[CANCER RESEARCH 57, 3486–3493, August 15, 1997]

MCF7/LCC9: An Antiestrogen-resistant MCF-7 Variant in Which Acquired Resistance to the Steroidal Antiestrogen ICI 182,780 Confers an Early Cross-Resistance to the Nonsteroidal Antiestrogen Tamoxifen[1]

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ABSTRACT

Acquired resistance to antiestrogens is a major problem in the clinical management of initially endocrine responsive metastatic breast cancer. We have shown previously that estrogen-independent and -responsive MCF7/LCC1 human breast cancer cells selected for resistance to the triphenylethylene tamoxifen produce a variant (MCF7/LCC2) that retains sensitivity to the steroidal antiestrogen ICI 182,780 (N. Brünner *et al.*, Cancer Res., *53:* 3229–3232, 1993). We have now applied stepwise selections *in vitro* from 10 pM to 1 μM ICI 182,780 against MCF7/LCC1 and obtained a stable ICI 182,780-resistant variant designated MCF7/LCC9. In contrast to 4-hydroxytamoxifen-selected MCF7/LCC2 cells, MCF7/LCC9 cells exhibit full cross-resistance to tamoxifen, despite never having been exposed to this drug. Significantly, tamoxifen cross-resistance arose early in the selection, appearing following selection against only 0.1 nM ICI 182,780. Although limited resistance to ICI 182,780 also was observed, full ICI 182,780 resistance was not detected until the selective pressure increased to 100 nM ICI 182,780. Cross-resistance to tamoxifen persisted throughout these additional selections. Despite their antiestrogen cross-resistance, MCF7/LCC9 cells retain a level of estrogen receptor expression comparable to that of their parental MCF7/LCC1 cells. Whereas MCF7/LCC1 cells retain an estrogen-inducible expression of progesterone receptors, MCF7/LCC9 cells exhibit an up-regulated expression of both progesterone receptor mRNA and protein that is no longer estrogen responsive. Estrogen-independent and -responsive components of the MCF7/LCC9 phenotype are apparent *in vivo*. These cells form slowly growing tumors in ovariectomized athymic nude mice but respond mitogenically upon estrogenic supplementation. The *in vivo* growth of MCF7/LCC9 tumors is not affected by treatment with ICI 182,780. Although there is some evidence of tamoxifen stimulation of tumor growth, this did not reach statistical significance. If this pattern of cross-resistance occurs in some breast cancer patients, administering triphenylethylene antiestrogens as a first-line therapy with a cross-over to steroidal compounds upon recurrence may be advantageous.

INTRODUCTION

Most drug-responsive tumors in patients with metastatic breast cancer, either spontaneously or following the selective pressure of systemic therapies, acquire a phenotype characterized by multiple metastatic lesions that are resistant to all endocrine and cytotoxic therapies. It is the development of this multiple resistance phenotype that is the primary reason for the failure of current breast cancer therapies.

The mechanisms that confer a multiple resistance phenotype are unclear but are likely to involve several molecular mechanisms. For cytotoxic drugs, the gp170 product of the *MDR1* (multidrug resistance) gene (1), the multidrug resistance-associated protein (2), and the altered expression of detoxification (*e.g.*, superoxide dismutases and glutathione transferases; Refs. 3 and 4) and stress [*e.g.*, heat shock proteins (5) and other enzymes, *e.g.*, topoisomerases (6)] likely contribute. Although the mechanisms that confer resistance to endocrine therapies are less well understood, a multiple hormone resistance phenotype may exist, analogous in some ways to the multidrug resistance phenotype.

The precise contribution of each potential endocrine resistance mechanism is unclear, and it is likely that more than one resistance mechanism is involved in conferring a multiple hormone resistance component (7). Thus, treating the multiple hormone resistance phenotype may ultimately require the development of novel therapies targeting several resistance mechanisms. A better understanding of how cells become resistant and how this resistance can be prevented, delayed, or overcome is a prerequisite for the development of such strategies.

Antiestrogens are the most common type of endocrine therapy in breast cancer treatment. The primary growth-inhibitory activities of antiestrogens are likely to be mediated, to a significant degree, through their competitive antagonism of estrogen binding to ERs[4] (8, 9). However, other mechanisms that operate essentially independently of ER-mediated events also may contribute. These include the ability of TAM to inhibit the activity of the intracellular signal transduction molecules protein kinase C (10) and calmodulin (11) and to induce changes in membrane structure and function (12). Endocrine perturbations (13) and the *de novo* production of estrogens (14) also may induce resistance in target tissues. The extent to which each or all of these contribute to the diverse effects of antiestrogens in patients largely remains to be established.

A new series of steroidal antiestrogens has been reported within the last few years. These compounds, which are C7-acyl-substituted analogues of E_2, are substantially different from the triphenylethylenes and potentially represent a major development for the management of endocrine-responsive breast cancer (15). Two analogues have received considerable attention: ICI 182,780, which has already begun clinical trials, and its predecessor, ICI 164,384. These compounds have a higher affinity for ER than TAM and are lacking significant agonist activities in most models studied to date (15, 16).

With the potential for diverse mechanisms of antiestrogenic action (9, 17), particularly for triphenylethylene antiestrogens and the new steroidal antiestrogens (18, 19), it is likely that a single mechanism of action will inadequately account for all biological responses. Even in

Received 1/30/97; accepted 6/18/97.

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[1] This study was supported by United States Public Health Service Grants R01-CA58022 and P30-CA51008 from the National Cancer Institute (to R. C.) and Grant 95-010 from the Danish Cancer Society (to N. B.).

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[4] The abbreviations used are: ER, estrogen receptor; PgR, progesterone receptor; TAM, tamoxifen; 4OH-TAM, 4-hydroxy-TAM; IMEM, improved MEM; CCS-IMEM, IMEM supplemented with charcoal-stripped calf serum; TD, tumor doubling time; E_2, 17β-estradiol; nt, nucleotide.

the growth regulation of breast cancer, it seems most likely that several mechanisms will operate, each with its own dose-response relationship or K_i. Thus, the overall inhibition of proliferation will likely reflect the effects of multiple overlapping mechanisms (15).

To investigate the possible patterns of cross-resistance among antiestrogens and to identify potential mechanisms of resistance, we have generated a series of MCF-7 cell variants. The initial variants were selected by *in vivo* passage in ovariectomized, athymic, nude mice. The MCF7/MIII (20) and MCF7/LCC1 cells (21) proliferate both *in vivo* and *in vitro* without estrogens but retain sensitivity to steroidal and nonsteroidal antiestrogens (21, 22). An *in vitro* selection of the MCF7/LCC1 cells against increasing concentrations of 4OH-TAM produced the MCF7/LCC2 cells, which retain estrogen-independent growth but have acquired resistance to TAM *in vitro* and *in vivo* (23). However, these cells are not cross-resistant to the steroidal antiestrogens ICI 182,780 and ICI 164,384 (23, 24).

To determine the consequences of an acquired resistance to a steroidal antiestrogen, we have now selected the MCF7/LCC1 cells *in vitro* against ICI 182,780. Our data indicate that cells acquiring resistance to a steroidal antiestrogen become cross-resistant to 4OH-TAM. Resistance to ICI 182,780 is induced following exposure to a relatively low concentration of the drug. Furthermore, cross-resistance to 4OH-TAM emerges early in the acquisition of this phenotype, which is not associated with the apparent presence of mutant ER. If applicable to the human disease, these observations have important implications for the endocrine management of breast cancer and the application and scheduling of steroidal and nonsteroidal antiestrogens.

MATERIALS AND METHODS

Cell Lines. MCF-7 cells were obtained originally from Dr. Marvin Rich (Michigan Cancer Foundation), and the cells were propagated routinely in IMEM (Biofluids, Bethesda, MD) supplemented with 5% FCS. Establishment and characterization of the two estrogen-independent but -responsive MCF-7 sublines, MCF7/LCC1 and MCF7/LCC2, have previously been described (21, 23). Both sublines and the MCF7/LCC9 cells were passaged serially in phenol red-free IMEM supplemented with 5% CCS-IMEM. Serum was stripped of endogenous steroids as described previously. This treatment reduces the concentration of estrogen in culture media to ≤10 fM (25). All of the cell cultures were maintained at 37°C in a humidified 5% CO_2/95% air atmosphere and demonstrated to be free of contamination with *Mycoplasma* species as determined by solution hybridization to *Mycoplasma*-specific, radiolabeled, rRNA riboprobes (Gen-Probe, Inc., San Diego, CA).

Selection Procedure. MCF7/LCC1 cells in their 18th passage were selected stepwise against increasing concentrations of ICI 182,780 (provided generously by Dr. Alan Wakeling, ZENECA Pharmaceuticals, Macclesfield, Cheshire, United Kingdom). Selection was initiated against a concentration of 0.01 nM ICI 182,780. The concentration of ICI 182,780 was increased by half a decade after three successive passages of cells at each dose. The highest concentration used was 1 μM ICI 182,780. At each dose level, several aliquots of cells were frozen and stored in liquid nitrogen. Cells proliferating in 0.1 nM, 10 nM, and 100 nM ICI 182,780 were studied for their response to 1 nM E_2, 1 μM 4OH-TAM, and 1 μM ICI 182,780. Cells proliferating in 1 μM ICI 182,780 were designated MCF7/LCC9 cells and were maintained for an additional 10 passages in the presence of 1 μM ICI 182,780, followed by 10 passages in 5% CCS-IMEM without the drug. Cells were refed with fresh growth medium every 3 days. Subsequently, the responses of MCF7/LCC9 cells to E_2, 4OH-TAM, and ICI-182,780 were reexamined.

The MCF-7 origin of MCF7/LCC9 was confirmed by both karyotype and isoenzyme analyses, as described previously (20). The polymorphic enzymes analyzed were lactate dehydrogenase, glucose-6-phosphate 1-dehydrogenase (EC 1.1.1.49), phosphoglucomutase-1 (EC 5.4.2.2), phosphoglucomutase-3 (EC 5.4.2.2), esterase D (EC 3.1.1.1), mitochondrial malic enzyme (EC 1.1.1.40), adenylate kinase (EC 2.7.4.3), and glyoxalase (EC 4.4.1.5). For karyotype analyses, 100 metaphases were examined. The isozyme and karyotype analyses were performed by the Cell Culture Laboratory at the Children's Hospital of Michigan (Detroit, MI).

Steroid Hormone Receptor Analyses. Levels of the ER and PgR proteins were measured using a whole-cell competitive binding assay as described previously (26). Briefly, cells were grown in 24-well dishes (Costar, Cambridge, MA) and incubated for 60 min at 37°C with increasing concentrations of radiolabeled steroids, 0.2–0.6 nM [^{3}H]E_2 (specific activity, 95 Ci/mmol; Amersham Corp., Arlington Heights, IL) for ER and 0.1–2.5 nM [^{3}H]ORG 2058 (specific activity, 50.6 Ci/mmol; Amersham) for PgR determinations, in the absence or presence of a 200-fold excess of unlabeled competitors. Cells were incubated at 37°C with 100 nM hydrocortisone for 30 min before determining PgR to reduce progestin binding to glucocorticoid receptors (26). Radioactivity was extracted into ethanol and measured in a liquid scintillation spectrometer. Data were analyzed using the LIGAND receptor binding software (27).

RNase Protection Analysis of PgR mRNA Expression. Three independent experiments were performed. Where appropriate, cells were treated with 1 nM E_2 for 24 h prior to isolation of total RNA. MCF-7 and MCF7/LCC9 cells were depleted of endogenous estrogens by daily washing and refeeding with CCS-IMEM for 3 days prior to the isolation of total RNA. RNA was obtained using the Trizol Reagent (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions. The PgR riboprobe was made by *in vitro* transcription of a 250-bp fragment of the PgR cDNA, the 36B4 loading control riboprobe being obtained similarly from a 220-bp fragment of the 36B4 cDNA. Riboprobes were labeled by the addition of [^{32}P]UTP (Amersham) in the transcription buffer. To achieve bands for the two genes with similar intensities in the E_2-treated cells, the 36B4 riboprobe was made with a specific activity of approximately one-fifth that of the PgR riboprobe. The RNase protection assays were performed as described previously (28). Briefly, RNA (15 μg), the PgR probe, and the 36B4 probes were hybridized overnight at 50°C followed by digestion with RNase A. The protected fragments were analyzed on a 6% acrylamide Tris-borate EDTA-urea minigel (Novex, San Diego, CA). The gels were dried and the respective signals measured by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA) and visualized by autoradiography performed at −70°C between two Chronex Quanta III intensifying screens.

Cell Growth *in Vitro*. Cell proliferation *in vitro* was studied by determining the anchorage-dependent growth of cells propagated in CCS-IMEM. Cell growth was determined using a crystal violet dye uptake assay, in which dye uptake is related directly to cell number (29). Cells were stained with the crystal violet stain [0.5% (w/v) crystal violet in 25% (v/v) methanol] for 5 min at 25°C. Following two gentle rinses with distilled H_2O, the cells were allowed to dry, and the dye was extracted into 0.1 M sodium citrate in 50% (v/v) ethanol by incubation at room temperature for 10–15 min. Absorbance was read at 540 nm, and the data are presented as the mean and SD of four determinations, and the absorbance on day 6 is expressed as a percentage of that for untreated cell populations on the same day.

To determine the concentration at which the cells gained full resistance to ICI 182,780 and/or 4OH-TAM, a frozen ampule of cells from each of the selection steps was thawed; grown for 5 days without any treatment; and then exposed to 1 nM E_2, 1 μM 4OH-TAM, or 1 μM ICI 182,780 for 6 days. Cell number was assessed on days 2, 4, and 6 using the crystal violet staining assay.

Cell Growth *in Vivo*. The tumorigenicity of MCF7/LCC9 cells was determined by inoculating 2×10^6 MCF7/LCC9 cells into each flank of 6–8-week-old untreated ovariectomized female NMRI nude mice (Bomholtgaard, Ry, Denmark). *In vivo* hormone sensitivity was evaluated by administration of E_2, TAM, or ICI 182,780 starting on the day of cell inoculation. Estrogen supplementation was provided by the s.c. intrascapular implantation of a 0.72-mg, 60-day-release E_2 pellet (Innovative Research, Toledo, OH). TAM treatment was administered by daily injection of 0.1 mg of TAM, and ICI 182,780 was given as 0.5 mg *i.p.* once weekly. These doses and schedules significantly inhibit growth of the parental MCF-7 and/or MCF7/LCC1 tumors (23, 30). Tumor area was recorded three times weekly, and TDs were estimated by application of the Gompertz function (31).

Identification of Mutant ER. To identify mutant ER, RNA was isolated from MCF-7, MCF7/LCC1, MCF7/LCC2, and MCF7/LCC9 cells using a model 340A nucleic acid extractor from Applied Biosystems, Inc. (Foster City, CA). PCR amplification of cDNA was performed as described (32). We used 10 different primers that divided the coding domain (33) into five overlapping PCR products: AB1 (TTCTGAGCCTTCTGCCCTGC; nts, −56 to −37, sense); AB2 (GACTC-

CATAATGGTAGCCTG; nts, 578–597, antisense); AB3 (TGCAGTAGCATCAGCGGGCT; nts, 316–335, antisense); AB4 (AGGATCTACGGTCAGACCGGCCTCCCCTA; nts, 212–239, sense); ERY2 (CGAAGCTTCACTGAAGGGTC; nts, 1004–1023, antisense); ERY4 (CCGGCATTCTACAGGCCAAA; nts, 439–458, sense); ERY6 (TGCAAGGAATGCGATGAAGT; nts, 1720–1739, antisense); HB10 (GGAGACATGAGAGCTGCCAAC; nts, 850–870, sense); HB11 (CCAGCAGCATGTGGAAGATC; nts, 1269–1288, antisense); HB18 (GTAGAGGGCATGGTGGAG; nts, 1250–1270, sense). *Eco*RI restriction endonuclease sites were added onto the primers to facilitate cloning.

The amino-terminal region of ER (34) was amplified using primers AB1 and AB3, and AB4 and AB2. DNA binding to the ER hinge region (34) was amplified using primers ERY4 and ERY2. The COOH-terminal hormone-binding domain of the ER (34) was amplified using primers HB10 and HB11, and HB18 and ERY6. The products of two individual PCR amplifications from each cell line, and for each of the five regions, were cloned into the *Eco*RI site of pGEM 7zf+ (Promega, Madison, WI). Standard Sanger dideoxy sequence analysis of PCR products was performed as described (35). Five clones from each PCR reaction were analyzed to ensure that we would identify any altered ER transcript(s) present as minor subpopulation(s).

RESULTS

***In Vitro* Selection of MCF7/LCC1 Cells against ICI 182,780 to Generate the MCF7/LCC9 Variant.** The cellular lineage of the MCF7/LCC9 line is shown in Fig. 1, as is the relationship with its more immediate predecessor variants, MCF7/MIII (20) and MCF7/LCC1 cells (21). These latter cells were selected following orthotopic inoculation of MCF-7 and MCF7/MIII cells, respectively, into ovariectomized NCr *nu/nu* athymic nude mice. This also provided an endocrinologically relevant host environment, because the circulating steroid hormone profile of these mice is similar to that seen in postmenopausal women (36–38). The common parental cell line

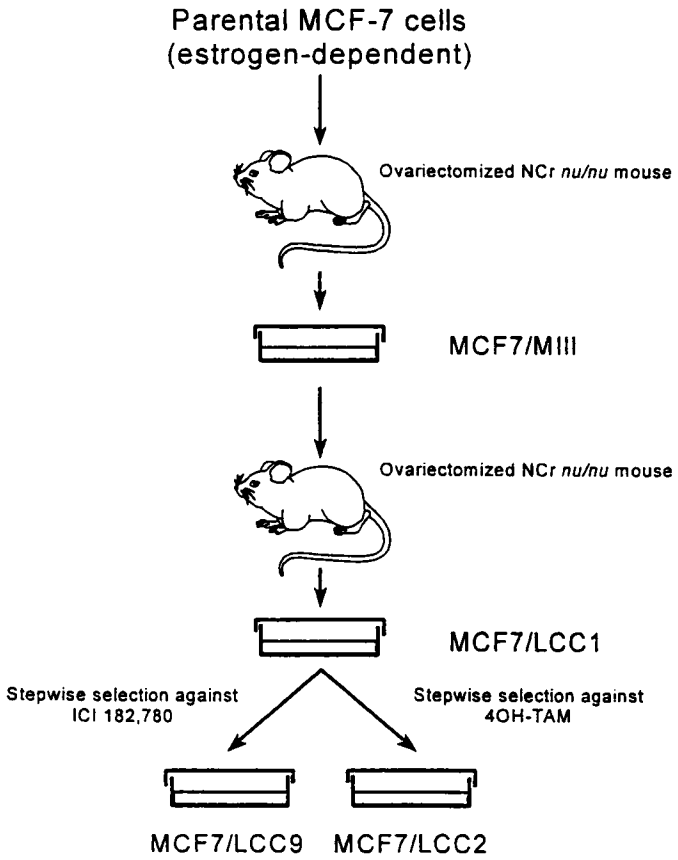

Fig. 1. Derivation of the MCF-7 variant cells, showing the relationship among the variant and parental cells.

(MCF-7) was isolated originally from a malignant pleural effusion in a postmenopausal breast cancer patient (39). The MCF7/LCC2 cells were derived from the MCF7/LCC1 cells by *in vitro* selection against 4OH-TAM in a stepwise manner equivalent to that used to derive MCF7/LCC9 (23).

The response of the MCF7/LCC1 cells prior to initial selection against ICI 182,780 is shown in Fig. 2*A*. As is apparent, the cells are growth inhibited by both 1 μM 4OH-TAM and 1 μM ICI 182,780, consistent with their previously reported phenotype (21). The greater sensitivity to ICI 182,780 at equimolar concentrations, relative to 4OH-TAM, probably reflects its higher affinity for ER and greater antiestrogenic potency (15).

Following three passages in 0.1 nM ICI 182,780, the cells have acquired cross-resistance to 4OH-TAM, despite having never been exposed to this drug. There also is some evidence of a small level of resistance to ICI 182,780 (Fig. 2*B*). When the selective pressure is increased to 10 nM ICI 182,780, there is more evidence of acquired resistance to ICI 182,780, and the 4OH-TAM-cross-resistance remains apparent. However, the cells have not become fully ICI 182,780 resistant (Fig. 2*C*). The response to other triphenylethylene antiestrogens has not been determined but is currently under investigation.

Complete resistance is evident upon selection against 10 nM ICI 182,780 (Fig. 2*D*). Not surprisingly, the cells also retain their cross-resistance to 4OH-TAM. These cells were allowed to proliferate in 1 μM ICI 182,780 for 10 passages and an additional 10 passages without selective pressure, at which point they were designated MCF7/LCC9.

When retested for their growth responses to E_2, 4OH-TAM, and ICI 182,780, MCF7/LCC9 cells exhibit a pattern equivalent to that seen in Fig. 2*D*, indicating that MCF7/LCC9 has a stable phenotype (not shown). Indeed, this phenotype has remained stable in our hands since the preliminary characterization (40). The antiestrogen cross-resistance phenotype of MCF7/LCC9 contrasts with the 4OH-TAM- and ICI 182,780-sensitive proliferation of MCF7/LCC1 cells (Fig. 2*A*; Ref. 21) and the 4OH-TAM-resistant and ICI 182,780-sensitive growth of the MCF7/LCC2 cells (23).

Origin of MCF7/LCC9. The isoenzyme phenotype of MCF7/LCC9, lactate dehydrogenase (human), glucose-6-phosphate dehydrogenase (B isoform), phosphoglucomutase-1 (isoform 1), phosphoglucomutase-3 (isoform 1), esterase D (isoform 1), mitochondrial malic enzyme (absent), adenylate kinase (isoform 1), and glyoxalase (isoforms 1 and 2), is identical to that of MCF-7, MCF7/LCC1, and MCF7/LCC2 cells (20, 21, 23). The estimated frequency of this phenotype is 0.1014.

The MCF7/LCC9 karyotype is that of a female with varying representations of normal chromosomes. Thus, chromosome 9 is absent; there are zero or one copy of chromosomes 7, 20, and 22; and there is one copy each of chromosomes 1, 2, 4, 5, 8, 12, and 14. One or two copies of chromosomes 6, 11, 15, 16, 17, 19 and 21 are present, with two copies of chromosome 3, two or three copies of chromosomes 10 and 18, and one to three copies of chromosome 13.

There are 32 marker chromosomes (Table 1). The origins of marker chromosomes M1–M5 are reported by Nelson-Rees *et al.* (41) and M6–M30 by Whang-Peng *et al.* (42); those of the others are provided in Table 1. MCF7/LCC9 cells have acquired seven marker chromosomes not apparent in their immediate MCF7/LCC1 ancestor. Of these, three are present and four are absent in the triphenylethylene resistant/ICI 182,780-responsive MCF7/LCC2 cells. Those unique to MCF7/LCC9 cells are M2A [t(12qter→12q11::4q)], M30 [del(4)(p11:)], M33B (14p+), M34 [del(12)(q23:)], and M39(16q+). The marker chromosome M21 [amp(6p21)] is absent in both resistant variants but present in the MCF7/LCC1 cells.

MCF7/LCC1 and MCF7/LCC2 cells have previously been identified as variants of MCF-7 cells (21, 23). The chromosome and

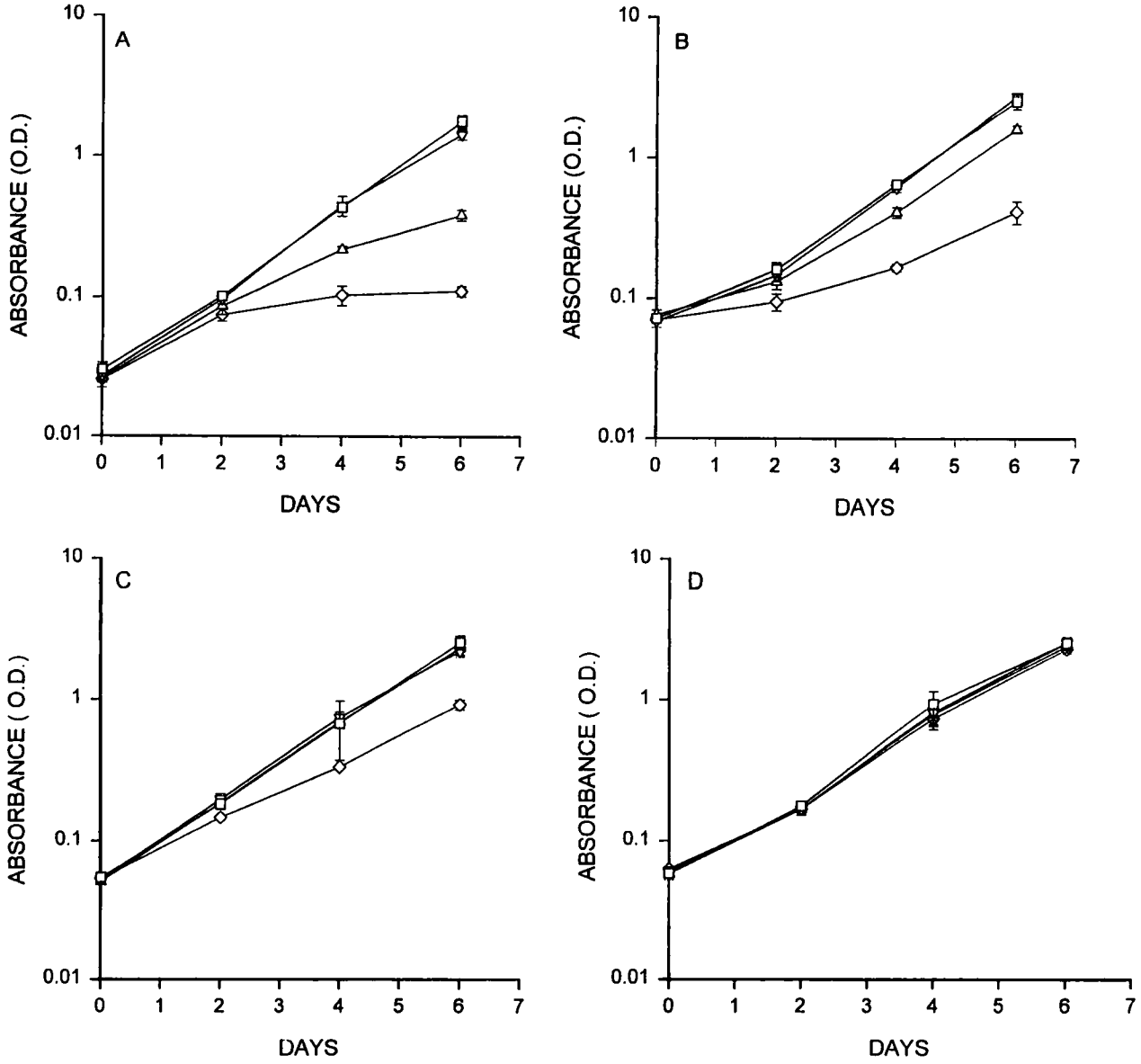

Fig. 2. Representative growth response relationships for MCF7/LCC1 cells during the generation of the MCF7/LCC9 variant. Selected cells were designated MCRF7/LCC9 after 10 passages in 1 μM ICI 182,780 and an additional 10 passages in the absence of the drug. *A*, MCF7/LCC1; *B*, following three passages in 0.1 nM ICI 182,780; *C*, following three passages in 10 nM ICI 182,780; *D*, following three passages in 100 nM ICI 182,780. Data are means (*bars*, SD) of three or more determinations. Three or more independent experiments were performed as described in "Materials and Methods." ◇, ICI 182,780; △, 4OH-TAM; ▽, E_2; □, vehicle.

karyotype analyses are consistent with MCF7/LCC9 also being a variant of MCF-7 cells. On the basis of their overall patterns of marker chromosome expression, MCF7/LCC9 cells appear more directly related to MCF7/LCC1 than MCF-7, closely reflecting their immediate derivation (Fig. 1). The chromosome number range, as estimated from 100 metaphases, is 66–73 for MCF7/LCC1 (21) and 60–69 for MCF7/LCC9. This suggests the elimination of some subpopulations from MCF7/LCC1 cells, perhaps through clonal selection. However, it is not immediately clear whether this reflects either the adaptation of a subpopulation or the presence of a preexisting MCF7/LCC1 subpopulation.

Steroid Hormone Receptor Expression. Steroid hormone receptor binding studies demonstrate the presence of both ER and PgR in the MCF7/LCC9 cells (Table 2). The levels of ER and PgR for the immediate predecessor cells (MCF7/LCC1), the 4OH-TAM resistant (MCF7/LCC2) and the parental (MCF-7), are provided for comparison. The levels of ER expression are essentially comparable among MCF-7, MCF7/LCC1, MCF7/LCC2, and MCF7/LCC9 cells. The ER and PgR dissociation constants for ligand/receptor binding are comparable to those published previously for MCF-7 and other cells (not shown).

MCF-7, MCF7/LCC1, and MCF7/LCC2 all exhibit an E_2-inducible PgR protein expression. Relative to MCF-7 cells, the immediate predecessor of MCF7/LCC9 (MCF7/LCC1) has an increased level of PgR protein (Table 2) but not mRNA expression (Fig. 3) when growing without E_2. However, expression of both protein and mRNA are increased by E_2 (21). In marked contrast, PgR protein and mRNA expression are significantly up-regulated in the MCF7/LCC9 cells growing without estrogenic supplementation. Several individual experiments show a small E_2 induction of PgR mRNA levels (approximately 50%).

***In Vivo* Growth Response to Estrogen and Antiestrogens.** MCF7/LCC9 cells, like MCF7/LCC1 (21) and MCF7/LCC2 cells (23), are tumorigenic in ovariectomized female nude mice without estrogenic supplementation (Table 3). Untreated MCF7/LCC9 tumors grow more slowly (mean, 79 days) than untreated MCF7/LCC1

Table 1 *The distribution of marker chromosomes in MCF7/MIII, MCF7/LCC1 and MCF7/LCC9 cells*[a]

Marker	MCF7/LCC1	MCF7/LCC2	MCF7/LCC9
M1	−	−	−
M2A	−	−	+
M3	+	+	*
M4	+	+	+
M5	−	−	*
M5A	+	+	+
M6	+	+	+
M6A	−	−	−
M7	+	+	*
M7A	+	+	+
M8	−	+	*
M9	+	+	+
M10	−	+	+
M11	+	+	+
M11A	−	−	*
M12	+	+	+
M13	−	−	−
M14	−	−	−
M15	−	−	−
M15A	−	−	−
M16	+	+	+
M16A	−	−	−
M17	−	+	+
M18	−	*	−
M19	−	−	*
M20	−	−	−
M21	+	−	−
M22	−	−	−
M23	−	−	−
M23A	−	−	−
M25	+	−	+
M26	−	−	−
M27	−	−	*
M27A	−	−	*
M28	+	+	+
M29	+	+	*
M30	−	−	+
M30A	−	−	*
M31 = del (1) (p21:)	+	+	+
M32 = t(5qter → 5p11::?::1q21 → 1qter)	+	+	+
M33 = 14p++	−	−	*
M33B = 14p+	−	−	+
M34 = del (12) (q23:)	−	−	+
M37 = 17p+	−	−	*
M38 = 13p+	*	+	*
M39 = 16q+	−	−	+

[a] +, present in ≥50% of the karyotypes; *, present in <50% of the karyotypes; −, absent (as performed by Dr. Ward Peterson, Children's Hospital of Michigan, Detroit, MI). Origin of marker chromosomes M1–M5 are reported by Nelson-Rees *et al.* (41) and M6–M30 by Whang-Peng *et al.* (42). The karyotype of the MCF7/LCC1 was reported previously (21) but not those of the MCF7/LCC2 or MCF7/LCC9 cells.

(mean, 39 days; Ref. 21) and MCF7/LCC2 cells (mean, 33 days; Refs. 21 and 23). Although the rate of proliferation is relatively slow, this is in contrast to the parent MCF-7 tumors, which do not produce tumors in ovariectomized nude mice without hormonal supplementation (20, 36). Table 3 also shows the effect on tumor growth of E_2 supplementation, daily injections of the antiestrogen TAM, or weekly injections of the antiestrogen ICI 182,780. MCF7/LCC9 tumors have retained *in vivo* the E_2 responsivity of the parental MCF-7 tumors and their immediate predecessor MCF7/LCC1 cells (21).

TDs were estimated from the tumor growth data as described by Rygaard and Spang-Thomsen (31). Consistent with the *in vitro* data, and in common with the MCF7/LCC2 cells (23), no inhibitory effect is seen following TAM treatment of MCF7/LCC9 tumors. Indeed, there is evidence of a 2-fold TAM-induced stimulation of tumor growth, but this did not reach statistical significance. However, the number of tumors is small (n = 5/group) and provides a limited statistical power to identify small changes in doubling times. Significant TAM-stimulated growth has been reported in TAM-selected MCF-7 xenograft models and may reflect the estrogenic properties of TAM (43, 44).

Treatment with ICI 182,780 does not affect MCF7/LCC9 tumor growth (Table 3). The growth of E_2-treated MCF7/LCC9 tumors (12 days) is significantly more rapid than any of the tumors in the other treatment groups ($P < 0.01$). This mitogenic response to E_2 *in vivo* also is seen in the MCF7/LCC1 and MCF7/LCC2 cells, which have mean E_2-stimulated TDs of 14 days (21) and 18 days (23), respectively.

The apparent discordance with the *in vitro* data may reflect differences in the metabolism of cells growing *in vitro* compared with *in vivo*. The cells may already be proliferating at their metabolic/proliferative maximum *in vitro* under baseline conditions ($-E_2$), whereas their rate of proliferation *in vivo* under similar conditions is significantly slower. This would enable a mitogenic response to E_2 *in vivo* but not *in vitro*.

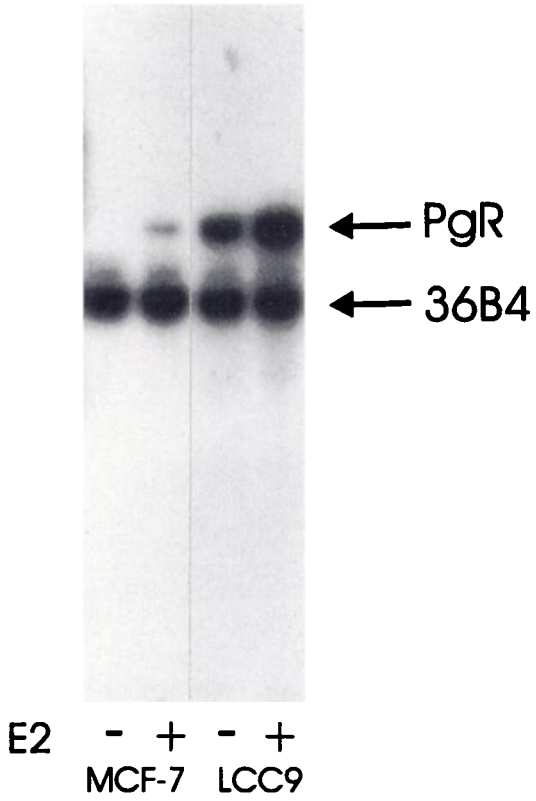

Fig. 3. Representative RNase protection assay for PgR expression in MCF-7 and MCF7/LCC9 cells. The regulation of PgR mRNA in the immediate predecessor (MCF7/LCC1) can be found in Ref. 21. The internal loading control for RNA is provided by the signal for 36B4, a constitutively expressed acidic ribosomal protein (62). Three independent experiments were performed as described in "Materials and Methods."

Table 2 *ER and PgR levels in MCF-7 variants*[a]

Cell line	ER	PgR ($-E_2$)	PgR ($+E_2$)	Reference
MCF-7	120,540 ± 20,000	9,841 ± 4,680	47,520 ± 12,000	21
MCF7/LCC1	112,270 ± 19,440	36,770 ± 12,420	90,330 ± 950	21
MCF7/LCC2	91,290 ± 1,330	6,420 ± 1,130	16,740 ± 1,720	23
MCF7/LCC9	133,200 ± 21,140	103,270 ± 41,500	111,420 ± 6,870	NA

[a] Data represent the number of receptor sites/cell expressed as the mean ± SD of three or more experiments. NA, not applicable. Data for the appropriate parental and other related MCF-7 variants are provided for comparison.

Table 3 *Growth of MCF7/LCC9 cells in ovariectomized mice treated with vehicle, E_2, TAM, or ICI 182,780*[a]

Tumor	Mean TD (days)	ANOVA		
		Vehicle	E_2	Tamoxifen
Vehicle	79.03			
E_2	11.60	$P < 0.01$		
TAM	43.17	NS[b]	$P < 0.01$	
ICI 182,780	73.59	NS	$P < 0.01$	NS

[a] Data estimated from tumor growth curves (not shown) in five or more mice/group. Comparisons of TDs among groups were performed by ANOVA. MCF-7 cells do not grow in ovariectomized mice (20, 36). The TDs for MCF-7/LCC1 cells growing as tumors in these mice are approximately 39 days without E_2 and 14 days in E_2-supplemented mice (21).
[b] NS, not significant.

Expression of Mutant ER. We examined the ER mRNA species present in the MCF7/LCC9 cells by using a series of five PCR primer pairs that produce overlapping fragments of the mRNA encompassing nts −37 to 1739. This strategy ensures that all of the major domains are analyzed for potential mutations. All of the clones sequenced from each of the PCRs were wild-type (MCF-7) sequences. We found no evidence of deletions, point mutations, or alternative splice variants in the MCF7/LCC9 cells.

DISCUSSION

Until recently, there were few reasons to be concerned with the potential for cross-resistance among antiestrogens, because most drugs available for clinical use were triphenylethylene analogues representing relatively minor modifications to the structure of TAM, *e.g.*, Toremifene (chloro-TAM). There has been little compelling evidence that these drugs have any consistently significant differences in either their potencies or response patterns in experimental models and breast cancer patients, or that they have substantially different mechanisms of action. Patients who respond and then recur while receiving TAM generally show poor responses to a second triphenylethylene (45).

The new generation of steroidal antiestrogens appears to exhibit significant differences from the triphenylethylenes not only in their structural characteristics but also in their potencies and molecular mechanisms. However, the pattern of cross-resistance between triphenylethylenes and steroidal antiestrogens has not been widely studied in the clinic.

We have addressed the issue of acquired cross-resistance to antiestrogens in our experimental *in vitro* and *in vivo* models. The MCF7/LCC2 cells were selected for resistance to 4OH-TAM. When screened for their responsiveness to ICI 182,780 (23) and ICI 164,384 (24), we found that they are not cross-resistant and exhibit a dose-response relationship similar to their parental cells (MCF7/LCC1). This suggested that patients who responded to and then failed on TAM could respond to a steroidal antiestrogen (23). There is now clinical evidence to support this prediction. Patients who responded initially to TAM and subsequently failed have been crossed over to ICI 182,780. No cross-resistance between TAM and ICI 182,780 was seen in 69% of these patients (46).

We now report on cells selected for resistance to the steroidal antiestrogen ICI 182,780. These cells also acquire a cross-resistance to 4OH-TAM that emerges early in the selection. 4OH-TAM resistance is evident following three passages in only 0.1 nM ICI 182,780. In contrast, we began our selection against 4OH-TAM (generation of MCF7/LCC2) at 1 nM and obtained a reproducible pattern of 4OH-TAM resistance following selection in 1 μM 4OH-TAM. These cells (MCF7/LCC2) did not acquire cross-resistance to ICI 182,780 (23).

Because the ER is the primary target for antiestrogens, alterations in its expression, ligand-binding characteristics, and/or function could influence responsivity to antiestrogens. ICI 182,780 can change the rate of ER turnover (18) and might be expected to inhibit ER expression in the resistant cells. The ER levels in MCF7/LCC9 cells are unchanged relative to their parental cells when growing without E_2 or ICI 182,780. This may indicate either that cells become resistant by preventing ICI 182,780-induced effects on ER turnover, or that these effects are not central to its mechanism of antiestrogenic action. Alternatively, cells may acquire resistance by altering events "downstream" of the ER, thereby bypassing effects on receptor turnover.

Mutations in the *ER* gene have been reported in breast tumors (47–50) and could influence antiestrogen sensitivity. However, determining the likely functional significance of ER mutants has been difficult. Almost without exception, when mutant receptors are found, they are coexpressed with significant levels of wild-type receptors (47, 51). However, dominant negative mutant ERs are likely to have biological function. Such mutants could function either by binding wild-type receptor and/or squelching/preventing transcriptional activation even when bound to DNA, or be transcriptionally inactive and compete with wild-type receptor for DNA binding. A truncated ER (clone 4) has been associated with progression from estrogen dependence to estrogen independence (51).

An ER mutant that converts the ER's perception of TAM from partial agonist to agonist has been described (52). Because we detect a potential TAM stimulation of proliferation *in vivo*, we looked for ER mutant mRNAs by PCR, using a strategy that should detect this and any other previously reported ER mutants. However, we found no evidence of ER mutant expression. All of the mRNAs detected encode wild-type ER. The dissociation constants for ER binding also are unchanged (not shown). These observations suggest that altered receptors are unlikely to be responsible for the TAM/ICI 182,780 cross-resistance apparent in MCF7/LCC9 cells.

The mechanism(s) driving the early emergence of 4OH-TAM cross-resistance, which arises before full resistance to ICI 182,780, is unclear. Both 4OH-TAM and ICI 182,780 function primarily by influencing the gene transcription-activating functions of the ER. We have suggested previously that the effects of estrogens and antiestrogens, and perturbations in cellular responsiveness to ER-mediated events, reflect the regulation of one or more gene networks (17). These networks consist of groups of genes that are normally induced or repressed by E_2 in estrogen-dependent cells and may be driven by estrogens of either systemic or intratumor origin in estrogen-dependent or estrogen-responsive tumors. In estrogen-independent or antiestrogen-resistant cells, these networks may be regulated by a ligand-independent activated ER, perhaps reflecting differences in the pattern of ER coregulator expression and/or recruitment into ER-regulated transcriptional events (7, 53). The regulation of each network component (gene) may be either direct, *e.g.*, an estrogen-responsive element-like sequence in its promoter, such as pS2, or indirect, *e.g.*, because of the activities of another directly E_2-regulated component, such as transforming growth factor α/epidermal growth factor receptor-regulated genes. This hypothesis neither requires, nor precludes, ER mutations or a loss of ER expression to explain the

acquisition of antiestrogen resistance, cellular proliferation being driven by a ligand-dependent or -independent activation of ER (7).

The steroidal antiestrogens ICI 182,780 and ICI 164,384 are more potent than TAM, probably reflecting their higher affinity for ER (15). An analysis of the MCF7/LCC9 phenotype alone could be interpreted as indicating a single, E_2-regulated gene network, sensitive to either a TAM- or ICI 182,780-occupied ER. As the cells are selected against the more potent antiestrogen (ICI 182,780), they first become resistant to the less potent compound (TAM).

A single common network hypothesis would predict that selection against ICI 182,780 would induce some degree of cross-resistance to TAM. This is seen clearly in the emergence of the MCF7/LCC9 cells, in which the 4OH-TAM cross-resistance is accompanied by a decrease in sensitivity to ICI 182,780. The reverse association also might be predicted. However, 4OH-TAM-selected and -resistant MCF7/LCC2 cells do not exhibit any alteration in their sensitivity to ICI 182,780 (23). This suggests that selection against 1 μM 4OH-TAM may not sufficiently perturb the activities of a single network to affect its regulation by ICI 182,780.

TAM can induce ER dimerization and DNA binding and perhaps enable regulation of those functions mediated through the activity of only one of the two putative transcriptional activation functions of ER (estrogens activate both transcriptional activation functions 1 and 2). In contrast, the steroidal antiestrogens may inhibit ER dimerization (19), alter receptor turnover (18), and differentially recruit ER coregulator proteins into ER-driven transcription complexes. These differences could result in some genes within a single network being regulated differentially by triphenylethylene and steroidal antiestrogens.

Although constructing other network models to account for the various phenotypes is possible, the single-network hypothesis is the simplest and can explain both the MCF7/LCC2 and MCF7/LCC9 phenotypes and the early emergence of acquired resistance to TAM. We have described previously how such models may explain the apparent independence of the antiestrogen-resistant and estrogen-independent phenotypes by invoking a ligand-independent activation of the transcriptional regulatory properties of ER (7). This model also allows for the mitogenic response to E_2 seen *in vivo*, because any ligand-independent or weak ligand-dependent ER activation could be overridden by E_2 to produce a more potent regulation of the gene network.

The regulation of PgR expression may be one E_2-regulated gene that differentiates between the molecular events associated with 4OH-TAM resistance (MCF7/LCC2) and cross-resistance (MCF7/LCC9). PgR is induced by E_2 in MCF-7, MCF7/LCC1, and MCF7/LCC2 cells. However, the fold induction in MCF7/LCC2 (2.6-fold) is lower than that seen in MCF-7 cells (5.8-fold), more closely reflecting that seen in MCF7/LCC1 cells (2.5-fold). The PgR levels in E_2-treated MCF7/LCC2 cells are approximately one-third that seen in MCF-7 cells, the levels without E_2 treatment being essentially equivalent in both cell lines (Table 2). In marked contrast, PgR protein expression is not E_2 regulated in MCF7/LCC9 cells, and the mRNA levels are only weakly E_2 regulated. The PgR protein levels in MCF7/LCC9 cells are similar to, or higher than, those seen E_2-treated MCF-7 or MCF7/LCC1 cells.

The functional relevance of the up-regulation of PgR in MCF7/LCC9 cells is unclear. The essentially constitutive up-regulation of PgR may indicate that other members of an associated gene network also are constitutively up-regulated. It may be a subset of these other members of the network that are driving the MCF7/LCC9 phenotype. Many PgR-expressing breast tumors are sensitive to progestins and antiprogestins, and secondary clinical responses to these compounds are often seen in patients who initially responded to and subsequently failed on TAM (54). This may be evidence of a switch from an ER-activated to a PgR-activated gene network for the regulation of proliferation. Thus, the elevated PgR expression in MCF7/LCC9 cells could render these cells sensitive to antiprogestins. Studies to this effect are currently in progress.

The marked intratumor heterogeneity of steroid hormone receptor expression (55, 56) indicates that many tumors already contain ER-negative, antiestrogen-resistant cells. Thus, one mechanism of resistance could be a selection by antiestrogens for the ER-negative subpopulations (9). There is some evidence in support of this hypothesis (57, 58). However, the majority of breast cancer patients who respond to and subsequently fail on TAM do so while still expressing both ER and PgR (58). Both the MCF7/LCC2 and MCF7/LCC9 cells have acquired different patterns of antiestrogen resistance while retaining expression of ER and PgR.

The MCF7/LCC2 phenotype (23) predicted a clinical pattern of resistance that has only recently been observed (46). The acquisition of antiestrogen resistance and retention of ER and PgR expression in the MCF7/LCC9 cells also appear to reflect the biology of a proportion of patients with ER-positive breast tumors. The clinical relevance of a concurrent acquisition of TAM resistance with ICI 182,780 therapy awaits the data from appropriate clinical trials. However, the cross-resistance phenotype of the MCF7/LCC9 cells suggests that primary treatment with the more potent steroidal compounds may preclude subsequent response to a triphenylethylene.

There are other reasons to consider scheduling a triphenylethylene as the first-line antiestrogen. It seems likely that the Goldie-Coldman hypothesis (59) could apply to endocrine therapies as it does to cytotoxic therapies. Day's (60) modification of this hypothesis suggests that the sequential rather than alternating administration of drugs may be more effective. Furthermore, it is predicted that the greatest benefit would be derived by administering the lesser potent of two noncross-resistant drugs first, followed by the more potent drug (60, 61). If TAM and ICI 182,780 are considered to exhibit some degree of functional noncross-resistance, *e.g.*, a MCF7/LCC2 phenotype, patients may be better served by initial treatment with TAM, from which they also will obtain the benefits on cardiovascular performance and bone resorption, with a cross-over to ICI 182,780 upon relapse/progression. If some cells switch from an ER- to a PgR-driven phenotype, the inclusion of an antiprogestin/progestin-based third-line therapy might be worthy of consideration.

ACKNOWLEDGMENTS

We thank Drs. Ward Peterson, Joseph Kaplan, and Bharati Hukku of the Cell Culture Laboratory of the Children's Hospital of Michigan (Detroit, MI) for performing the karyotype and isozyme analyses.

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