Resistance to Tamoxifen with Persisting Sensitivity to Estrogen: Possible Mediation by Excessive Antiestrogen Binding Site Activity

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ABSTRACT

The growth of a large proportion of estrogen receptor-positive breast tumors is stimulated by estrogen and can often be controlled through antiestrogen therapy. Resistance to antiestrogen (AE) therapy can occur while tumors retain the expression of estrogen receptors (ERc) and remain functionally responsive to estrogens. The ability of specific antiestrogen binding sites (AEBS) to prevent AE from interacting with ERc has been examined as a possible mechanism through which this appropriation of AE could interfere with antiestrogen action. Comparisons were performed between uterine preparations where ERc activity exceeded AEBS binding and liver preparations where AEBS binding predominated. Identical estimates of ERc activity were obtained in uterine preparations using either [3H]estradiol or [3H]-4OH-tamoxifen and radioinert diethylstilbestrol (α,α'-diethyl-4,4'-stilbenediol) to estimate nonspecific binding. AEBS binding was observed only when [3H]-4OH-tamoxifen was used, while binding to Type II sites was resolved using both [3H]estradiol and [3H]-4OH-tamoxifen. When excess AEBS activity predominated, analyses with radiolabeled estrogen and antiestrogen present simultaneously showed that virtually all of the antagonist was bound to AEBS with little of the antagonist available to associate with ERc. In an effort to relate these observations to AE resistance per se, ERc and AEBS were measured in MCF-7 human breast cancer cells (ERc-positive, responsive to estrogens and antiestrogens) and in variant AE-insensitive LY-2 human breast cancer cells (ERc-positive, responsive only to estrogens). In AE-resistant LY-2 cells, the ratio of AEBS:ERc was approximately three times greater than in MCF-7 cells. Examination of 128 human breast carcinomas revealed that AEBS activity was present and could exceed ERc activity. Importantly, the partition of significant AE away from ERc was observed in human specimens. These observations identify a biochemical mechanism for antiestrogen resistance through which AE access to ERc can be totally incapacitated while sensitivity to estrogens continues. These observations indicate that AEBS activity, in addition to ERc activity, may provide helpful information for predicting the response of certain cancers to hormonal therapy.

INTRODUCTION

Estrogens initiate an array of events which program molecular responses that can culminate in cellular reproduction. The proliferation of certain tumors is induced by estrogen and in the case of breast cancer can be controlled through the use of estrogen antagonists (1–3). Decisions to treat breast cancer with estrogen antagonists are based upon the presence of intracellular ERc, with the expectation that tumors lacking ERc activity are unlikely to respond to pharmaceuticals known as "antiestrogens," which inhibit the action of estrogen (Ref. 4, pp. 118–130; 5, 6). Tamoxifen (trans-1-[p-(dimethylamino)ethoxy]phenyl]-1,2-diphenyl-1-buten) has a nonsteroidal triphenylethylene structure, is the most widely used AE, and has been shown to increase both the recurrence-free interval (7, 8) and overall survival (9) in patients with breast cancer. When antiestrogen responses are not observed in ERc-positive tumors, it has been suggested that estrogen independence results from the presence of some ERc-negative tumor cell elements in these tumors. However, this hypothesis is not supported by the fact that only rarely have ERc-negative metastases originated from ERc-positive primary tumors (10–12), indicating that the resistance manifest in recurrence is not associated with the appearance of ERc-negative cells. Even more compelling evidence against the origination of AE resistance through a loss in ERc expression is the observation that ERc-positive cell lines result after selection for AE resistance and the fact that these cell lines are sensitive to estrogens while lacking responsiveness to estrogen antagonists (13, 14). Thus, there is significant evidence that resistance to AE is able to occur while ERc remains expressed, and functional responsiveness to estrogens is retained. Clear perceptions of how AE responsiveness can be lost without affecting estrogen sensitivity have been difficult to develop. It has been tempting to speculate that antiestrogenic action is mediated independently of ERc by specific AEBS. AEBS are competitively reduced by antiestrogens, but not estrogens, and have been characterized as physically distinct from ERc (15–19). AEBS also occur in microsomes and have been reported to be ubiquitous in normal as well as tumor tissue, including both breast and endometrial tumors (20–24). Physical characteristics of AEBS have been limited to crude preparations (22). In breast tissue, AEBS exist as unusually larger holomers which are not disrupted by elevated salt, and are commonly found in high-speed supernatants (22). AEBS are more thermostable than ERc and can be distinguished by elution from DEAE with lower salt concentrations than ERc. Physical characteristics of uterine and breast AEBS are remarkably similar (22). However, there are several pieces of evidence that indicate that AEBS do not directly mediate AE action. First, the affinity of AE for AEBS does not closely parallel the biopotency of these antiestrogens (25–27). Second, a ligand (β-butyloxynediethylnyl) that occupied AEBS while showing no affinity for ERc, did not directly mediate AE action. First, the affinity of AE for AEBS does not closely parallel the biopotency of these antiestrogens (25–27). Second, a ligand (β-butyloxynediethylnyl) that occupied AEBS while showing no affinity for ERc, did not directly mediate AE action. Finally, a non-ERc molecule which binds estradiol, but not tamoxifen, has been termed a Type II binding site (Ref. 4, pp. 52–58). These Type II binding sites are chromatographically distinct, eluting after ERc from the columns utilized in the present report. In the present report, the problem of AE insensitivity in estrogen responsive, ERc-positive tumor cells has been approached by determining if AEBS can act to limit antagonists from interacting with ERc. The findings reported here indicate...
that AEBS appear to be capable of acting as molecular “sponges,” which appropriate AE so that AE is not available to interact with ERc and thus cannot inhibit the action of estrogen. SEH-PLC (30–33) has been used so that all components which bind estrogens and AE can be simultaneously studied under rapid separation conditions which minimize the effects that different ligand dissociation rates might have on individual binding components. Using this approach, it has been possible to precisely study the partition of [3H]-4OH-tamoxifen, a high-affinity AE, between ERc and AEBS under varied conditions of AEBS expression and to identify a restriction of AE from ERc by AEBS, which has been termed “appropriation.”

**MATERIALS AND METHODS**

**Materials.** (Z)-4-Hydroxy[α-methyl-3H]tamoxifen (80 Ci/mmol) and 17β-(2,4,6,7-3H)estradiol (95–115 Ci/mmol) were purchased from Amersham Corp. Bovine serum albumin, dextran blue 2000, DES, estradiol 17β, ferritin, tamoxifen, and thyroglobulin were obtained from Sigma Chemical Co. Other chemicals were sodium molybdate (reagent grade; Mallinckrodt), Tris (ultrapure grade; Schwarz/Mann, Inc.), and dimethylformamide (Burick and Jackson Chemicals). Female mice were used between the ages of 20 and 23 days (NSA] CF-1/HSD strain: Harlan Sprague-Dawley, Indianapolis, IN). MCF-7 and LY-2 human breast cancer cells were provided by Diane Bronzert and Marc Lippman while at the NIH.

**Preparative Procedures.** Mice were sacrificed by cervical dislocation and decapitaiton. Tissues were collected and transferred to Hanks’ balanced salt solution (Ca2+ and Mg2+ free) on ice. Homogenization was carried out in P100 buffer (5 uteri/ml or 1 liver/ml). Phenylmethylsulfonyl fluoride (0.2 mm), aprotonin (0.5 μg/ml), leupeptin (0.5 μg/ml), EDTA (1 mM), and pepstatin (0.7 μg/ml) as multiple protease inhibitors and Na2MoO4 (5 mM) were routinely included in preparatory buffers. Preparation of cytosols included a low-speed supernatant (1,500 x g for 15 min) which was centrifuged (105,000 x g for 45 min) to yield the high-speed supernatant. Resuspension of the high-speed pellet in P100 buffer demonstrated low and equivalent activity for ERc, AEBS, and Type II binding sites, while KCl extraction of the high-speed pellet (0.4 M KCl, 60 min, 0–4°C) predominantly released additional AEBS activity. This extracted microsomal AEBS activity was approximately equivalent to cytosol AEBS activity. While repeated KCl extractions of the high-speed pellet did not release appreciable additional AEBS, ethanol extraction yielded considerable [3H]-4OH-tamoxifen. Since ethanol-extracted activity increased with ligand concentration, while bound ligand remained constant, and because this activity was quite variable, ethanol-extracted activity probably represents lipid-associated radioligand. In this preparative format, the yield of cytoplasmic AEBS and ERc is taken to be equivalent because little of either AEBS or ERc is found in the supernatant that results when the high-speed pellet is resuspended in P100. Moreover, since repeat KCl extraction of the high-speed pellet yields little additional AEBS, quantitative recovery is approached. Quantitations of specific binding were as described previously (30–33). When [3H]estradiol was used to determine ERc activity, radioinert DES (2 μM) was used as the competitor to assess nonspecific binding. To determine AEBS activity, [3H]-4OH-tamoxifen was used with radioinert tamoxifen (2 μM) as the competitor to assess nonspecific binding. Human breast tumors were homogenized (13.3 ml/g tumor wet weight) and prepared as described previously (30).

Parallel human preparations were charged with [3H]estradiol (10 nM) to determine ERc activity and with [3H]-4OH-tamoxifen (10 nM) to determine AEBS activity. Nonspecific binding was determined using 2 μM radioinert competitor.

**Ligand Binding Determinations by Size-Exclusion Liquid Chromatography.** All procedures have been previously described in detail (30–33). Cytosols were charged 18–20 h at 0–4°C, and equilibrium binding of [3H]estradiol and [3H]-4OH-tamoxifen was observed for both ERc and AEBS. For the estimation of equilibrium association constants, preparations were charged with ten concentra-

**RESULTS**

Estrogen and Antiestrogen Binding to AEBS, ERc, and Type II Sites in Uterus and Liver. Simultaneous identification of binding molecules for estrogens and AES was rapidly accomplished using SEHPLC. ERc and Type II binding sites (35) were defined using [3H]estradiol and were each reduced by excess radioinert DES or tamoxifen (Fig. 1A). Specific AE binding sites were only observed using [3H]-4OH-tamoxifen, were not subject to competition from DES, and demonstrated extreme competition by AE (Fig. 1B). Thus, AEBS only bind estrogen antagonists, while ERc bind both agonists and antagonists. During the course of a ligand saturation analysis, most [3H]estradiol bound to uterine ERc sites at lower concentrations, while upon reaching saturation, binding to Type II sites increased (Fig. 2A). Using [3H]estradiol and excess radioinert DES, no AEBS-specific binding was observed, although specific binding for ERc and Type II sites was significant (Fig. 2B). When [3H]-4OH-tamoxifen was substituted, little ligand associated with Type II sites, even though considerable activity was associated with AEBS (Fig. 2C). Most of the AE binding was associated with ERc. When excess DES was used to obtain the specific binding estimate, an equivalent saturation of both the [3H]estradiol (Fig. 2B) and the [3H]-4OH-tamoxifen (Fig. 2D) profile indicated that AEBS do not affect saturation estimates of specific ERc binding in uterine preparations where ERc binds more ligand than AEBS. Little or no specific binding of [3H]-4OH-tamoxifen by Type II or AEBS was observed when radioinert DES was used. These data show that identical estimates of ERc activity were obtained when either [3H]estradiol or [3H]-4OH-tamoxifen and a radioinert estrogen were used to estimate nonspecific binding. When radioinert tamoxifen was used to estimate nonspecific binding, estimates of specific uterine AEBS binding of [3H]-4OH-tamoxifen saturated at about

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Levels of antagonist were contrasted by the liver, where AEBS binding capacity was considerably greater than one-half the level of uterine ERc activity. However, in liver preparations, AEBS binding was considerably greater than binding to ERc or Type II sites (Fig. 44). Liver AEBS activity (Fig. 3B, Δ) also was observed to be much greater than uterine AEBS activity (Fig. 3B, +), as has been reported (36–38). Thus, liver preparations present a model in which more [3H]-40H-tamoxifen is bound by AEBS than by ERc.

Restrictive Appropriation of Antiestrogen by Excess AEBS. Antiestrogenic effectiveness occurs when, in the presence of estrogens, the antagonist successfully competes for ERc binding through which hormone action is promoted. In preparations exposed to both [3H]estradiol and [3H]-40H-tamoxifen, appropriation of AE by AEBS was evaluated using TLC analysis of SEHPLC fractions which demonstrated ligand binding. These determinations focused on the extent to which AE was bound to ERc in the presence of estrogens. In uterine preparations, where the binding capacity of ERc predominates over AEBS, thin-layer chromatography of ERc-bound radioligand separated by SEHPLC analysis identified estrogen receptors (ERc) and Type II binding sites (II). Elution of antiestrogen binding sites (AEBS) is shown. Insets, specific binding profiles; arrows, elution of standard proteins: blue dextran 2000, thyroglobulin, ferritin, and bovine serum albumin (left to right). Spline fitting was used to connect data points.

Restrictive Appropriation of Antiestrogen by Excess AEBS. Antiestrogenic effectiveness occurs when, in the presence of estrogens, the antagonist successfully competes for ERc binding through which hormone action is promoted. In preparations exposed to both [3H]estradiol and [3H]-40H-tamoxifen, appropriation of AE by AEBS was evaluated using TLC analysis of SEHPLC fractions which demonstrated ligand binding. These determinations focused on the extent to which AE was bound to ERc in the presence of estrogens. In uterine preparations, where the binding capacity of ERc predominates over AEBS, thin-layer chromatography of ERc-bound radioligand separated by SEHPLC analysis identified estrogen and AE when present simultaneously were bound equivalently above 10 nM by ERc (Fig. 44). When increasing concentrations of antagonist were presented against a fixed concentration of agonist, antagonist progressively occupied more uterine ERc (Fig. 4B). The expected easy access of antagonist to ERc in uterine preparations was contrasted by the liver, where AEBS binding capacity is greater than ERc binding. In liver preparations, virtually all antagonist was bound to AEBS and little antagonist was observed in association with liver ERc when either equal concentrations of agonist and antagonist were used (Fig. 4C) or when increasing antagonist concentration challenged a fixed agonist concentration (Fig. 4D). When affinity was considered, the equilibrium association constants (Ks) for [3H]estradiol binding to uterine (0.4–1.5 × 10^8 liters/mole) and liver (1.2–2.5 × 10^8 liters/mole) ERc were similar to those obtained with [3H]-40H-tamoxifen (uterus, 1.7–2 × 10^8 liters/mole; liver, 0.5–2 × 10^8 liters/mole) and were very similar to Ks for [3H]-40H-tamoxifen binding to uterine (0.6–1.1 × 10^8 liters/mole) and liver AEBS (0.7–5 × 10^8 liters/mole). Thus, while AEBS have little affinity for estrogen, both ERc and AEBS have virtually the same affinities for [3H]-40H-tamoxifen. In a binding equilibrium sense, it can be concluded that (a) AEBS can bind 40H-tamoxifen at levels that are as low as levels of endogenous estradiol and (b) both AEBS and ERc possess a similar high affinity for 40H-tamoxifen. In the simplest case, the equivalent affinities of ERc and AEBS for 40H-tamoxifen imply that the binding to either will be random and not skewed by affinity. Consequently, the explanation for failed antagonist binding to liver ERc is an increased binding of [3H]-40H-tamoxifen to the AEBS in liver preparations. As an organ, the liver contains ~18 times more AEBS activity (9 × 10^{-12} mol/liver) than the uterus (0.5 × 10^{-12} mol/uterus); however, liver preparations are so enriched in their protein content that the liver has ~3 times less AEBS specific activity (1.8 × 10^{-13} mol/mg protein) than the uterus (6.3 × 10^{-13} mol/mg protein). When ERc activity is considered, the liver, although larger than the uterus, contains a little less ERc (0.9 × 10^{-12} mol/liver versus 1.2 × 10^{-12} mol/uterus), with the specific activity being ~80 times lower in liver (1.8 × 10^{-14} mol/mg protein) than in uterus (1.5 × 10^{-12} mol/mg protein). Because the AEBS:ERc ratio is 10 for liver and 0.4 for uterus, it appears that it is not the absolute AEBS activity, but the relationship to ERc activity, that actually governs appropriation. When intact liver fragments were exposed to increasing concentrations of [3H]estradiol and [3H]-40H-tamoxifen (1:1, 100 nm), almost all (>99%) of the activity bound to ERc was [3H]estradiol. Thus, under both cell-free and intact cell exposure conditions, AEBS successfully partitioned [3H]-40H-tamoxifen, so that little antagonist was able to bind to ERc. Consequently, in the liver model where AEBS binding capacity exceeds ERc capacity, AEBS so effectively binds antagonist that AE becomes restricted from ERc. Such a restriction is capable of disabling the process in which AE inhibits receptor action. Since molecules which chromatograph similarly to AEBS have not been observed in blood, these observations cannot be explained by a vascular introduction of serum AE binders into the liver.

Occurrence of Excess AEBS in Human Tumors. AEBS capacity was compared with ERc binding in 128 primary human breast carcinoma preparations (Fig. 5). The overall pattern of AE activity was independent of ERc activity. Significant AEBS binding capacity was observed in ERc-negative (~5 fmol ERc/mg protein), borderline (5–20 fmol ERc/mg protein), and ERc-positive (>20 fmol ERc/mg protein) tumors. More than one-half of the ERc-positive samples (58%) demonstrated AEBS activity >5 fmol/mg, while ~20% exceeded 20 fmol/mg protein. AE activity was observed in ~86% of the ERc-positive specimens. Finally, negligible or negative AEBS binding (<5 fmol/mg) was observed in 14 breast carcinomas (~11%), indicating that AEBS expression is not ubiquitous. In addition to the absolute activity levels of AEBS and ERc in...
Fig. 2. Binding of [3H]estradiol and [3H]-4OH-tamoxifen in uterine preparations. Cytosol was charged overnight in the presence of 5-100 nM radioligand as indicated. A, total binding of [3H]estradiol to estrogen receptors (ERc, +), Type II binding sites (T(II), *), and AEBS (AEBS). B, specific binding, determined using 2 uM DES as competitor. Total binding of [3H]-4OH-tamoxifen (O) is shown, as well as specific binding determined using 2 uM DES (D). Binding to ERc, Type II sites, and AEBS was determined by SEHPLC on TSK-G4000SW columns.

Fig. 3. Estrogen and antiestrogen binding sites in liver and uterine preparations. Cytosol was prepared and charged using [3H]-4OH-tamoxifen as described in Fig. 2, at 1 liver/ml (A). Binding was determined by SEHPLC. Preparations were made at a concentration (45-50 mg protein/ml) that ensured the detection of ERc activity (20-25 fmol/mg protein). Specific binding was used to determine the relative abundance of AEBS in liver and uterine preparations as the difference between total binding, estimated with [3H]-4OH-tamoxifen alone, and nonspecific binding, which also included 2 uM radioinert tamoxifen (B). Specific AEBS activity in liver (+) and uterus (O) preparations was determined. Protein concentrations were 3-4 mg/ml (uterus) and 45-50 mg/ml (liver), with receptor specific activity being about 130 times higher in uterus preparations.

Final analyses compared AEBS and ERc binding activity in LY-2 cells, an ERc-positive and estrogen-responsive variant of MCF-7 cells, which was selected for on the basis of tamoxifen resistance (13). Different receptor analyses were performed on multiple cell harvests in order to adequately describe growth-related variations that might influence the expression of AEBS and ERc. Using this approach, the ratio of AEBS:ERc was almost 3 times greater for LY-2 cells (2.32 ± 0.39; n = 9; x ± SEM) than for MCF-7 cells (0.81 ± 0.13; n = 7) and was significantly different (P < 0.001). The resistance of LY-2 cells to 4OH-tamoxifen is greatest at 1 nM. At this concentration, AEBS appropriate 70-80% of [3H]-4OH-tamoxifen. These observations demonstrate that LY-2 cells are characterized by an AEBS binding capacity which exceeds the ERc capacity and indicate that AE resistance in this variant could occur when AEBS become able to restrictively appropriate antagonist so that opportunities to influence receptor action cannot occur.

DISCUSSION

The restrictive appropriation of AE by AEBS, combined with demonstrations of excess AEBS in tumor cells, illustrates a straightforward mechanism through which insensitivity to AE can occur while estrogen sensitivity is maintained. Assuming equivalent binding above 10 nM (as demonstrated in Fig. 4), we have made estimates of AEBS and ERc occupancy by AE that breast carcinoma, the extent to which AE may be bound by AEBS at the expense of binding to ERc was determined. Examination of the distribution of [3H]-4OH-tamoxifen binding between AEBS and ERc showed that more of the hormone antagonist could be bound by the AEBS than by ERc, even when ERc was >20 fmol/mg (Fig. 6). Taken together, these data show that AEBS activity and ERc activity are present in a significant fraction of ERc-positive human breast tumors and that the AEBS can partition AE away from ERc.
42). Thus, comparisons have been made between two ligands.

AEBS activity can appropriately AE to completely AE by microsomal suspensions or the possibility that high salt with cytosol components in order to avoid lipid sequestration of estradiol:pH\-4OH-tamoxifen at the concentrations indicated (A) or with a fixed concentration of 5 nM [\(\text{H}\)]estradiol and increasing concentrations of [\(\text{H}\)-4OH-tamoxifen as indicated (B). Liver cytosol was similarly incubated with 1:1 increasing concentrations of both radioligands (C) or a fixed 5 nM [\(\text{H}\)]estradiol augmented with increasing [\(\text{H}\)-4OH-tamoxifen (D). Preparations were subjected to SEHPLC, and collected fractions were split for scintillation counting to determine the activity profile: the remainder in the receptor elution region were used for TLC. Percentage binding of [\(\text{H}\)]estradiol (C) and of [\(\text{H}\)-4OH-tamoxifen (C) to ERc is shown.

Fig. 4. ERc binding of antiestrogens in the presence of estrogen and antiestrogen. Preparations from uterus and liver were prepared as described in previous figures. Uterine cytosols were incubated overnight at 4°C in a 1:1 ratio of [\(\text{H}\)]estradiol: [\(\text{H}\)-4OH-tamoxifen at the concentrations indicated (A) or with a fixed concentration of 5 nM [\(\text{H}\)]estradiol and increasing concentrations of [\(\text{H}\)-4OH-tamoxifen as indicated (B). Liver cytosol was similarly incubated with 1:1 increasing concentrations of both radioligands (C) or a fixed 5 nM [\(\text{H}\)]estradiol augmented with increasing [\(\text{H}\)-4OH-tamoxifen (D). Preparations were subjected to SEHPLC, and collected fractions were split for scintillation counting to determine the activity profile: the remainder in the receptor elution region were used for TLC. Percentage binding of [\(\text{H}\)]estradiol (C) and of [\(\text{H}\)-4OH-tamoxifen (C) to ERc is shown.

AEBS activity is present, AE and estradiol are both equally available, so that the AE can engage in antagonistic interaction with ~50% of the ERc. With low AEBS activity (AEBS:ERc = 0.8), some AE is bound by AEBS, so that less AE interacts with ERc, allowing less than 50% of ERc to be involved in antagonistic interactions with AE. As more AEBS activity is expressed, even less AE can interact with ERc, until virtually no AE can interact with ERc when AEBS activity is high (AEBS:ERc = 10). Thus, it is quite clear that AEBS activity can appropriate AE to completely restrict antagonist from ERc interaction. When this appropriation occurs, only agonist is available to interact with ERc, so that hormone action remains unimpeded. The exact levels of AE restriction to which AEBS contributes can only be approximated at this time; however, the data presented here indicate the following: (a) When AEBS:ERc values are ~0.4, as in uterine preparations, the binding of AE by AEBS appeared to have little consequence. (b) With AEBS:ERc values of ~10 in liver preparations, remarkably complete restriction of AE from ERc occurred. (c) AEBS:ERc values in AE-resistant LY-2 cells are approximately 3 times greater than in AE-sensitive MCF-7 cells. (d) AEBS:ERc values of 3 or greater were observed in some human breast cancer specimens.

An important aspect of these studies was the use of 4OH-tamoxifen, which is the active antiestrogen metabolite in vivo and has an affinity for ERc that is equivalent to estradiol (39–42). Thus, comparisons have been made between two ligands with high affinity for ERc over similar concentration ranges. It should also be kept in mind that the present studies are unique in that they focus exclusively on AEBS in the cytosol. Microsomal AEBS account for roughly equivalent AEBS activity and consequently would further contribute to the restriction of AE from ERc. We have elected to perform the present studies solely with cytosol components in order to avoid lipid sequestration of AE by microsomal suspensions or the possibility that high salt extraction of the AEBS from microsomes disrupts cellular components in a way that can create an environment where there is greater AE binding than occurs within the intact system. Thus, our demonstrations of appropriation in the same fraction in which ERc occurs are significant and essential for eliminating the possibility that compartmentalization might prevent AEBS from influencing AE binding by ERc or that salt extraction might introduce AEBS to an environment where they could influence AE binding to ERc. When the additional contributions of microsomal AEBS are considered, appropriation of AE should be even more extreme. Importantly, AEBS appropriation has been documented using native cytosols and is not a phenomenon arising in the context of mixing or reconstitution. In future studies it will be important to resolve the issue of any differences between cytoplasmic and microsomal AEBS and to
determine the extent to which both forms contribute to a precise identification of appropriation.

It is noteworthy that in vivo the liver, high in AEBS activity, is estrogen sensitive but does not demonstrate antiestrogen inhibition of estrogen sensitivity (43, 44). Thus, elevated AEBS expression is common to one AE-insensitive normal tissue. It is the process of AEBS expression which is most relevant to advancing our understanding of when resistance might be anticipated. It will be important to determine how effectively different levels of AEBS activity predict AE insensitivity in a large cohort of subjects on AE therapy. These determinations will be relevant to individuals afflicted with two major cancers, since both breast (45) and endometrial (46, 47) cancers can be responsive to AE therapy. It will also be valuable to determine if human AEBS expression is stimulated by estrogen (48) or other inducers. Research focused at the control of AEBS expression and how AEBS expression is related to the stage of the advancing disease will yield valuable information. Since N,N-diethyl-2-[(4-phenethylmethyl)-phenoxy]-ethanamine, certain unsaturated fatty acids, some phenothiazines, and certain thioxanthenes have been reported to reduce AEBS activity by competition (49–51), it may be possible to use nonestrogenic pharmaceuticals to overcome AE resistance by reducing antagonist AEBS appropriation in vivo.

It has been proposed that resistance to endocrine therapy could occur through more than one mechanism (6, 52). Consequently, appropriation may account for only a portion of hormone-resistant cancers. Thus, the present work should not be taken to indicate that AE appropriation provides the only mechanism for AE resistance. In fact, if the AEBS are capable of catalytic activity, in addition to high affinity for the most active form of tamoxifen, then restrictive appropriation might be further enhanced by the generation of forms with lower affinity for ERC. Such catalytic possibilities are illustrated by reports of the isomerization of trans-4OH-tamoxifen in resistant breast cancer cells to the cis form, which functions as an agonist (53), and of binding to P-450 isozymes and flavin-containing monooxygenases (54). AE appropriation does provide a straightforward framework for a therapeutic incapacitation which is compatible with a persisting sensitivity to estrogens. Since AE resistance without loss of ERC appears to be a major avenue taken by breast cancer clinically, the real significance of AE appropriation mediated by AEBS becomes intensified by a potential for reversal. At present, it is quite feasible to augment radiochemical receptor determinations with a radiolabeled AE for the estimation of AEBS. By so doing, it should be possible to define the extent to which AEBS expression can forecast resistance to AE therapy.

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