. The importance of ERα expression in predicting response to antiestrogens was described in Section I.C.

ERβ mRNA has been detected by polymerase chain reaction in breast tumors (Leygue et al., 1998; Dotzlaw et al., 1999; Speirs et al., 1999b), but ERα may be the predominant species in many ER-positive breast tumors (Leygue et al., 1998; Speirs et al., 1999b). This reflects an apparent increase in ERα expression in neoplastic versus normal mammary tissues (Leygue et al., 1998). When present in tumors, ERβ is associated with a poorer prognosis, absence of PgR, and lymph node involvement (Dotzlaw et al., 1999; Speirs et al., 1999b). Thus, it may be important to separate any effects on response to antiestrogens from an association of ERβ expression with this more progressed phenotype. In contrast, ERα expression is generally associated with a better prognosis.

The relative binding affinities of ERα and ERβ for 17β-estradiol are comparable. Similar effects are seen in the regulation of transcription in simple promoter (estrogen-responsive element; ERE)-reporter assays (Kuiper et al., 1997). However, there are notable differences in the molecular pharmacology of these two receptors. Agonists and antagonists exhibit opposite effects on ERα versus ERβ-mediated transcription at AP-1 sites in a promoter-reporter assay (Paech et al., 1997). The ability of ERβ to activate the retinoic acid receptor promoter is driven by antiestrogens. Estradiol alone is inactive, but can block the activities of antiestrogens. The effect of 4-hydroxyTAM appears to be mediated through SP1 sites in the retinoic acid receptor promoter and is conferred by the 3‘ region of ERβ [i.e., independent of the two transactivating domains (Zou et al., 1999)].

Compounds that are antagonist for ERα may be agonists for ERβ, at least at AP-1 and SP-1 sites (Paech et al., 1997; Zou et al., 1999). An increase in ERβ expression, acting through genes with AP-1 and/or SP-1 sites in their promoters, could produce the TAM-stimulated phenotype seen in some MCF-7 xenografts and cell lines. Binding ICI 182,780 targets ERα for degradation (Dauvois et al., 1992). Since it is transcriptionally activated upon binding ICI 182,780 (Paech et al., 1997), ERβ may not be so targeted. ERβ’s transcriptional activation could contribute to the apparent agonist-like effects of ICI 182,780 seen in some tissues (Paech et al., 1997).

The ratio of ERα:ERβ also may be important in predicting response, particularly in those tumors that express ER, but do not respond to antiestrogens. When both receptors are present, transcriptionally active heterodimers can be formed (Pettersson et al., 1997). 4-HydroxyTAM can act as an agonist through ERα/ERβ heterodimers, but the effect is promoter- and cell context-dependent (Tremblay et al., 1999). Although the effects on proliferation were not evaluated, these agonist effects on transcription could affect the expression of genes induced by estrogens and responsible for proliferation. Thus, in breast cancer cells where adequate concentrations of functionally active ERα and ERβ proteins are present, TAM could induce, rather than inhibit, cell proliferation. This could explain some of the endogenous and acquired resistance seen in ER-positive breast tumors. Generally, the agonist effects of TAM are cell- and promoter context-dependent and related to the ER sub-
types expressed in the target cells (Clarke and Brüninger, 1996; Watanabe et al., 1997).

Data from clinical material are still somewhat limited and the role of ERβ in antiestrogen-resistant and responsiveness requires further study. One small study of nine TAM-resistant and eight responsive tumors found 2-fold higher median levels of ERβ versus ERα mRNA expression by polymerase chain reaction in the TAM-resistant biopsies (Speirs et al., 1999a). However, protein levels were not reported. The association with TAM resistance may reflect the poor prognosis associated with ERβ expression (Speirs et al., 1999a).

The role of ER mutants has been most widely studied for ERα. Several mutant ERα genes have been reported, but the consequence of this expression is unclear. For example, it is often not known whether the mutant mRNA is translated, although some mutant ER proteins clearly are produced (Murphy et al., 1998). Most tumors that express mutant ER concurrently express the wildtype receptor, with the mutant representing a relatively small proportion of total ER proteins. Thus, only dominant negative mutants have a substantial chance of affecting transcription. A mutant ER that perceives TAM as an agonist has been described in some MCF-7 cell variants (Jiang et al., 1992). It is not clear whether this, or functionally similar mutant proteins, occur in breast tumors in patients.

At least five isoforms of ERβ have been identified, with three full-length isoforms exhibiting the ability to bind DNA as homodimers and heterodimers with ERα (Moore et al., 1998). A tyrosine mutant of ERβ has been reported, but is sensitive to the actions of antiestrogens and is likely not involved in antiestrogen resistance (Tremblay et al., 1998). An exon 5 deletion mutant of ERβ also has been reported (Vladusic et al., 1999). Whether this mRNA is translated, and its likely role in antiestrogen resistance, remain to be elucidated.

There is little compelling evidence that ER mutant proteins directly confer resistance in a significant proportion of breast tumors (Karnik et al., 1994). However, it would be premature to exclude the possibility that mutated ER confer resistance in some breast cancers. It is likely that a better understanding of the role of such ER mutants, whether these be of the ERα and/or ERβ genes, will likely emerge in the relatively near future.

### B. Coregulators of Estrogen Receptor Action

Recently, several investigators have identified coregulator proteins that can significantly influence ER-mediated transcription; for an excellent recent review, see McKenna et al. (1999). These can be most easily thought of as being either coactivators (increase transcription, e.g., SRC-1) (Xu et al., 1998) or corepressors (inhibit transcription, e.g., N-CoR, SMRT) (Jackson et al., 1997; Soderstrom et al., 1997). Binding of the SRC family of proteins is mediated by a conserved LXXLL motif that facilitates interactions with ligand-occupied ER (Ding et al., 1998). One likely consequence of receptor-coactivator binding is the activation of SRC-1’s histone acetyltransferase activity (Spencer et al., 1997), which would be expected to unwind and expose the adjacent promoter DNA. This should facilitate the binding of additional transcription factors and the initiation of transcription. In contrast, complexes containing corepressors such as N-CoR can exhibit deacetylase activity (Heinzel et al., 1997; Spencer et al., 1997), which would be expected to inhibit transcription (Pazin and Kadonaga, 1997). Whereas most studies of coregulator action have been done with ERα, ERβ function also appears to be affected by coregulators (Tremblay et al., 1997).

The ability of a liganded receptor to recruit coregulators is at least partly dependent on its conformation. Shiau et al. (1999) have recently shown that 4-hydroxy-TAM induces a conformation that blocks the coactivator recognition groove in ER. The consequences of coregulator binding can be complex (McKenna et al., 1999). SRC-1 inactivates ER bound to pure antagonists, enhances the agonist activity of partial agonists like 4-hydroxyTAM, is involved in a ligand-independent activation, and interacts synergistically with cAMP response element-binding protein in regulating ER-mediated transcription (Smith et al., 1996, 1997; Jackson et al., 1997). The corepressor SMRT binds ER, inhibits the agonist activity of 4-hydroxyTAM, and blocks the agonist activity of 4-hydroxyTAM induced by SRC-1 (Smith et al., 1997). N-CoR binds TAM-occupied, but not ICI 182,780-occupied ER (Jackson et al., 1997).

These observations suggest that changes in coregulator expression or recruitment into an ER-antiestrogen-driven transcription complex could produce a resistance phenotype (Clarke and Brüninger, 1996; Horwitz et al., 1996; Smith et al., 1997). However, mice lacking SRC-1 exhibit only partial hormone resistance (Xu et al., 1998). Overexpression of SRC-1 in MCF-7 cells may not significantly alter response to 4-hydroxyTAM (Tai et al., 2000), although data presented in this study are somewhat limited in this regard. The partial agonist (estrogenic) properties of 4-hydroxyTAM are increased by the coregulator L7/SPA (Jackson et al., 1997). In contrast, TAM’s estrogenic activity is inhibited when SMRT is recruited into an ER-TAM complex (Smith et al., 1997). Thus, an increase in L7/SPA concurrent with reduced SMRT expression could generate a TAM-stimulated phenotype. A change in antiestrogen-ER complex conformation (e.g., through mutation or posttranslational modification) could either eliminate recruitment of corepressors or allow a preferential recruitment of coactivators. Either could contribute to antiestrogen resistance by influencing the regulation of ER-regulated gene networks that alter signaling to proliferation/differentiation/cell death.

Whether such effects occur and are biologically relevant clearly requires further study. MCF-7 xenografts that are TAM-stimulated express lower levels of N-CoR
interrelated (e.g., intracellular Ca\(^{2+}\), 1999; Singh et al., 1999). Some of these activities are
1999), and MAPK (Migliaccio et al., 1996; Nuedling et
and Weber, 1999), protein kinase C (PKC) (Kelley et al.,
cAMP (Farhat et al., 1996; Picotto et al., 1996; Schaffer
C. Estrogenic and Antiestrogenic Regulation of
Mitogen-Activated Protein Kinase
Estrogens can activate, rapidly, specifically, and at
physiological concentrations, several well characterized
signaling molecules/pathways, including intracellular Ca\(^{2+}\) (Mermelstein et al., 1996; Picotto et al., 1996),
cAMP (Farhat et al., 1996; Picotto et al., 1996; Schaffer
and Weber, 1999), protein kinase C (PKC) (Kelley et al.,
1999), and MAPK (Migliaccio et al., 1996; Nuedling et
al., 1999; Singh et al., 1999). Some of these activities are
interrelated (e.g., intracellular Ca\(^{2+}\) (Burgering et al.,
1993; Albert et al., 1997; Improta-Brears et al., 1999),
PKC (Kazlauskas and Cooper, 1988; L'Allemain et al.,
1991), and cAMP can each affect MAPK activation (Qian
et al., 1995; D'Angelo et al., 1997)). Thus, an estrogenic
and/or growth factor activation of MAPKs could play a
key role in ER-mediated signaling.
MAPK signaling is generally through one or more of
the three MAPK modules (Fig. 2), each comprising one
or more MEK kinases (activate MEK), a MEK (activates
MAPK), and a MAPK (Cobb and Goldsmith, 1995; Mar-
shall, 1995). Two additional, but less well defined, mod-
ules also exist; one where the MAPK is ERK3 and the
other using ERK5 as the MAPK (Schaffer and Weber,
1999). The first of the three defined MAPK modules is
dependent upon ras/raf activation, which regulates
MEK1,2 activity, with the subsequent activation of
ERK1,2 (Cobb and Goldsmith, 1995). This module is
often associated with differentiation/proliferation and
can be activated by receptor tyrosine kinases. The sec-
ond module [stress-activated protein kinase (SAPK)
module] is ras-independent and is primarily regulated
by rac (Lopez-Ilasaca, 1998; Vojtek and Cooper, 1999),
rac being overexpressed in many breast cancers (Fritz et
al., 1999). Subsequently, JNK/SEK/MKK4 activates
JNK/SAPK (Cobb and Goldsmith, 1995). The third mod-
ule activates the p38/HOG1 MAPK and is associated
with phosphorylation of HSP27 (Pelech and Charest,
1995). The latter two modules are often associated with
signals arising from exposure to stressors and cytokines
(Marshall, 1995; Woodgett et al., 1996; Vojtek and Coo-
per, 1999). Despite the complexity of cellular conse-
quences of MAPK activation (see Schaffer and Weber,
1999, for recent review), cross-talk among modules can
be effectively regulated. Activation of one module could
produce contrasting effects in diverse cell types, or in the
same cell type under different conditions.
MEK1,2 activities are increased in up to half of all
breast cancers (Sahl et al., 1999). There also is evidence
for a preferential activation of ERK1/MAPK (Xing and
Imagawa, 1999). ERK/MAPK activities are elevated in
experimental mammary tumor models driven by c-myc,
c-erb-B2, and v-Ha-ras, but not those driven by ei-
ther transforming growth factor (TGF)-\(\alpha\) or heregulin
(Amundadottir and Leder, 1998). Overexpression of raf
can induce an estrogen-independent phenotype in
MCF-7 breast cancer cells (El-Ashry et al., 1997).
Estrogen increases MAPK activity in some MCF-7
cells (Migliaccio et al., 1996; Improta-Brears et al.,
1999), with this activity being constitutively elevated in
estrogen-independent cells (Coutts and Murphy, 1998).
Estrogenic activation of MAPK apparently signals
through activation of src and ras. Blockade of MAPK
activation eliminates estrogen signaling in primary cor-
tical neurons (Singer et al., 1999). The rapidity and
nonantiestrogen reversibility in some models are consist-
tent with the widely reported nongenomic effects of ste-
roids. Where antiestrogens reverse the effects of estro-
gens, the ER may be required. Thus, the ability of
estrogens to activate MAPKs is probably multifactorial,
with both ER-dependent and ER-independent events occurring.

Determining the precise contribution of signaling through the MAPKs is complex. For example, FGF-2 inhibits breast cancer cell growth, but induces both ERK1 and ERK2, which are generally associated with mitogenic signals (Fenig et al., 1997). TAM can inhibit MAPK activation, an effect that may be related to TAM’s ability to influence PKCε (Luo et al., 1997). However, TAM can increase both ERK2 activity and activate JNK1 (Duh et al., 1997). In rat cardiomyocytes, TAM activates ERK1/ERK2, but not p38 MAPK (Nuedling et al., 1999). The ability to concurrently activate both the MAPK and SAPK signaling modules could contribute to TAM’s tissue-specific partial agonism. The importance of cellular context for downstream signaling from MAPKs is well established (Day et al., 1999b; Schaffer and Weber, 1999). In tissues where TAM initiates signaling only through the MAPK module, TAM might function as a partial agonist. Where only the SAPK module is activated, or where this activation predominates over any potentially mitogenic signaling from the MAPK module, TAM’s apoptosis/growth inhibition-inducing properties could predominate (Fig. 3).

The ability of some cells to perceive TAM as an agonist (TAM-stimulated phenotype) may reflect a preferential activation/predominance of signaling through the MAPK module. Other resistant cells may no longer be able to either activate a SAPK pathway, change the way in which MAPK/SAPK signaling is perceived (e.g., by modifying expression of downstream signaling targets), and/or switch to alternative pathways to maintain cell proliferation/survival.

Ultimately, the role of MAPKs may be determined by the balance between their activation and inactivation. For example, PP2A is a major phosphatase for the deactivation of protein kinases (Millward et al., 1999), and inhibition of PP2A blocks the decay of epidermal growth factor-stimulated MAPK activity (Flury et al., 1997). PP2A activity is higher in estrogen-dependent, compared with estrogen-independent, breast cancer cell lines. Furthermore, it is induced by estrogens in a manner that is blocked by antiestrogens (Gopalakrishna et al., 1999). These effects are most consistent with the endocrine control of PP2A activity being required to regulate mitogenic signaling [e.g., to prevent an excessive or prolonged activation of MAPKs (Fig. 4)]. Since PP2A expression is lower in ER-negative cells (Gopalakrishna et al., 1999), estrogen-independent growth and/or an antiestrogen-resistant phenotype might require lower PP2A levels.

D. Regulation of Gene Networks by Receptor Cross-Talk: Mitogen-Activated Protein Kinase Activation and Estrogen Receptor Function

Inhibition of breast cancer cell proliferation by either antiestrogens or estrogen withdrawal produces cell cycle arrest in G0/G1. Cells that are resistant to these endocrine manipulations are no longer subject to the late G1 restriction, a cell cycle check point that can be overcome by estrogens and/or several mitogenic growth factors alone or in combination. These growth factors can produce estrogenic effects in ER-positive cells in the absence of estrogenic stimuli (Bunone et al., 1996; Curtis et al., 1996; El Tanani and Green, 1996). Thus, signaling from growth factor receptors may play a critical role in regulating the proliferative response of some breast cancer cells to estrogens and antiestrogens. Perhaps the most widely studied signal cascade is the ability of growth factor receptor tyrosine kinases to activate MAPKs (Fig. 2).

MAPK activity is induced downstream of the receptor in an epidermal growth factor-receptor (EGF-R) signaling pathway (Tari et al., 1999; Xing and Imagawa, 1999). Blockade of MAPK activation can reduce EGF-induced mitogenesis (Reddy et al., 1999). The estrogenic effects of EGF are lost in ERα knockout mice (Curtis et al., 1996), suggesting that ERα but not ERβ is required. EGF-stimulated cell proliferation, in the absence of estrogen, is inhibited by TAM (Vignon et al., 1987). ICI 182,780 can block the abilities of EGF and TGF-α to increase expression of the otherwise estrogen-regulated pS2 mRNA (El-Tanani and Green, 1997).

The ability of EGF to induce estrogenic effects is dependent on the AF-1 (ligand independent), but not AF-2 domain of ERα, and is closely associated with EGF’s
activation of MAPK and ultimate alteration of the ER’s phosphorylation state (Bunone et al., 1996; El-Tanani and Green, 1998). ER phosphorylation occurs on both Ser118 (Bunone et al., 1996) and, as a consequence of pp90rsk1 (ribosomal S6 kinase), on Ser167 (Joel et al., 1998a), consistent with the abilities of EGF to induce ERK1,2 in breast cancer cells (Xing and Imagawa, 1999). As with Ser118, phosphorylation of Ser167 is associated with ER’s transcriptional activation (Castano et al., 1997). Whereas EGF partially reverses the growth inhibitory effects of antiestrogens (Koga and Sutherland, 1987), the mechanism(s) producing EGF’s and TGF-α’s mitogenic effects in breast cancer cells are not identical to that of estrogen (Novak-Hofer et al., 1987).

Activation of MAPK can phosphorylate ER on Ser118, a phosphorylation that is required for activation of ER’s AF-1 (Kato et al., 1995). The extent to which such cross-talk occurs is difficult to assess because others have reported Ser118 phosphorylation independent of ERK1,2 (Joel et al., 1998b). It seems likely that MAPK is not the only kinase capable of phosphorylating ER on this serine. However, MAPK appears important in the ability of growth factor receptor signaling to lead to ER phosphorylation, an event that may require ras (Patrone et al., 1998). Furthermore, when MAPK does phosphorylate this residue, it produces a sufficiently active conformation to initiate transcription (Kato et al., 1995). Thus, external stimuli that signal to an activation of MAPK, or that phosphorylate ER at Ser118 through their activation of other kinases, could produce a ligand-independent activation of ER-mediated transcription. Growth factor cross-talk with the ER will occur when these intracellular signals are initiated by their receptor tyrosine kinases (see Fig. 6).

Several other intracellular messenger systems can affect MAPK activation and ER function. For example, the intracellular concentration of cAMP affects MAPK activity (Qian et al., 1995; D’Angelo et al., 1997) and may determine isof orm specificity in signaling to mitogenesis (Schaffer and Weber, 1999). The transcriptional activities of ER are also affected by cAMP (Aronica and Katzenellenbogen, 1993; Ince et al., 1994), an effect that may be primarily confined to the ligand-dependent AF-2 transactivation domain (El-Tanani and Green, 1998). Estradiol and TAM can increase cAMP levels in some cells (Ince et al., 1994; Picotto et al., 1996), although compounds that increase intracellular cAMP levels are generally growth inhibitory toward breast cancer cells (Fontana et al., 1987). The ability of estrogens to increase cAMP levels seems to be primarily nongenomic in several systems (Farhat et al., 1996; Gu et al., 1999). ER is an estrogen-regulated gene (Saceda et al., 1988), and cAMP produces a biphasic effect on ER mRNA expression (Ree et al., 1990). Together, these observations implicate changes in cAMP occurring in response to estrogens/antiestrogens. The consequences potentially include cAMP-driven perturbations in ER function and the expression of ER-specific estrogen-regulated genes. If these are primarily restricted to AF-2 activities, antiestrogen resistance could accompany changes in the cAMP/ER interactions that eliminate TAM’s antiproliferative signals and/or cAMP-mediated changes in the function of a TAM/ER complex.

E. Mitogen-Activated Protein Kinases in Mediating the Effects of Estrogens and Conferring Antiestrogen Resistance

Many estrogen-regulated growth factors, including members of the EGF, FGF, IGF, and TGF-β families, activate tyrosine kinase receptors that are directly linked to activation of MAPK signaling. Consequently, activation of one or more of the MAPK signaling modules (Fig. 2) could provide a common integration point for signaling from both ER and growth factor receptors. Since MAPK can activate ER (Kato et al., 1995), a possible perpetual cycle between ligand independently activated ER and growth factor signaling could arise (see Fig. 6). Some of the inhibitory effects of antiestrogens could be derived from their abilities to either disrupt, or redirect, the downstream signaling from this MAPK-centered cycle.

Whether ligand-independent activation of ER AF-1 functions contribute to antiestrogen resistance is unknown. This activation does not produce a fully estrogenic response, in that not all estrogen-regulated genes are induced (Clarke and Brünnner, 1996). This “weaker” estrogenicity may reflect the effects of ligand activation on the association of coregulators with ER (Parker, 1998). Estrogen-independent growth can be induced in breast cancer cells by selection either in vitro or in vivo in a low estrogen environment (Katzenellenbogen et al., 1987; Clarke et al., 1989). It seems likely that this estrogen independence is associated with increased MAPK activity in some cells (Shim et al., 2000). However, many estrogen-independent cells retain a fully antiestrogen-responsive phenotype (Katzenellenbogen et al., 1987; Clarke et al., 1989; Brünnner et al., 1993a) and TAM can inhibit MAPK activation (Luo et al., 1997). In most experimental systems where ligand-independent ER activation occurs, antiestrogens block this activity. This is not surprising for the steroidal antiestrogens, since a major consequence of their interaction with ER is to down-regulate ERα expression. The ability of antiestrogens to block growth factor-induced mitogenesis is also predictable because ER expression appears essential for EGF to induce its estrogenic effects (Fig. 5). However, the ability of some growth factors to induce mitogenic signals through MAPK modules, in a manner independent of ER/antiestrogen signaling, could contribute to antiestrogen resistance. This might explain how some growth factors overcome the antiproliferative effects of antiestrogens.

Events apparently regulated by MAPKs are reversed/ prevented by antiestrogens in some, but not all, studies.
FGFs inhibit MCF-7 cell proliferation despite activation of MAPK (Johnson et al., 1998; Liu et al., 1998) and the potential for a ligand-independent activation of ER with a consequent induction of ER-mediated transcription (Kato et al., 1995). However, FGF overexpressing cells do not increase transcription of an ERE-reporter construct (McLeskey et al., 1998). Similar evidence is apparent from studies of TGF-β signaling. TGF-β secretion is induced by antiestrogens, producing a potentially inhibitory autocrine loop (Clarke et al., 1992b). Generally, treatment with exogenous TGF-β inhibits breast cancer cell proliferation (Knabbe et al., 1987), but activates MAPK (Frey and Mulder, 1997a,b; Visser and Themmen, 1998). The apoptosis-inducing effects of TGF-β cannot be blocked by activation of the ras/MAPK pathway (Chen et al., 1998). Melatonin also inhibits MCF-7 cell proliferation, although it can cooperate with EGF to activate MAPK, phosphorylate ER, and activate ER’s transcriptional regulatory functions (Ram et al., 1998).

Overexpression of a constitutive raf-1 kinase or activated c-erbB2 would be expected to activate MAPK. However, these transfectants significantly down-regulate ER expression. Thus, high levels of MAPK activation may be sufficient to fully produce estrogen-independent and antiestrogen resistant growth (Liu et al., 1995; El-Ashry et al., 1997). Whether activation of MAPKs produce a ligand-independent activation of ER or down-regulate ER expression, may be related to the level of MAPK activation and/or the MAPKs activated.

These observations suggest that the activation of MAPK alone is not sufficient to determine/predict the full nature of the cellular response to estrogens or antiestrogens. A necessary, but not sufficient, role for MAPK activation in signaling to mitogenesis could include its ability to phosphorylate/activate ER (Fig. 6). However, the direction/outcome of other downstream signaling also appears critical (i.e., cellular context). Unfortunately, cellular context is highly plastic and readily affected by many external signals (e.g., autocrine, paracrine, endocrine, and immunologic). Modifications in adjacent stromal populations and the tumor matrix are also likely to affect signaling within the tumor cells (Clarke et al., 1992b; Ronnov-Jessen et al., 1996; Cunha, 1999). These observations raise the possibility that individual cells or subpopulations within a single tumor may respond differently under various conditions. Thus, cells may exhibit cyclic changes in their responses to antiestrogens, perhaps reverting to responsiveness after a period of resistance.

Measuring the activity of ER, MAPK, or any other protein in isolation, as a means to assess its contribution to antiestrogen responsiveness or resistance, may be suboptimal. For example, measuring a combination of ER and PgR fails to predict response in approximately 30% of breast cancers that express these proteins. For MAPK studies, the situation may be complicated by the association of its activation with such divergent processes as initiation of mitogenesis, cell death, differentiation, activation of proto-oncogene expression (Hafner et al., 1996; Bornfeldt et al., 1997; Johnson et al., 1998) and both activation and repression of ER function (Kato et al., 1995; El-Ashry et al., 1997). The importance of cellular context to ER function (Clarke and Brunner, 1996) and MAPK signaling (Cobb and Goldsmith, 1995; Day et al., 1999b; Schaffer and Weber, 1999) are now becoming more clear. One of the challenges in the future will be to better understand the regulation of cellular context and how this can be manipulated to affect signaling through the ER and MAPKs. An understanding of cellular context and how this can be manipulated to affect signaling through the ER and MAPKs. An understanding
of these interactions may lead to novel approaches for the modification of responsiveness and resistance to antiestrogens.

**F. Estrogen Receptor Signaling through AP-1 and Antiestrogen Resistance**

AP-1 is a transcription complex comprising either c-*jun* homodimers, c-*jun/c-fos* heterodimers, or heterodimers among other members of these families (Angel and Karin, 1991). Expression and activation of AP-1 are regulated by many extracellular signals, including those initiated by growth factors and steroid hormones, and in response to oxidative stress (Schultze-Osthoff et al., 1995; Xanthoudakis and Curran, 1996). Intracellular signals that result in the activation of AP-1 include those initiated by PKC, cAMP, calmodulin kinase (Angel and Karin, 1991), MAPK, and Janus kinases (Karin, 1995). However, the consequences of AP-1 activation appear cell context-dependent. AP-1 is induced by TGF-β in cells that are growth inhibited or stimulated by this growth factor (Angel and Karin, 1991). AP-1 expression has also been implicated in the induction of programmed cell death (Colotta et al., 1992; Smyeene et al., 1993). These differential responses to AP-1 activation likely reflect, at least partly, the composition of the AP-1 complex and other differences in cellular context.

We will consider three interactions between AP-1 and steroid hormone receptors. First, we described the ability of estrogens to regulate the expression of AP-1 components. This may affect AP-1 function by influencing composition of the AP-1 complex (e.g., altering the relative abundance of specific members of c-*jun/c-fos* family members). Second, we will consider the effects of AP-1 activation on ER expression/function. Finally, we will discuss recent evidence suggesting that ER can signal through direct ER/AP-1 interactions to affect transcriptional regulation regulated by AP-1 response elements. Data clearly demonstrate the ability of estrogens to up-regulate expression of c-*jun/c-fos* family members (Chiappetta et al., 1992). In ERβ-transduced Chinese hamster ovary cells, estradiol induces c-*jun* N-terminal kinase activity. This activity is inhibited when cells are transduced with ERα (Razandi et al., 1999). The c-*fos* protein is readily detected in breast tumors, but its role is unclear. Some investigators describe estradiol activation of AP-1-mediated transcriptional events in breast cancer cells (Chen et al., 1996). Antisense-mediated inhibition of c-*fos* expression can inhibit MCF-7 tumorigenicity (Arteaga and Holt, 1996). Since MCF-7 growth in nude mice requires estrogenic supplementation (Clarke et al., 1989), inhibition of c-*fos* may block estradiol-ER signaling in vivo. TAM can activate an ER/AP-1 pathway in uterine cells, which are generally growth stimulated by the antiestrogen. In MCF-WES cells, TAM-stimulated growth is associated with increased AP-1 activity (Dumont et al., 1996). However, van der Burg et al. (1995) found AP-1 activity to be significantly reduced after 1 to 4 days of TAM treatment, and Webb et al. (1995) found no AP-1 regulation by TAM. These data suggest that not all MCF-7 cells may respond to TAM by affecting AP-1 expression/activity.

An enhancer element in the ER promoter has been described that requires AP-1 and might be expected to increase ER transcription (Tang et al., 1997). However, several ER-negative cell lines exhibit higher levels of AP-1/DNA binding than MCF-7 cells (van der Burg et al., 1995). Activation of AP-1 results in a down-regulation of ER expression (Martin et al., 1995), and might be expected to antagonize ER function and produce antiestrogen resistance. These latter observations may partly explain the associations of an up-regulation of AP-1, a down-regulation of ER, and the TAM-stimulated, but ICI 182,780, cross-resistant phenotype of the MCF-WES cells (Dumont et al., 1996). Overexpression of c-*jun* or c-*fos*, but not c-*jun*-D, inhibits ER activity in MCF-7 cells (Doucas et al., 1991). Consistent with these observations is the ability of transfection with c-*jun* to down-regulate ER, producing the consequent TAM-resistant phenotype (Smith et al., 1999).

Steroid hormone receptors can directly interact with AP-1 and affect its function (Pont et al., 1992; for reviews, see Pfahl, 1993). The consequences of these interactions are strongly receptor, promoter, and cell-type specific (Shemshedini et al., 1991). The most widely reported interaction is the ability of the glucocorticoid receptor (GR) to antagonize the activities of AP-1. This appears to be the result of GR/AP-1 protein-protein interactions (Pfahl, 1993). AP-1/ER interactions also occur. The model described for the ER/AP-1 interactions (Webb et al., 1995), in which AP-1 is bound to both its response element and ER protein, is equivalent to those previously proposed by both Pfahl (1993) and Miner et al. (1991) to explain the GR/AP-1 interactions. The transcriptional response from an ER/AP-1 complex is dependent on the ER and its ligand. Estradiol induces transcription through AP-1/ERα, but inhibits transcription through AP-1/ERβ. In general, ligands elicit opposing effects through AP-1/ERβ, compared with AP-1/ERα (Paech et al., 1997).

These studies were performed using promoter/reporter constructs, and AP-1 activity is known to be highly context sensitive (Angel and Karin, 1991; Shemshedini et al., 1991). It remains unclear how many endogenous promoters are estrogen-regulated through this mechanism. ICI 164,384 is as potent a transcription inducer through AP-1/ERβ in Ishikawa cells (endometrial carcinoma) as are both TAM and Raloxifene (Paech et al., 1997). However, only TAM is believed to have a significant mitogenic effect in the endometrium. In one study, TAM could not active AP-1 in breast cancer cells (Webb et al., 1995), despite other evidence of a TAM-stimulated phenotype associated with increased AP-1 expression (Dumont et al., 1996). Nonetheless, the apparently estrogenic effects of ICI 182,780 on mouse...
mammary gland development (Hilakivi-Clarke et al., 1997) and KPL-1 human breast cancer cell proliferation in vivo (Kurebayashi et al., 1998) might reflect activation of genes through an ERα/AP-1 interaction.

One problem in evaluating the role of AP-1 in antiestrogen resistance is that, in many cell systems, AP-1 protein expression and DNA binding activity are poor predictors of its transcriptional activity. For example, phorbol esters can increase AP-1 binding, but not transactivation of AP-1/reporter constructs in ER-negative cell lines (van der Burg et al., 1995). Thus, directly establishing the functional relevance of altered AP-1 expression/DNA binding in patients' tumors is difficult. One study could not correlate c-fos expression with either proliferation or differentiation (Walker and Cowl, 1991), whereas another found a significant association with proliferation, but not differentiation (Gee et al., 1995). A more recent study by the latter group reports reduced fos expression in the tumors of TAM responders and increased expression in proliferating and de novo-resistant tumors (Gee et al., 1999).

A borderline association (p = 0.09) of higher phosphorylated c-jun expression is seen in patients with ER-positive tumors that exhibited progressive disease versus CR+PR+stable disease (Gee et al., 2000). The duration of responses is significantly shorter in tumors with high c-jun expression, but no association with the expression of known estrogen-regulated genes is observed. Thus, the association does not seem to be related to ER-mediated events (Walker and Cowl, 1991), whereas another found a significant association with proliferation, but not differentiation (Gee et al., 1995). In another study, AP-1 DNA binding activity correlated with acquired TAM resistance in ER-positive tumors (Johnston et al., 1999). In neither study was it clear that this association reflected transcriptionally active AP-1, although the studies measured active (Ser^{63} phosphorylated) c-jun. These studies also did not clearly exclude the possibility that the associations identified reflect the high incidence of metastatic disease from tumors with high AP-1 activity (Gee et al., 2000). Other phosphorylation sites on c-jun can inhibit its activity and could be concurrently present with phosphorylation of the Ser^{63} site (Gee et al., 2000). Jun-jun homodimers may be the prevalent AP-1 complex in breast tumors, and these are 25-fold less active in regulating transcription (Gee et al., 2000).

Although certainly encouraging, further studies are clearly warranted to better define the role of AP-1 in TAM responsiveness/resistance. Some observations are potentially confounded by the importance of cell context on outcome, and the often poor abilities of AP-1’s protein expression and DNA binding activities to consistently reflect its transcriptional regulatory effects. In future studies, it will be important to establish that any altered AP-1 expression/DNA binding is reflecting altered transcriptional activity. Perhaps it will be necessary to correlate changes in AP-1 expression/DNA binding with the regulation of several downstream target genes and response to antiestrogens. However, it is unclear which targets are appropriate, since many target genes can be regulated by factors independently of AP-1. Adjusting for the possibility that tumors with high AP-1 activity can be more aggressive, also may be necessary.

AP-1 is an important molecule in signaling to both proliferation and apoptosis, and it is likely that perturbations in its gene regulation activities may explain some antiestrogen resistant phenotypes. One possible mechanism is through AP-1’s inhibition of ER expression (Doucas et al., 1991; Martin et al., 1995). However, several other mechanisms also can reduce/eliminate ER expression, including growth factors (Stoica et al., 2000) and methylation of the ER gene (Ferguson et al., 1995; Iwase et al., 1999). Conversion to ER negativity is not a particularly common form of acquired resistance (Johnston et al., 1995). Nonetheless, lack of ER expression is clearly a major de novo resistance mechanism. Perhaps the most important contribution of AP-1 is as one of the mechanisms that either initiate and/or maintain the de novo, ER-negative, resistance phenotype. A possible contribution to resistance in some ER-positive tumors also seems likely but remains to be established.

G. Signaling to Mitogenesis or Apoptosis in Antiestrogen Resistance

The consequences of affecting ER signaling in responsive cells is to alter the cell’s choice to proliferate, differentiate, or die. The survival benefit some patients derive from antiestrogens implies that, in some cells, these drugs are cytotoxic. Whereas antiestrogens certainly reduce the rate of proliferation (cytostasis), it is likely that their cytotoxicity is at least partly a consequence of an increased rate of apoptosis (Zhang et al., 1999). Thus, altered signaling to apoptosis is one potential mechanism of resistance.

Proving cause and effect is often difficult. For example, cells that are resistant to the induction of apoptosis may already have changed the regulation of key effector molecules in the apoptotic signaling cascade. This may be a direct effect on specific genes in the cascade or altered signaling that ultimately could initiate the cascade at any one of several points. Since additional responses to other endocrine and cytotoxic therapies are common, a total loss of apoptotic signaling is most unlikely. Rather, cells seem to have considerable plasticity in adapting to selective pressures, and there is some redundancy in apoptotic signaling.

Several studies have focused on alterations in signaling through the bcl-2 family. TAM can down-regulate bcl-2, but not bax, bcl-XL, or p53 (Zhang et al., 1999). The down-regulation of bcl-2 appears to reflect the relative potency of antiestrogens (Diel et al., 1999) and may be mediated through multiple enhancer elements in the bcl-2 promoter. Direct binding of ER is not required (Dong et al., 1999). It might be expected that downregulation of bcl-2’s antiapoptotic activities would be
associated with response to TAM. However, several studies have reported that a down-regulation or loss of bcl-2 expression is associated with a poor response to TAM (Gasparini et al., 1995; Silvestrini et al., 1996; Daidone et al., 2000). This somewhat unexpected association may more closely reflect the ability of TAM (Gasparini et al., 1995; Silvestrini et al., 1996; bcl-2 levels are reduced in responders, but not nonresponders. The changes in bcl-2 levels also are associated with changes in apoptotic index (Cameron et al., 2000).

The clinical studies with p53 and bcl-2 demonstrate some of the difficulties in clearly attributing clinical observations to biological function and cell signaling. Nonetheless, it seems likely that several forms of antiestrogen resistance are closely linked to the altered regulation of the gene networks that control signaling to proliferation, differentiation and apoptosis. Precisely which networks are involved may well be first identified using experimental models.

VII. Growth Factors as Mediators of Antiestrogen Resistance

A. Gene Networks: Growth Factors, Their Receptors, and Cellular Signaling

The role of growth factors in the biology of the normal and neoplastic breast has been widely reviewed (Clarke et al., 1992b; Dickson and Lippman, 1995). Thus, this text will focus primarily on the potential role for growth factors in affecting ER function and as candidate components in a broad ER-regulated gene network associated with estrogen responsiveness and antiestrogen resistance.

De Larco and Todaro (1978) initially suggested that some tumor cells may produce the factors they require for continued proliferation. These factors could subsequently function in an autostimulatory or “autocrine” manner. Thus, cells would secrete ligands that then bind to their receptors on the surface of the same cell from which they were secreted. Internal autocrine stimulation may also result from ligand-receptor interactions that occur intracellularly, perhaps at the endoplasmic reticulum-Golgi complexes or within secretory vesicles (Browder et al., 1989).

Expression of several growth factors and their receptors is regulated by estrogens (Clarke et al., 1992b). These are prime candidates for inclusion in a key ER-driven gene network. Estrogen-dependent breast cancer cells might be expected to secrete increased levels of mitogenic growth factors, and lower levels of inhibitory growth factors, in response to estrogenic stimuli (Lippman et al., 1986). Furthermore, additional cross-talk may arise from the ability of signaling downstream of growth factor receptors to influence ER activation [e.g., through changes in MAPK activity (Kato et al., 1995)]. Antiestrogens should increase the production of inhibitory factors, concurrently decreasing the production of mitogens. Antiestrogen-resistant cells would be expected to produce an estrogenic pattern of gene expression, with its regulation perhaps uncoupled from antiestrogen signaling from the ER. However, estrogen signaling pathways from the ER could remain intact in resistant cells.

B. Epidermal Growth Factor, Transforming Growth Factor-α, and Other Family Members

The EGF family of proteins contains several structurally and functionally related molecules, including EGF, TGF-α, amphiregulin, and cripto. All four can bind EGF-R, are coexpressed with this receptor (LeJeune et al., 1993; Ma et al., 1998; Niemeyer et al., 1998), and are implicated in the control of normal breast development and in the maintenance of malignant phenotype (Clarke et al., 1989a; Niemeyer et al., 1998). TGF-α seems important in the formation of the terminal-end bud structures in rodent mammary glands (Hilakivi-Clarke et al., 1997; Tsunoda et al., 1997), where it can mimic some of the effects induced by estradiol (Hilakivi-Clarke et al., 1997). TGF-α transgenic mice develop mammary adenomas and adenocarcinomas (Matsui et al., 1990).

TGF-α secretion is induced by estradiol in most estrogen-dependent human breast cancer cell lines (Bates et al., 1988). TGF-α is constitutively expressed in many estrogen-independent cells (Perroteau et al., 1986; Bates et al., 1988), and EGF can induce the estrogen-dependent MCF-7 human breast cancer cells to form small transient tumors in ovariectomized nude mice (Dickson et al., 1987). Similarly, administration of EGF to castrate female mice produces estrogenic effects in the normal uterus (Ignar-Trowbridge et al., 1992). EGF-stimulated cell proliferation, in the absence of estrogen, is inhibited by TAM (Vignon et al., 1987). EGF, TGF-α, and IGF-I increase pS2 mRNA expression, which can be blocked by ICI 182,780 (El-Tanani and Green, 1997) and partially reverse the growth inhibitory effects of antiestrogens (Koga and Sutherland, 1987). Antisense TGF-α sequences reduce the estrogenic response in MCF-7, ZR-75–1 (Kenney et al., 1993), and T47D cells (Reddy et al., 1994). Together, these data are consistent with a contribution of EGF family members to estrogenic signaling and imply an ability of growth factors to initiate estrogenic signaling in the absence of estrogens. One possible pathway is through activation of MAPK activity (Fig. 5), which appears to be downstream of the receptor in an EGF-R signaling pathway (Tari et al., 1999; Xing and Imagawa, 1999).
To more directly address the role of TGF-α in estrogen independence and antiestrogen resistance, MCF-7 cells were transfected with the TGF-α cDNA. Transfectants secrete sufficient TGF-α to down-regulate EGF-R, but retain a fully estrogen-dependent and antiestrogen-responsive phenotype (Clarke et al., 1989a). These data suggest that the estrogenic regulation of TGF-α may be necessary, but is not sufficient, to produce a full estrogenic response in some estrogen-dependent cells. This interpretation is consistent with the observations that estradiol and EGF interact synergistically in stimulating the proliferation of human breast epithelial cells in primary culture (Gabelman and Emerman, 1992), that the effects of TGF-α in the mammary gland are similar but not identical to those induced by estradiol (Hilakivi-Clarke et al., 1997), and that blockade of either ligand (Kenney et al., 1993) or receptor (Arteaga et al., 1988) is not sufficient to consistently and fully eliminate the estrogen-induced growth of estrogen-dependent cells in vitro.

C. Epidermal Growth Factor-Receptor and c-erb-B2

Although the effects of the EGF family of ligands are mediated by their receptors, studies of the receptors alone have also shown association with both response and resistance to antiestrogens. EGF-R and c-erb-B2 are estrogen regulated, and both are implicated in morphogenesis of the mammary ducts during development. This role appears to involve EGF-R heterodimerization with c-erb-B2 in the mammary stroma (Sebastian et al., 1998). In neoplastic cells, estrogen produces opposing effects on the regulation of EGF-R and c-erb-B2 expression. EGF-R expression is induced (Yarden et al., 1996), whereas c-erb-B2 expression is inhibited (Dati et al., 1990).

In addition to its ligands, the EGF-R also is hormone regulated. Both estrogens and progestins increase EGF-R expression in hormone-responsive tissues (Leake et al., 1988; Lingham et al., 1988). Estrogen-independent breast cancer cell lines express high levels of EGF-R relative to hormone-dependent cells (Fitzpatrick et al., 1984; Davidson et al., 1987). Antisense to EGF-R reduces the tumorigenicity of three breast tumor models (Ma et al., 1998). Since estrogens increase the levels of both secreted ligand and receptor in breast cancer cells, the contribution of any estrogenic signaling mediated by EGF-R may only be sufficient where there are adequate levels of both EGF-R and its ligand(s).

A consistent inverse relationship between ER and EGF-R expression has been widely reported in breast cancer cell lines and tumors. Primary breast tumors that have either low ER content, or lost the ability to express ER, frequently express high levels of EGF-R (Davidson et al., 1987; Cattoretti et al., 1988). This partly explains the association of high EGF-R expression and poor response to TAM. However, there is some evidence that a poor response rate to TAM is seen in ER-positive tumors that also express EGF-R (Nicholson et al., 1994).

c-erb-B2 is a member of the EGF-R gene family, but no specific ligand has been identified. Signaling from c-erb-B2 may be a consequence of heterodimerization with other liganded members of the family (Chang et al., 1997). Amplification of the c-erb-B2 gene is detected in approximately 25% of human breast tumors (Revillion et al., 1998). High levels of protein may be expressed in up to 70% of tumors with an amplified gene (de Cremoux et al., 1999). However, active signaling by this receptor, as determined by the use of an activation-state specific monoclonal antibody, may only occur in one-third of invasive tumors that overexpress c-erb-B2 (DiGiovanni et al., 1996). In univariate analyses, c-erb-B2 expression is associated with a more aggressive phenotype, a high rate of cellular proliferation, ER negativity and worse histological grade, nuclear grade, and prognosis. Its prognostic significance is less clear in multivariate analyses because of c-erb-B2’s association with several other strong prognostic indicators (see Revillion et al., 1998, for a recent review).

In vitro, antiestrogen-responsive cells transfected with the c-erb-B2 gene exhibit estrogen-independent growth and reduced responsiveness to TAM (Benz et al., 1993; Liu et al., 1995; Pietras et al., 1995). This effect may be related to the ability of c-erb-B2 to up-regulate Bel-2 and Bel-XL, and suppress TAM-induced apoptosis in MCF-7 cells (Kumar et al., 1996). Addition of a c-erb-B2 blocking antibody increases the antiproliferative effects of TAM in BT474 human breast cancer cells (Witters et al., 1997). Paradoxically, TAM increases (Antoniotti et al., 1992) and estrogens decrease (Dati et al., 1990) c-erb-B2 expression, despite this gene’s expression being associated with a poor prognosis and increased proliferation (Revillion et al., 1998). These effects might be expected to reduce TAM’s antiproliferative activity. In transfection studies, down-regulation of ER expression, which would be expected to confer some degree of antiestrogen resistance, is seen inconsistently. Reduced ER expression occurs in some c-erb-B2 transfectants (Pietras et al., 1995), not in others (Benz et al., 1993), and both increases and decreases in ER expression have been described in different clones from the same transfection (Liu et al., 1995).

Although data from in vitro studies provide some evidence for an association of c-erb-B2 expression and resistance to TAM, the levels of overexpression in transfectants are generally higher than that seen in patients' tumors. Data from clinical studies provide a less clear indication of the putative role of c-erb-B2 in conferring antiestrogen resistance. Several studies suggest a poorer response rate to TAM in patients with c-erb-B2 expressing tumors (Wright et al., 1992; Borg et al., 1994; Car lamagnolo et al., 1996; Yamauchi et al., 1997). However, other studies have not confirmed this association (Archer et al., 1995; Elledge et al., 1998). Since ER-
negative tumors exhibit little response to TAM but are more frequently c-erb-B2 positive, a major problem with many of these studies is the small number of c-erb-B2-positive/ER-positive tumors. In one of the largest studies of ER-positive tumors (Elledge et al., 1998), no significant association between c-erb-B2 positivity and either TAM response rate, time to treatment failure, or survival was found. Furthermore, when (Newby et al., 1997) c-erb-B2 expression was measured before TAM treatment and at recurrence, they found no change in c-erb-B2 expression, regardless of whether the tumors responded or were resistant. Overall, current data are inconclusive, providing little in the way of compelling evidence of a strong association of c-erb-B2 expression and TAM resistance.

D. Transforming Growth Factor-β Family

There has been considerable interest in the possible role of the TGF-βs in antiestrogen responsiveness and resistance since the first report of the ability of estrogens and antiestrogens to differentially regulate TGF-β secretion in breast cancer cells (Knabbe et al., 1987). Both 4-hydroxyTAM and ICI 182,780 increase the secretion of TGF-β2 by human breast cancer cells (Koli et al., 1997; Muller et al., 1998). In one small study, 11 of 15 breast tumors responding to TAM exhibited increased TGF-β2 mRNA expression (MacCallum et al., 1996). Serum TGF-β2 levels are also higher in TAM responders (Kopp et al., 1995). Although some cells exhibit resistance to both TAM and TGF-β (Herman and Katzenellenbogen, 1996), several MCF-7 cell lines that are resistant to TGF-β are not resistant to antiestrogens (Kalkhoven et al., 1996; Koli et al., 1997). Cells that are resistant to TAM often overexpress TGF-β (Herman and Katzenellenbogen, 1996; Arteaga et al., 1999), but their antiestrogen responsiveness cannot be restored in vitro by inhibiting TGF-β function with blocking antibodies (Arteaga et al., 1999). In responsive cells, the growth inhibitory effects of antiestrogens are not consistently blocked by the addition of anti-TGF-β antibodies (Koli et al., 1997).

In patients who do not respond to TAM, TGF-β2 levels increase before clinical evidence of disease progression (Kopp et al., 1995). This implies that the tumor cells have become resistant to any possible growth inhibitory effects of TGF-β2 and may even obtain an advantage from this increased expression. Overexpression of TGF-β2 can suppress natural killer (NK) cell function. Inhibition of TGF-β2 activity restores both NK cell function and response to TAM in vivo (Arteaga et al., 1999). Thus, some of the effects of TGF-β may be immunologic.

Clearly, the involvement of TGF-β2 in antiestrogen-mediated signaling is complex. The ability of TGF-β to inhibit the proliferation of some breast cancer cells, and to be induced by antiestrogens but inhibited by estrogens, suggests that some breast tumors may initially respond through an autocrine inhibitory pathway. This may occur early in treatment, consistent with the increased tumor TGF-β mRNA expression and TGF-β2 serum levels seen in some responders. If this is a direct autocrine effect on the cancer cells, any reduced immunosurveillance would have little effect. However, once the tumor cells become resistant to TAM/TGF-β, the TGF-β2-induced immunosuppression could predominate. This changing response pattern would be consistent with the initial reduction in TGF-β2 serum levels, followed by an increase before clinically detected recurrence, seen in TAM nonresponders (Kopp et al., 1995). Other TGF-β response patterns probably also occur, because not all responding tumors exhibit increased TGF-β2 expression (MacCallum et al., 1996), and the antiestrogenic responsiveness of some cells is not directly associated with their sensitivity to TGF-β2 (Koli et al., 1997).

E. Insulin-Like Growth Factors, Their Receptors, and Binding Proteins

IGF-I is a 70 amino acid polypeptide and IGF-II a 67 amino acid polypeptide, both proteins sharing structural and functional homologies with insulin. IGF-I increases the rate of proliferation of some breast cancer cells (Furlanetto and DiCarlo, 1984; Mayal et al., 1984; Leake et al., 1988) and can induce the transient formation of estrogen-independent MCF-7 tumors in ovariectomized athymic nude mice (Dickson et al., 1987). Although some breast cancer cell lines produce an estrogen-regulated IGF-like material (Huff et al., 1988), this does not appear to be authentic IGF-I (Yee et al., 1989b). IGF-II mRNA or protein has been observed in breast cancers (Peres et al., 1987), and this can be induced by estrogen in some cells (Parisot et al., 1999). Generally, the proportion of human breast cancer cell lines and tumor cells that express IGF-I and/or IGF-II mRNA appears to be small (Travers et al., 1988; Yee et al., 1989b). In contrast, significant IGF-I and IGF-II mRNA expression is observed in the stromal components of a number of breast tumors, implying a potential paracrine role for the IGFs (Yee et al., 1989b).

Several investigators have shown that the serum levels of IGF-I are moderately reduced in patients receiving TAM (Lonning et al., 1992a; Ho et al., 1998; Pollack, 1998). This may primarily reflect an effect of TAM on hepatic IGF secretion. Nonetheless, lower serum levels, and any reduction in local stromal production, could result in lower intratumor levels of the IGFs. This would reduce the ability of these proteins to induce/maintain tumor proliferation. Some, but not all, studies report a concurrent increase in the levels of IGF-II in antiestrogen-treated patients (Helle et al., 1996b; Ho et al., 1998). Increases in either the serum and/or stromal production of mitogenic IGFs could significantly impair the action of antiestrogens and produce an apparent resistance.

Determining the precise role of the IGFs is complicated by apparently concurrent changes in the levels of
several IGF-binding proteins (IGF-BPs) and the two IGF receptors. Both IGF-I receptors (IGF-I-Rs) and IGF-II receptors (IGF-II-Rs) are expressed in breast tumors (Papa et al., 1993; Zhao et al., 1993). Of these, IGFs' activities are primarily mediated through IGF-I-Rs. The IGF-II-R is the mannose-6-phosphate receptor, which is also involved in the activation of the TGF-β (Dennis and Rifkin, 1991). There are no direct intracellular signaling consequences for ligand binding to the IGF-II-R, which is primarily an extracellularly exposed membrane protein.

In the context of antiestrogen action and resistance, most interest has focused on the IGF-I-R. Growth of the estrogen-unresponsive MDA-MB-231 human breast cancer cells, both in vivo and in vitro, is partly inhibited by an antibody that blocks ligand binding to the IGF-I-R (Rohlik et al., 1987; Arteaga and Osborne, 1989). This antibody also inhibits proliferation of a number of other human breast cancer cell lines in vitro (Arteaga and Osborne, 1989). Growth of estrogen-dependent MCF-7 cells is inhibited in vitro, but not in vivo (Rohlik et al., 1987; Arteaga et al., 1989). Several groups have shown the ability of activation of the IGF-I-R to regulate the expression of otherwise estrogen-regulated genes (Hafner et al., 1996; Lee et al., 1997). These data imply cross-talk between the IGF-I-R and ER, and are consistent with the ability of ICI 182,780 to decrease the rate of IGF-I-R transcription (Hunyh et al., 1996a), and of estrogen to induce IGF-I-R expression (van den Berg et al., 1996; Parisot et al., 1999). TAM inhibits IGF-I's ability to phosphorylate the insulin receptor substrate-1 of the IGF-I-R in some studies (Guvakova and Surmacz, 1997), but not in others (Lee et al., 1997). Nonetheless, estrogen withdrawal produces a reduction in insulin receptor substrate-1 expression in MCF-7 xenografts (Lee et al., 1999; Salerno et al., 1999). Thus, either overexpression (Salerno et al., 1999), and/or a constitutive activation of insulin receptor substrate-1, could contribute to cross-talk with ER-mediated signaling to produce antiestrogen resistance.

There are several IGF-BPs that exhibit a high affinity for both IGF-I and IGF-II and generally inhibit IGF function. Breast cancer cell lines secrete significant levels of these IGF-BPs (Yee et al., 1989a; Adamo et al., 1992). Addition of IGF-BPs to cell culture media can inhibit the mitogenic effects of IGFs in human breast cancer cells (van der Burg et al., 1990). Since breast cancer cells secrete multiple IGF-BPs (Clemmons et al., 1990), it seems likely that the cumulative effect of IGF-BP secretion is to partly antagonize the mitogenic effects of IGFs in breast cancer cell growth. Both IGF-BP-3 (Nickerson et al., 1997) and IGF-BP-5 (Hunyh et al., 1996b) are induced by ICI 182,780. IGF-BP-3 alone can induce apoptosis, perhaps by sequestering IGF-I-R ligands (Nickerson et al., 1997). TAM-resistant cells secrete lower levels of IGF-BP-2 and IGF-BP-4 (Maxwell and van den Berg, 1999). In patients, triphenylethylene therapy is associated with increased levels of IGF-BP-1 (Helle et al., 1996a; Ho et al., 1998) and IGF-BP-3 (Helle et al., 1996a). However, there is no clear association between plasma sex steroids and either IGF-I or IGF-BP-1 levels (Lonning et al., 1995).

Cumulatively, these observations are consistent with a reduction in the secretion of IGF-I and a possible increase in secretion of selected IGF-BPs, within the tumor or from other sources, as being associated with antiestrogen treatment. Antiestrogen resistance could be produced by changes in IGF-I-R signaling, either directly or through downstream interactions with ER function, by changes in systemic IGF/IGF-BP secretion, and/or by autocrine/paracrine interactions mediated by IGFs. In addition, or alternatively, cells could become resistant to the loss of IGF-induced mitogenesis by becoming more dependent on the proliferative activities of other growth factors or mitogenic signaling pathways.

VIII. Estrogen Receptor-Independent Targets for Mediating Antiestrogen Action and Resistance

Several ER-independent targets have been described for TAM. These are often called nongenomic because they do not require interaction of TAM with ER and/or do not directly affect the transcriptional regulatory activities of ER. These targets have received considerable attention, primarily in an attempt to explain the apparent clinical responses occasionally seen in some patients with ER-negative tumors. However, the nongenomic (ER-independent) activities of antiestrogens may also be important in ER-positive tumors. For example, these may be necessary, but not sufficient, to induce a growth inhibitory effect in response to antiestrogen exposure. Although an initial interaction may be independent of ER, the downstream consequences of this could affect ER expression and/or function by altering cellular context. Some ER-independent interactions have already been discussed (e.g., binding to AEBs). Other targets may involve both direct ER interactions and nongenomic effects. For example, AP-1's transcriptional activity can be directly influenced by an occupied ER (direct genomic effect), whereas AP-1 activity can also be regulated downstream of an oxidative stress and/or cytokine/growth factor signaling that regulates Jun N-terminal kinases (ER-independent; nongenomic for ER involvement). The following sections focus on the more widely studied of the ER-independent targets for TAM.

A. Oxidative Stress

The generation of an excess of reactive oxygen species has been implicated in many diseases, including cancer. The mutagenic properties of these species is primarily associated with the production of DNA strand breaks, base modification, and DNA-protein cross-linkages (Toyokuni et al., 1995). However, the generation of an oxidative stress also has significant effects on the regu-
lation of several genes (Morel and Barouki, 1999), and can, therefore, substantially alter the cellular context of affected cells. The ability of reactive oxygen species to regulate gene expression is likely multifactorial. The promoter of some genes contain an electrophile response element that is sensitive to changes in redox state. Many of these genes are associated with a potentially general stress response, encoding proteins associated with cellular detoxification [e.g., glutathione-S-transferase, quinone reductase (Montano and Katzenellenbogen, 1997)].

TAM has been widely implicated as an antioxidant, potentially consistent with its ability to influence plasma membrane structure and function (Garcia et al., 1998). However, such activities, might also initiate an antioxidant cascade (Gundimeda et al., 1996). 4-HydroxyTAM is a scavenger of peroxyl radicals in several cells and experimental systems. For example, 4-hydroxyTAM inhibits lipid peroxidation in sarcoplasmic reticulum membranes (Custudio et al., 1994) and Fe(III)-ascorbate-induced lipid peroxidation in rat liver microsomes (Wiseman, 1994). Endogenous and UV light-induced oxidative damage to DNA, protein, and lipids is inhibited by TAM in mouse epidermis (Wei et al., 1998). In human neutrophils, TAM inhibits hydrogen peroxide formation in response to treatment with triphenylethylene antiestrogen (TPA) (Lim et al., 1992). The ability of TAM and 4-hydroxyTAM to inhibit Cu^{2+}-induced peroxidation of low-density lipoprotein has been suggested to contribute to the putative cardioprotective effects of these antiestrogens (Wiseman et al., 1993a).

Paradoxically, whereas both estradiol and TAM can act as antioxidants (Garcia et al., 1998; Schor et al., 1999), there is clear evidence that TAM is associated with intracellular oxidative stress. The membrane association of PKC induced by TAM appears to reflect its ability to partition into membranes and initiate an oxidative stress. This effect is largely eliminated upon administration of antioxidant (Gundimeda et al., 1996). TAM-induced lipid peroxidation has been described in which the generation of superoxide is implicated (Duthie et al., 1995). Both TAM and 4-hydroxyTAM can induce 8-hydroxy-2'-deoxyguanosine formation in rat liver microsomes (Ye and Bodell, 1996), potentially through changes in redox cycling (Okubo et al., 1998). In marked contrast, TAM inhibited the formation of this intermediate in HeLa cells treated with TPA (Bhimani et al., 1993). More recently, TAM has been shown to induce oxidative stress in ovarian and T-leukemic cells (Ferlini et al., 1999). TAM also induces TPA-induced AP-1 activity (van der Burg et al., 1995), NFκB (Ferlini et al., 1999), quinone reductase (Montano and Katzenellenbogen, 1997), and other genes associated with oxidative stress. These data clearly suggest that, despite its antioxidant properties, some cells respond to TAM as they would to an oxidative stressor.

Why should there be this apparent contradiction in pro-oxidative versus antioxidative activities is unclear. It is possible that, like many other events, cellular context is critical in determining response. The ability of TAM and its metabolites to generate an oxidative stress is likely related, at least partly, to their intracellular metabolism to species that can generate reactive intermediates. Day et al. (1999a) compared the one-electron activation of 4-hydroxyTAM and 3-hydroxyTAM by several enzymes. Although generation of the phenoxyl radical by myeloperoxidase was weak, several other enzymes effectively generated the species. The substrate specificity of the (myelo)peroxidases determined whether a phenolic substrate generated a reactive phenoxyl radical or an antioxidant. Thus, the ability of TAM to generate either a pro-oxidant or antioxidant response may depend on the levels and activities of activating enzymes in the target cells.

Another possibility is that TAM has antioxidant properties at the cell’s surface, but acts as a pro-oxidant when metabolically activated within the cell, or when partitioned into specific membrane domains. This would appear consistent with antioxidant effects on some membrane lipids, but pro-oxidant effects on gene transcription. Although this might occur in the short term, intracellular activation could produce sufficient concentrations of reactive intermediates that even some membrane lipids and phospholipids eventually become peroxidized.

It is also possible that the oxidative stress is a result of TAM’s effects on cellular metabolism. Preliminary data from our laboratory has implicated altered cytochrome C oxidase and NFκB activity with antiestrogen resistance. These changes could reflect differences in mitochondrial function and oxidative metabolism, the consequences of which could lead to free oxygen radical production, in excess of cells’ abilities to scavenge these reactive metabolites.

B. Perturbations in Membrane Structure/Function

It is clear from their structures that most of the TPAs are relatively lipophilic and would be predicted to partition predominately into the hydrophobic domains of cellular membranes. Membrane partitioning will affect the physicochemical properties of the membrane domain(s) into which the drug partitions. This latter effect could significantly impact the function of adjacent or nearby proteins that are dependent upon the properties of their lipid environment for function (Lenaz et al., 1978). Such proteins could include growth factor receptors, membrane ER (Nelson et al., 1987; Watson et al., 1999), and other membrane-associated signaling molecules, such as G-proteins, phosphoinositides, and members of the PKC family. For example, TAM induces a selective membrane association of PKCε (Cabot et al., 1997).

TAM alters the physical attributes of breast cancer cells by decreasing membrane fluidity (Clarke et al., 1990). Fluidity was estimated by determining the
steady-state polarization of fluorescence of the probe 1,6-diphenyl-1,3,5-hexatriene, which reflects the rotational ability of the probe resulting from the molecular packing of the lipids comprising the membrane domains into which the probe is inserted. The reduced fluidity occurs regardless of ER status, as would be expected for an effect independent of ER. Similar effects have subsequently been reported in artificial membranes (Custodio et al., 1993b) and liposomes (Custodio et al., 1993a; Kayyal et al., 1994).

In breast cancer cells, these changes in membrane structure are associated with increasing cytotoxicity (Clarke et al., 1990). TAM has been reported to affect other membrane-associated events, including calcium ion influx (Morley and Whitfield, 1995), P-glycoprotein-mediated drug efflux (Leonessa et al., 1994), and membrane phospholipid metabolism (Cabot et al., 1995). Although potentially nonspecific, in terms of ER expression, there may be some degree of specificity conferred by the physicochemical characteristics of the domains into which TAM is inserted. If these domains are functionally linked to the activity of key membrane proteins, resistance could arise by cells switching to other pathways that do not require these membrane-dependent events, or by altering local membrane structure to reduce the stabilizing effects of TAM. The possibility that TAM-induced changes in membrane function are necessary, but not sufficient for its antiestrogenicity or antiproliferative effects, cannot be excluded. For example, these events might interact with specific ER-mediated signaling events that do not occur in ER-negative cells.

C. Protein Kinase C

PKC is a membrane protein that has been implicated as an important signal transduction molecule in several cellular systems. There are at least 10 isoforms that fall into one of three families. The classical family contains PKC isoforms α, β, and γ; the novel family comprises isoforms δ, ε, η, θ, and μ; and the atypical family contains isoforms ζ and λ (Datta et al., 1997). PKC is activated by the diacylglycerol produced following the hydrolysis of membrane inositol phospholipids by phospholipase C (Nishizuka, 1992; Olson et al., 1993). The hydrolytic activities of phospholipases D and A₂ may enhance this activation (Nishizuka, 1992).

Like many membrane-associated proteins, the function of PKC is probably dependent upon its lipid environment. The ability of TAM to alter the structural properties of membranes could indirectly alter PKC function. It also is apparent that TAM can bind directly to PKC (O’Brien et al., 1986, 1988). However, there is some controversy relating to whether TAM inhibits or activates PKC. TAM inhibits PKC activity with an IC₅₀ = 25 μM in studies performed on partially purified PKC (O’Brien et al., 1986). In intact cells, TAM does not inhibit PKC activity (Issandou et al., 1990), whereas others have reported PKC activation by triphenylethylenes (Bignon et al., 1991). More recent studies have shown that TAM causes both a membrane translocation and a down-regulation of the enzyme. This translocation is generally associated with PKC activation and appears to require release of arachidonic acid (Gundimeda et al., 1996). TAM can activate phospholipases C and D and translocate PKCε, but not the α, β, γ, δ, and ζ PKC isoforms, to the membrane (Lavie et al., 1998). These effects occur at concentrations similar to those affecting membrane fluidity (Clarke et al., 1990). Thus, the membrane signaling effects of TAM on PKC activation may be related to its ability to alter membrane structure/function.

Signaling from PKC is often complex and the end result can be cell specific. For example, overexpression of PKCα in MCF-7 cells has produced conflicting results. Manni et al. (1996) observed a less aggressive phenotype, whereas Ways et al. (1995) reported a more aggressive phenotype. The latter observation is more reflective of the abilities of PKC to influence attachment, motility, and invasiveness (Palmantier et al., 1996; Platel et al., 1998). The difference between these studies might be explained by the concurrent changes in expression of other PKC isoforms. Ways et al. observed increased expression of the δ- and η-isoforms, whereas their expression was not changed in the Manni et al. study.

There are several potential signaling pathways following PKC activation that could produce the responses seen in normal and neoplastic breast tissues. PKC has been implicated in mediating the mitogenic activity of the ras proto-oncogene (Lacal et al., 1987). PKC activation causes the formation of ras/raf-1 complexes, but activates ras in a manner that differs from its activation by receptor tyrosine kinases (Marais et al., 1998). Expression of p21waftcip1, which is associated with cell cycle arrest, is induced by PKC independently of p53 through a posttranscriptional mechanism (Akashi et al., 1999). In contrast, cleavage of PKCβ by caspase 3 induces apoptosis (Datta et al., 1997).

PKC activity is greater in neoplastic breast tissues when compared with normal breast (O’Brien et al., 1989). Most appear to be the Ca²⁺-dependent PKC isoforms (Gordge et al., 1995), which are more highly expressed in ER-negative tumors (Borner et al., 1987). Induction of PKC activity can inhibit ER function (Martin et al., 1995), whereas the ability of growth factors to alter ER function occurs independently of PKC (Ignar-Trowbridge et al., 1996). PKC affects ER signaling in osteoblasts (Migliaccio et al., 1993, 1998), similar to its effects in breast cancer cells (Martin et al., 1995). The consequences of PKC activation in breast cancer cells include cell cycle arrest (Seynaeve et al., 1993) and induction of prostaglandin E₂ synthesis (Boorne et al., 1998).

TAM can inhibit PKC activity following a transient activation (Gundimeda et al., 1996). If PKC activity
were rate-limiting for proliferation, any significant inhibition of its activity may be sufficient to induce a reduction in cellular proliferation. The importance of PKC in the regulation of mitogenic signals implies that, if TAM does regulate its function in vivo, this inhibition likely contributes to the overall effect on cellular proliferation. Perturbations in either the level of expression of PKC, or its sensitivity to inhibition by TAM, could contribute to acquired TAM resistance in some cells. The implications of altered PKC activation on ER function also require clarification, and these may differ among cells.

Any events related to TAM/PKC interactions could be most important in a subset of ER-positive cells. Since the effects of overexpression of PKCa appear cell-specific, additional studies are required to determine whether some isoforms are more important than others. Nevertheless, it seems likely that TAM’s ability to influence PKC activity is important in mediating the effects of antiestrogens in some breast cancer cells. Some of these effects may be mediated through the ability of PKC to activate AP-1 and/or influence ER activity at AP-1 sites.

D. Calmodulin

Estrogen can depolarize plasma membranes and initiate internal calcium signaling (Nadal et al., 1998). Calmodulin is an intracellular Ca^{2+} binding protein and an important signal transduction molecule that participates in the signaling to several endpoints in different cells (Means, 2000). A major intermediary in this signaling is the calmodulin-dependent kinase II. For example, calmodulin kinase II activates the protooncogene c-fos (Wang and Simonson, 1996), is implicated in signaling to fas-mediated apoptosis (Pan et al., 1996; Wright et al., 1997), and can affect ER-mediated signaling. Calmodulin can phosphorylate the ER protein on tyrosine (Migliaccio et al., 1984), an event that effects ligand binding (Migliaccio et al., 1989). More recently, Biswas et al. (1998) have shown that calmodulin binds directly to ER, is an integral component of an active ERE-ER complex, and is required for the formation of a productive transcription complex. Calmodulin also is involved in cyclic nucleotide metabolism. Some aspects of ER-mediated gene transcription can be regulated by cAMP (Aronica and Katzenellenbogen, 1993). Calmodulin antagonists can inhibit breast cancer cell proliferation, arresting cells in the same cell cycle phase as TAM (Musgrove et al., 1989).

TAM could indirectly influence ER function through its ability to inhibit calmodulin’s activities. A high-affinity interaction between TAM and calmodulin has been reported, with a $K_d$ value of approximately 6 nM (Lopes et al., 1990). A second, lower affinity, interaction occurs with an apparent $IC_{50}$ of 6 to 9 μM (Rowlands et al., 1995; Greenberg et al., 1987). 4-Iodination and elongation of the basic side chain length increase both the calmodulin and PKC antagonist activities of TAM (Rowlands et al., 1995).

An inhibition of calmodulin and/or calmodulin kinase II could contribute to the antiproliferative effects of antiestrogens. The extent of inhibition will be determined by the intratumor availability of TAM and its appropriate metabolites. The high-affinity TAM-calmodulin interaction occurs at concentrations well below those associated with an estrogen-reversible growth inhibition by the triphenylethylenes in vitro. These high-affinity sites should be occupied in the majority of TAM-treated tumors. A proportion of the low-affinity sites also may be occupied, since intratumor TAM concentrations in the range of their $K_i$ can be detected in human tumors. These observations raise the possibility that inhibition of calmodulin is necessary, but not sufficient for TAM’s activities. If calmodulin levels are dose-limiting for ER activation, a modest level of inhibition may be sufficient to influence ER function. It is tempting to speculate that one reason why TAM is a weak partial agonist is because it concurrently limits calmodulin’s ability to produce a fully productive ER-ERE transcription complex.

E. Comments on the Possible Role of Nongenomic Effects

Cellular context may substantially affect how a cell perceives and responds to an occupied ER protein. Thus, a major contribution of nongenomic effects may be to influence the cellular context, such that other key regulators of the antiestrogen-induced signaling network are appropriately expressed/repressed. It can readily be appreciated that this could be facilitated by perturbations in the activities of key intracellular signaling proteins such as calmodulin, PKC, or the various factors associated with the induction of an oxidative stress response. For example, cellular stress is often accompanied by changes in the expression of apoptosis modulating factors such as NFkB or AP-1. Preliminary data from our laboratory indicate that NFkB activity is significantly elevated in the antiestrogen-resistant MCF7/LCC9 cells, as are several other genes regulated by oxidative stressors.

Some of these events are likely to be regulated independently of the ER. Thus, there may be a necessary interaction between ER-mediated and nongenomic events for the full induction of an antiestrogenic response in cells expressing ER. It might be predicted that the expression of some of the nongenomic targets will be different in ER-positive cells because they are more responsive. The levels of calmodulin in breast tumors appear higher than in normal tissue (O’Brian et al., 1989), and ER-negative tumors tend to express higher levels than ER-positive cells (Borner et al., 1987). Ultimately, it should be clearly demonstrated that the concentrations at which nongenomic effects occur represent achievable intracellular TAM concentrations in tumors. Many of the nongenomic effects are observed at micro-
molar concentrations of TAM in vitro. The cell culture conditions used contain only low concentrations of serum, generally ≤10%, which may not reduce availability to the same degree as occurs in blood/tissues.

IX. Immunologic Mechanisms of Tamoxifen Resistance

The immunosuppressive activities of estrogens have been known for many years, and antiestrogenic effects on these endpoints might be expected to affect host immunity and tumorigenicity. Not surprisingly, there is considerable evidence demonstrating the ability of antiestrogens to influence many aspects of immunity. Some of these effects are likely to be ER-mediated, since expression of steroid hormone receptors is widely reported among some lymphoreticular cells. For example, peripheral blood mononuclear cells, thymus and splenic cells, and CD8+ T cells express ER (reviewed in Schguurs and Verheul, 1990). Other immunologic effects of antiestrogens may well reflect perturbations in the activities of the ER-independent targets described elsewhere in this review.

Tumors proliferating successfully in the presence of cytotoxic host cells clearly indicate that the cells have evaded cytolytic effectors. The precise mechanisms involved remain unknown, but modification or masking of surface antigens, the secretion of factors that inhibit effector function, and an altered sensitivity to the direct cytolytic effects of effector cells are probably involved (Key et al., 1982). Where antiestrogens can influence these events, they also may impact the immune status of the host and alter its response to the tumor. Thus, the immunomodulatory activities of antiestrogens have considerable potential to contribute to their mechanism(s) of action and resistance.

A. Cell-Mediated Immunity

Cell-mediated or adaptive immunity (CMI) is primarily conferred by the interactions between T lymphocytes and cells expressing the antigens they recognize. There are several key lymphoid cell populations implicated in the control of cancer, including NK and lymphokine-activated killer (LAK) cells. Both NK and LAK cells are distinct from cytotoxic T lymphocytes, lysing cells lacking significant expression of the MHC genes. NK and LAK cells can infiltrate solid tumors and malignant effusions (Blanchard et al., 1988). Macrophages, which are of myeloid lineage, also exhibit antitumor activity (Wheelock and Robinson, 1983). Changes in CMI and the infiltration of its effectors are evident in many breast tumors. A common component of the desmoplastic response to breast cancers is the infiltration of reticuloendothelial cells (Clarke et al., 1992b). The skin window procedure, which provides an estimate of the extent of CMI, correlates inversely with metastatic disease (Humphrey et al., 1980; Black et al., 1988). The functional competence of T lymphocytes is impaired in 58% of breast cancer patients, with a high proportion observed in those with lymph node involvement (Head et al., 1993).

B. Natural Killer Cells

NK cells make up approximately 1 to 2.5% of peripheral lymphocytes and have been widely demonstrated to possess antitumor activity (Wheelock and Robinson, 1983). Low levels of NK cell activity are associated with familial breast cancer (Strayer et al., 1986), with these levels also seen in patients with stage III/IV disease (Akimoto et al., 1986; An et al., 1987; Contreras and Stoliar, 1988). Some tumors can suppress NK activity (Mantovani et al., 1980), perhaps explaining why this activity is generally low or absent in the axillary lymph nodes of patients with demonstrable metastatic disease (Horst and Horny, 1987; Bonilla et al., 1988). Other tumors may become resistant to NK cell-mediated cytolyis (Arteaga et al., 1999). Since NK cell activity may contribute to the control of metastasis, the poor metastatic potential of many human xenografts growing in nude mice may reflect their elevated NK cells activities (Clarke, 1996).

Estrogens and endocrine therapies clearly affect NK cell activity. Aminoglutethimide, which reduces serum estrogen concentrations, increases NK activity in breast cancer patients (Berry et al., 1987b). In mice, estrogens induce a biphasic response on NK cell activity. An initial increase in activity is generally followed by a subsequent reduction of activity to below pretreatment/untreated levels (Seaman et al., 1978; Seaman and Talal, 1980; Hanna and Schneider, 1983; Screpani et al., 1987). TGF-α transgenic mice have lower NK cell activity, consistent with increases in their serum estrogens (Hi-lakivi-Clarke et al., 1992).

TAM stimulates NK activity both in vitro (Mandeville et al., 1984) and in vivo in rodents (Gottardis et al., 1989; Baral et al., 1995). In humans, TAM can produce estrogenic effects on lymphocyte function (Myers and Peterson, 1985). Short-term TAM treatment (1 month) increases NK activity (Berry et al., 1987a), whereas longer term treatment (1.5 to 2 years) reduces NK activity (Rotstein et al., 1988). TAM can also sensitize the target cells to lysis (Baral et al., 1995), an effect that does not appear to require ER expression (Baral et al., 1995). Long-term TAM-induced reduction in immunity, and/or changes in the susceptibility of the tumor cells to lysis, could contribute to the emergence of a TAM-stimulated phenotype by eliminating the cytolytic or inhibitory effects of tumor infiltrates.

A loss of responsiveness to TAM-induced NK cell activation could contribute to the appearance of resistance. Using the MCF7/LCC2 TAM resistance model (Brünner et al., 1993b), the potential importance of inhibiting NK cell activity as a mechanism of TAM resistance has been demonstrated. The MCF7/LCC2 cells secrete significant
amounts of the cytokine TGF-β, which can inhibit NK cell activity (Arteaga et al., 1999). TAM inhibits the growth of MCP7/LCC2 xenografts in nude mice, which have high NK cell activity (Clarke, 1996), when concurrently treated with antibodies that block TGF-β activity (Arteaga et al., 1999). These data suggest that the antitumor effects of TAM are partly conferred by increased NK cell activity and that one form of resistance is for cells to secrete growth factors or cytokines that can block this activity (Arteaga et al., 1999).

C. Macrophages

Macrophages are widely observed to infiltrate solid tumors and can kill tumor cells, perhaps recognizing some tumors on the basis of their abnormal growth (Hibbs et al., 1972) or by surface modifications (Key et al., 1982). Macrophages can produce both antigen-specific and nonspecific cytolysis. These tumoricidal properties are acquired following activation by contact with either the target cell and/or its secreted products (Fidler, 1988). Cell kill is produced both by phagocytic and nonphagocytic processes (Key et al., 1982), the latter cytolysis probably involving the release of lysosomal enzymes by exocytosis.

In some cases, macrophage infiltration is associated with tumor progression rather than inhibition, implying that macrophages may secrete factors mitogenic for tumor cells (Acero et al., 1984). One possibility is their apparent ability to produce estradiol (Mor et al., 1998), which might limit their mitogenic effects to ER-positive breast cancer cells. However, macrophages secrete many cytokines and growth factors, and focal macrophage infiltration in breast tumors is associated with increased angiogenesis and poor prognosis (Leek et al., 1999).

The effects of endocrine treatments on macrophage activity have not been widely studied. However, estrogens can significantly alter the expression of several cytokines implicated in the activation of macrophages (Hunt et al., 1998; Rogers and Eastell, 1998). TAM blocks the estrogen-induced release of the interleukin-6 soluble receptor (Singh et al., 1995), tumor necrosis factor (Zuckerman et al., 1995), and induction of JE/MCP-1 mRNA (Frazier-Jessen and Kovacs, 1995). TAM also blocks the inhibitory effects of estradiol on macrophage function (Savita and Rai, 1998) and modulates the antiproliferative signal of interferon-α on premacrophage proliferation (Balint et al., 1992). These observations are consistent with a potential role for perturbations in macrophage function in both responsiveness and resistance to TAM therapy.

D. Lymphokine-Activated Killer Cells, Cytotoxic T Cells, and Other Cell-Mediated Immunity Effector Cells

LAK cells are clearly distinct from NK cells, a determination initially derived from studies of mice bearing different immune-deficiency mutations [i.e., nu and bg (Andriole et al., 1985)]. LAK cells are capable of killing neoplastic cells and can kill tumor cells resistant to NK cytolysis (Grimm et al., 1982). Some tumors produce material capable of blocking the development of LAK cells (Ebert et al., 1990). LAK cells are often present in the axillary lymph nodes of patients with demonstrable metastatic disease (Bonilla et al., 1988). Both TAM and estradiol can increase the sensitivity of target cells to lysis by LAK cells (Albertini et al., 1992; Baral et al., 1996a). TAM and Toremifene increase the immunomodulatory effect of coadministered LAK cells both in vivo and in vitro (Baral et al., 1996b). Where such effects are lost, target cells could become resistant to cytolysis and appear TAM resistant.

Cytotoxic T cells are T lymphocytes that recognize surface antigens bound to MHC class I molecules. Binding to the T cell receptor causes the release of the effector molecules that induce lysis of the target cell. Infiltration of breast tumors (Kiriiri et al., 1998; Nguyen et al., 1999) and lymph nodes (Ito et al., 1997) by cytotoxic T cells has been clearly demonstrated. Whereas the full series of antigens recognized by these cells remains to be established, antigenic proteins with a mucin polypeptide core are clearly involved (Kiriiri et al., 1998). Cytotoxic T cells isolated from patients immunized with a synthetic MUC1 peptide exhibit class 1-restricted killing of MUC1-expressing cells (Reddish et al., 1998). Both TAM and estradiol increase the sensitivity of target cells to lysis by cytotoxic T cells (Baral et al., 1994). A combination of antiestrogens increased the cytotoxic effects of cytoktic T cells against the H2712 mouse mammary tumor (Baral et al., 1997). The proliferation of some cytoktic T cells is arrested in G1 following TAM treatment (Lyon and Watson, 1996).

Endocrine treatments also have been reported to affect less well defined mediators of CMI. For example, TAM increases TNF-α production by mononuclear cells (Teodorczyk-Injeyan et al., 1993). TAM, Toremifene, and ICI 164,384 exhibit immunosuppressive activities when their effects are measured on human mononuclear cells (Teodorczyk-Injeyan et al., 1993).

E. Humoral Immunity

Humoral immunity is conferred by the antibody-mediated response to antigens. There are cooperative interactions between humoral and CMI, since the interaction of tumor cells with CMI effectors likely alters the balance of cytokines such that the functional differentiation of CD4 T cells is affected (Janeway et al., 1997). Steroids are known to affect humoral immunity in several species (Leitner et al., 1996). For example, estrogens can increase IgM secretion (Myers and Peterson, 1985).

Generally, the ability of antiestrogens to affect specific aspects of humoral immunity are less well reported than their effects on CMI. TAM can block the effects of estrogens on an antigen-specific antibody response in vitro...
(Clerici et al., 1991) and improve the persistent proteinuria and immune complex deposition in the kidneys of mice with experimental systemic lupus erythematosus (Stoeger et al., 1994). The ability of pokeweed mitogen to induce IgG and IgM secretion is inhibited by ICI 164,384, TAM, and Toremifene (Teodorczyk-Injejyan et al., 1993). Long-term Toremifene therapy is associated with lower immunoglobulin levels, including IgA, IgM, and IgG, despite a short-term increase in the number of immunoglobulin-secreting cells (Paavonen et al., 1991a).

Antiestrogens can also inhibit the rate of DNA synthesis in peripheral blood lymphocytes (Paavonen et al., 1991b). Estrogen enhances B cell maturation (Paavonen et al., 1981), whereas a short TAM incubation reduces C3 complement receptor expression in B cells (Baral et al., 1985). A TAM-dependent platelet antibody response has been reported that may contribute to the thrombocytopenia that occurs in some patients (Candido et al., 1993).

Several proteins associated with estrogen independence and TAM resistance have recently been identified (Skaar et al., 1998). Autoantibodies to one of these proteins (nucleophosmin; NPM), which is induced by estrogens and inhibited by antiestrogens in estrogen-dependent cells, are produced in breast cancer patients. The levels of anti-NPM autoantibodies increase 6 months before recurrence (Brankin et al., 1998). The levels of other autoantibodies generally do not have substantial predictive and/or prognostic power in breast cancer (Lee et al., 1985; Ronai and Sulitzeanu, 1986). For example, autoantibodies to p53 are detected in a relatively small proportion of breast cancer patients (Schlichtholtz et al., 1992; Mudenda et al., 1994; Vojtesek et al., 1995; Regidor et al., 1996) and appear to be of little predictive/prognostic value (Regidor et al., 1996). Early studies suggesting an association between autoantibody levels and poor prognosis in breast cancer (Wasserman et al., 1975; Turnbull et al., 1978) have not subsequently been confirmed (Swissa et al., 1990).

The levels of anti-NPM autoantibodies are significantly reduced in patients that have received TAM, consistent with the antiestrogenic regulation of the antigen (Brankin et al., 1998). This suggests that monitoring anti-NPM levels could be a useful intermediate biomarker for assessing TAM responses and failures. It seems unlikely that TAM’s effects on autoantibodies reflect its ability to influence immunity. TAM does not affect the production of 16/6 idiotypic autoantibodies in experimental systemic lupus erythematosus (Stoeger et al., 1994).

X. Conclusions and Future Prospects

The precise mechanisms of resistance to antiestrogens remain to be established. Clearly, the most important mechanism driving de novo resistance is lack of ER expression, since >90% of ER-negative tumors will not respond to antiestrogens. For ER-positive tumors, it seems likely that no single mechanism predominates for either de novo or acquired resistance. Indeed, each tumor, or each subpopulation within a tumor, may utilize a different resistance mechanism (genomic and/or non-genomic). Nonetheless, some critical event(s) driving response and resistance to TAM are related to activities regulated, at least initially, through the ER signaling pathway(s). This may explain why so few ER-negative tumors respond to antiestrogens, and why a majority of initially responsive tumors acquiring resistance continue to express ER.

With the exception of pharmacokinetic or receptor mutational events, the precise contributions of which remain to be established, defects at, and/or downstream of, receptor-ligand interactions seem important. Modifications in the assembly/function of the ER-regulated transcription complex that drives different gene networks could be involved. The ability of cells to acquire an estrogen-independent phenotype without concurrently acquiring antiestrogen resistance, and the lack of a consistent cross-resistance between triphenylethylenes and steroidal antiestrogens, could reflect the differential regulation of interrelated and/or interdependent gene networks (Clarke and Brünner, 1995; Clarke and Lippman, 1996).

The biophysical events regulating these gene networks could be explained by the conformational changes induced in the ER protein when occupied by different ligands. The physical properties of the ER protein appear associated with its ability to recruit coregulator proteins and regulate reporter gene expression. These properties are dependent upon the occupying ligand and the composition of the transcription complex formed.

Resistance to one class of antiestrogens would not necessarily produce crossresistance to others if the regulated gene networks are interrelated but not interdependent. There may be several pathways that are concurrently influenced by the transcriptional activity of ER occupied by estrogen, but the end result of activation in terms of the choice to proliferate, differentiate, or die may be the same. Thus, cells could switch from one pathway to another as these are selectively blocked by the action of different receptor-ligand complexes (Clarke and Lippman, 1996).

The genes that make up the critical networks pathways involved in antiestrogen responsiveness and resistance may be identified in the next few years. The application of new molecular techniques like serial analysis of gene expression, gene microarray analyses, proteomics, and other state-of-the-art molecular techniques are proving powerful in the identification of molecular patterns associated with specific phenotypes. Already, some novel candidate genes have been identified.

One example is Bcarl/p130Cas. Identified as a putative resistance gene by insertion of a retrovirus into TAM-responsive cells, overexpression of this protein can
produce antiestrogen resistance in ZR-75–1 cells (Brinkman et al., 2000). The protein is clearly expressed in a significant proportion of breast cancers, and there is limited evidence that high levels of this expression are associated with poor response to TAM (van der Flier et al., 2000). Although more studies need to be done to further evaluate the possible contribution of Bcar1/p130Cas to clinical antiestrogen resistance, these studies provide an elegant example of one approach to identify potentially clinically useful molecular information.

The precise contribution of nongenomic effects to TAM’s inhibitory effects will probably remain controversial for the moment. A necessary but not sufficient role seems plausible, given the importance of cellular context in determining response to ER activation/inhibition. As our understanding of how antiestrogens affect the function of the ER and its signaling network, this contribution may become more apparent.

Other areas of investigation include searches for endpoints that can predict TAM responders versus nonresponders. These should provide clinically important information because useful second line endocrine and cytotoxic therapies are available for tumors that begin to fail TAM. For example, investigators are looking for serum or other intermediate biomarkers of response/resistance to endocrine therapies. In this regard, changes in the levels of pS2 and apolipoprotein D in nipple aspirate fluids from patients on TAM may have predictive value (Harding et al., 2000). Autoantibodies to the nuclear phosphoprotein NPM are significantly lower in patients who have received TAM (Brankin et al., 1998). Measuring changes in mammographic density, following initiation of TAM therapy, may also have predictive value (Atkinson et al., 1999).

Additional approaches are to find therapies that may modulate response to antiestrogens. For example, the addition of γ-linoleic acid to TAM may accelerate clinical response (Kenny et al., 2000). This may reflect the ability of polyunsaturated fatty acids to block TAM binding to AEBS (Hoh et al., 1990), which should increase intracellular availability to bind ER. Estrogens can activate telomerase expression through an imperfect ERE (Kyo et al., 1999). Thus, combinations of antiestrogens and telomerase inhibitors may have clinical value. Similarly, the association of increased angiogenesis with TAM resistance suggests that combinations of angiogenesis inhibitors with antiestrogens may be useful.

Our understanding of how the ER works, the complexity of its transcriptional regulatory apparatus, and the importance of cellular context are beginning to change how we think of antiestrogen action and the mechanisms of acquired and de novo resistance. The identification of new selective ER modulators, particularly those with reduced risk of increasing the incidence of endometrial carcinomas, also holds considerable promise for the development of new antiestrogen-based therapies. The pace of change in this field continues to increase, and has every prospect of providing exciting new developments in our ability to improve and refine antiestrogen-based therapeutic strategies for breast cancer.

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