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Caroline E. Connor 7/29/98
PI - Signature Date

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INTRODUCTION

Breast cancer is expected to afflict one in nine American women (1). It is the leading cause of cancer death among women and in most developed countries accounts for the most frequent type of cancer (2). Among primary breast tumors, 60% have been shown to express the estrogen receptor (ER), which confers to the cell the ability to respond to estrogen (3). Conversely, ER is poorly expressed in normal breast tissue. It remains to be determined whether in a tumor ER becomes expressed ectopically in a cell which does not normally contain receptor or if existing ER-positive cells are transformed. Although the role of ER in normal breast tissue remains unclear, it permits estrogen to exhibit a mitogenic effect on ER-positive breast cancer cells (4). Because of this action of estrogen, antiestrogens (synthetic compounds which block the actions of estrogen) have been used clinically to retard the progression of these tumors. The treatment of breast cancer patients with antiestrogens such as tamoxifen, albeit highly successful, unfortunately leads to unwanted side effects and, in most cases, resistance. This has led to a search for alternate treatment regimens in the areas of adjuvant therapy, tamoxifen-refractory cancers, and chemoprevention. In order to understand the effects of estrogen on various target tissues, it is important to better comprehend the complete biology of the estrogen receptor.

The estrogen receptor is a nuclear protein and member of the subfamily of steroid receptors which includes the progesterone, glucocorticoid, mineralocorticoid, and androgen receptors (5, 6). There exist several functional regions that are highly conserved among all members of this superfamily at the nucleotide and amino acid level (5, 6). At the carboxyl terminus lies the hormone binding domain, a large region of approximately 250 amino acids that is hydrophobic in character. The DNA binding domain, which exhibits the highest homology among receptors, consists of 66-68 amino acids and includes nine perfectly conserved cysteines. This domain forms two zinc fingers which confer sequence-specific DNA binding.

All steroid hormone receptors operate by a similar mechanism in which the latent receptor is bound in a multiprotein complex comprised of receptor, heat-shock protein 90 (hsp90), hsp70, p59, and other proteins (7). Upon ligand binding, these proteins are displaced and the receptor undergoes a conformational change followed by spontaneous dimerization (8). In this activated state the receptor can interact with specific DNA sequences (steroid response elements, or SREs) within target genes. The mechanism by which this event alters the transcription of target genes remains to be elucidated, although

several nuclear hormone receptor coactivators and corepressors have recently been cloned toward this end (9, 10).

The ability of ER to alter target gene transcription depends on the tissue context of the DNA-bound receptor. Tamoxifen, for example, enigmatically exhibits desirable antagonist activity in breast, partial agonist activity in the cardiovascular system and bone, but is unfortunately associated with uterine hypertrophy (11). A search for an improved structural analog of tamoxifen led to the discovery of GW5638 and its hydroxylated version, GW7604 (12). In animal studies, these compounds exhibit antiestrogenic effects in breast and offer bone and cardiovascular protection. Interestingly, GW5638 not only failed to demonstrate uterotrophic activity in ovariectomized rats but also opposed the partial agonist activity of tamoxifen in all cell and promoter contexts examined, suggesting that it is mechanistically distinct from previously studied antiestrogens (13). One hypothesis derived from these observations is that different compounds, working through the same receptor, can regulate different genes. In support of this, our laboratory has shown that ICI is able to upregulate a gene to a greater extent than estrogen. Preliminary data suggests that this gene is not, however, regulated by tamoxifen (J. Galluzzo, personal communication). Based on these observations, it is likely that GW5638 regulates a unique subset of genes, which would account for its distinct tissue-specific effects. These unique characteristics make GW5638 a likely candidate for the treatment of tamoxifen-refractory breast cancer patients. In order to evaluate this compound as a potential chemotherapeutic, its effects on two target organs (breast and bone) must be further assessed.

To address the use of this compound in breast cancer, we have chosen to exploit the MCF-7 athymic nude mouse tumor model. Animal models of human breast cancer can provide a valuable tool for studying not only the pathophysiology of the disease, but the effects of various endocrine manipulations as well. Athymic nude mice (14) are incapable of rejecting a tumor immunologically (15) and thus provide a good repository for human cancer cells. This type of model offers an advantage over an animal cancer model in that the malignancy is of human descent and retains many characteristics of the original tumor.

Athymic mouse models for human cancer have in the past provided an excellent system in which to gain information regarding endocrine therapies. The athymic mouse breast cancer model, which was inoculated with the estrogen receptor (ER)-positive (and thus estrogen-dependent) cell line MCF-7, was first demonstrated by Soule, et al (16). This type of breast cancer is hormone-dependent, receptor-positive, treatable with anti-hormone, and eventually fails treatment (develops resistance). Because the mice do not produce enough estrogen to support tumor growth, sustained release estradiol pellets are implanted to circumvent this problem.

The MCF-7 mouse model was well-characterized by Jordan's lab in the late 80's whose studies showed that tamoxifen caused an inhibition of estradiol-stimulated growth but did not cause tumor growth when implanted alone (4). These studies confirmed the tumoristatic (rather than tumoricidal) actions of tamoxifen and suggested that long-term treatment be given since tumor growth was consistently reactivated by estradiol. However, Gottardis, et al. (17) tried to create an *in vivo* model of tamoxifen failure and eventually saw the development of tamoxifen-dependent growth. Work by this laboratory and others confirmed that a novel, steroidal, pure anti-estrogen, ICI 164,384, could inhibit tamoxifen-stimulated growth of MCF-7 tumor variants in mice (18). This model was validated clinically with the subsequent use of ICI for the treatment of tamoxifen-resistant breast cancer, but its inability to protect against bone loss necessitates the exploration of superior compounds. Based on previous *in vitro* studies, we propose that, like tamoxifen, GW5638 will also be able to inhibit estrogen-dependent growth in the nude mouse model. Moreover, because this compound appears to be mechanistically distinct from tamoxifen, we expect no cross-resistance.

Our laboratory is also interested in examining the specific transcriptional effects mediated by GW5638 in bone. The protective benefits of estrogen on bone were clearly established with the observation of an extremely high incidence of osteoporosis upon menopause (19). Estrogen replacement therapy serves not only to prevent bone loss but to provide cardiovascular protection as well. Fortunately, tamoxifen also exhibits bone protection (20), hence bone loss is not a problem for breast cancer patients undergoing this type of endocrine therapy. Therefore, it is crucial that novel breast cancer therapeutics also be bone protective. The mechanism by which estrogen and partial agonists of its receptor manifest this protection remains unclear, yet may involve classical regulation of estrogen-responsive genes by the receptor. Our laboratory and others have shown that compounds which demonstrate varying degrees of partial agonist activity can all protect against bone loss in the ovariectomized rat model, with the exception of the pure antagonist ICI (13). This suggests that there is a feature common to these compounds which allows for bone protection but is insufficient for desirable activities in other tissues. To examine this, we are utilizing differential display PCR technology. This method allows the identification of differentially expressed genes based on various treatment regimens.

BODY

A. Tumor Inhibition Studies

Experimental Methods

All animal experimentation is done in collaboration with Dr. Mark Dewhirst at Duke University. The mice, which are from a genetically inbred nude mouse breeding colony designated as BALB/cUrd *nu nu*, are maintained in the Duke Comprehensive Cancer Isolation Facility.

Mice are housed in laminar flow hoods with sterile cages and bedding and fed *ad libitum* autoclaved Purina mouse chow and sterile water. As the MCF-7 cell line is estrogen-dependent, ovariectomized female mice are given 60-day sustained-release pellets containing 0.72 mg estradiol (Innovative Research of America, Toledo, OH) two days prior to cell injections or tumor implantation. Approximately five million MCF-7 cells per animal were required for tumorigenesis. Cells were suspended in 0.1 mL media and added to an equal volume of MATRIGEL Basement Membrane Matrix (Becton Dickinson Laboratories, Bedford, MA). Subsequently, cells were injected into the right flank of 10 animals, ranging in age from six to eight weeks. Tumors were measured using calipers, and when they reached a size of approximately 8mm x 8mm, they were removed from donor mice and homogenized. The homogenized tumors were injected (50 μ L per animal) into 50 mice. Tumors were measured daily in two dimensions (l and w) to monitor progress, and tumor area is calculated as $l/2 * w/2 * \pi (4)$. Animals not exhibiting tumor growth within two weeks were eliminated from the studies.

We initially performed a dose-response experiment in order to compare the efficacy of GW5638 to control- and tamoxifen-treated tumors. Once tumors were growing, groups (n=8) began receiving one of five possible daily injection regimens: Control, 0.3 mg GW5638, 0.6 mg GW5638, 1.0 mg GW5638, and 1.0 mg tamoxifen. All compounds were delivered in corn oil in a 0.1 mL injection. Tumor measurements continued throughout the experiment to assess tumor growth or inhibition.

At the end of this experiment, several groups of mice were continued on treatment: 0.6 mg GW5638, 1.0 mg GW5638, and 1.0 mg tamoxifen. Appropriate treatment was given every three days awaiting tumor outgrowth. Upon growth, tumors were passaged and reevaluated for both resistance to treatment and estrogen dependence. Ultimately, tamoxifen-resistant tumors were discovered and assayed for hormone dependence by passaging tumor out groups of mice receiving one of four treatments: Control, Estrogen

alone, Tamoxifen alone, and Tamoxifen + Estradiol. Estrogen was administered as estradiol pellets, and tamoxifen was administered via 1.0 mg injections. Control and estrogen groups received corn oil injections.

In a parallel experiment, while awaiting resistance, we began exploring the MCF-7/LCC2 cell line *in vivo* (21). These cells were selected *in vitro*, form tumors in nude mice, and are tamoxifen-resistant yet estrogen-sensitive. These tumors were examined in our mice to determine the ability of GW5638 to inhibit a tamoxifen-resistant tumor. The tumor line was injected into 32 mice as described for MCF-7 cells (without estradiol pellets). After tumors were established, four treatment groups were formed: Control, Estrogen, Tamoxifen, and GW5638. Estrogen mice received estradiol pellets, tamoxifen and GW5638 mice received 1.0 mg injections every three days, and control and estrogen mice received corn oil injections every three days. Tumors were measured with calipers and assessed for growth or inhibition.

Results and Discussion

Figure 1 represents the dose-response experiment after 6 weeks of treatment. Despite the death of a few mice from each group, the elimination of their data did not change the overall results. Statistical analyses revealed that each treatment group had a significant effect over control (ANOVA, $p < .05$) and that there was no significant difference between the two highest doses of GW5638 and tamoxifen. At this point, animals in the control group and the 0.3 mg GW5638 group were sacrificed. This experiment demonstrated that GW5638 can inhibit tumor growth in the MCF-7 athymic mouse model. Additionally, this compound has an efficacy equal to that of tamoxifen at the highest dose administered. This supports our hypothesis that GW5638 offers potential as a breast cancer therapeutic.

Upon the completion of the dose-response experiment, we were interested in two pieces of data: (1) the effect of treatment on estrogen receptor and progesterone receptor levels in these tumors and (2) the development and characterization of antiestrogen resistance. Choosing to explore the latter area first, remaining animals continued to receive treatment every three days with the expectation of tumor outgrowth due to treatment failure as described in the Experimental Methods section. Unfortunately, only a few of the tamoxifen-resistant tumors grew, but these were passaged and treated as described. The results of this experiment are shown in **Figure 2** with each tumor depicted individually. The data demonstrate an inherent heterogeneity of tumor as the tumor growth fluctuated within groups. Statistical analyses revealed that the tumors were in fact tamoxifen-dependent but estrogen-independent. Currently, the tumors that grew the largest are being

passed and are receiving continued treatment with tamoxifen. The next step will be to determine if GW5638 can inhibit the growth of this tamoxifen-resistant tumor, which will suggest its utility in tamoxifen-refractory breast cancer.

The LCC2 study was completed after 8 weeks of treatment. Results, as shown in **Figure 3**, were unexpected. Previous data suggested that the tumors should grow in the presence of tamoxifen and that estrogen potentiates this growth over control (21). However, we found this tumor to be completely estrogen independent. Additionally, tamoxifen and GW5638 were able to inhibit the growth of this tumor. This suggests that GW5638 can inhibit the growth of an estrogen-independent tumor.

Recommendations

The initial dose-response experiment was successful. Our next action is to determine the ability of GW5638 to inhibit tamoxifen-resistant tumors. We also intend to repeat the dose-response study and use the tumors for analyses of receptor levels, as mentioned previously. Recent data from our laboratory suggest that GW5638 causes estrogen receptor downregulation in MCF-7 cells *in vitro* (A. Wijyaratne, personal communication). Ultimately we will compare the time to resistance for GW5638 versus tamoxifen to determine if GW5638 has potential as a chemopreventive agent.

B. Differential Display PCR

Experimental Methods

All work was done in collaboration with Dr. Dave Morris and colleagues at Glaxo-Wellcome. Female Sprague-Dawley rats were ovariectomized at six months of age. On day 15 post-ovariectomy, animals were treated via two subcutaneous injections (one a.m., one p.m.) with Estradiol (10 µg/kg), Tamoxifen (100 µg/kg), GW5638 (5 mg/kg) or control vehicle (cotton oil). Animals were sacrificed on day 16 post-ovariectomy and tissues were harvested. Total RNA was extracted from pulverized bone using an RNA Isolation Kit (Stratagene, La Jolla, CA) and subsequently used in differential display PCR by means of the RNImage kit (GenHunter Corporation, Nashville, TN). This technique (22, 23) will allow us to examine genes which are expressed exclusively in response to GW5638 as compared to control.

Total RNA was obtained and reverse transcribed into partial cDNA sequences through PCR amplification. Reactions were performed in duplicate, using unique combinations of one base anchored poly-T and random 13 bp upstream primers. Three different RNImage kits were used, translating into 24 upstream primers in combination

with the three poly-T primers. Samples were run on 6% denaturing polyacrylamide gels after being labeled by radioisotope to permit autoradiographic detection of bands. Genes regulated uniquely by specific compounds were found by comparing different treatment lanes. The differential display PCR technique is diagrammed in **Figure 4**. These bands were cut from the gel, their cDNA extracted, and subsequently reamplified via PCR using the original set of primer conditions.

Potential cDNA bands were subcloned into Teasy vectors (Promega, Madison, WI). Once cloned and verified, plasmids were amplified for use in sequencing and Northern blot analysis. Northern blots containing samples of total RNA from each of the four treatment groups were probed with labeled cDNA bands.

Results and Discussion

We did not obtain a large number of putative bands from the differential display PCR technique. However, several potential bands were cloned which appeared to have differential regulation in bone by the various compounds. Unfortunately, Northern blot analysis did not reveal any differential regulation at the RNA level.

Recommendations

Differential display PCR permits the comparison of genes regulated in either a direct or indirect manner by certain hormones. False positives, however, are not uncommon in this technique. We have continued to screen cDNAs in anticipation of finding at least one differentially regulated transcript. The identification of GW5638-responsive genes in bone will provide a surrogate marker for the action of this compound. In addition, this would offer insight into the mechanism by which ER modulators protect against bone loss.

CONCLUSIONS

The MCF-7 athymic mouse model is now functional in our laboratory. The results of the dose-response experiment indicate that GW5638 is capable of inhibiting estrogen-induced tumor growth, as expected. Additional information from the MCF-7/LCC2 tumor experiment reveals that GW5638 can also inhibit the growth of estrogen-independent tumors, suggesting an alternate mechanism for inhibition. Through continued treatment with tamoxifen and passaging of tumors, we have finally obtained what we consider to be tamoxifen-resistant tumors. The treatment of these tumors with GW5638 will allow us to determine if this compound has utility as a therapy for tamoxifen-refractory breast cancer. Repeating the dose-response experiment will allow us to examine the levels of steroid hormone receptors in the tumors following antiestrogen therapy.

The search for genes regulated by antiestrogens in bone has not yet been completed. However, the technique is operational and minor modifications may provide better results. We have continued to screen for differentially regulated cDNAs and are in the process of Northern analysis.

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APPENDICES

Effect of antiestrogen treatment on MCF-7 breast cancer tumors in nude mice

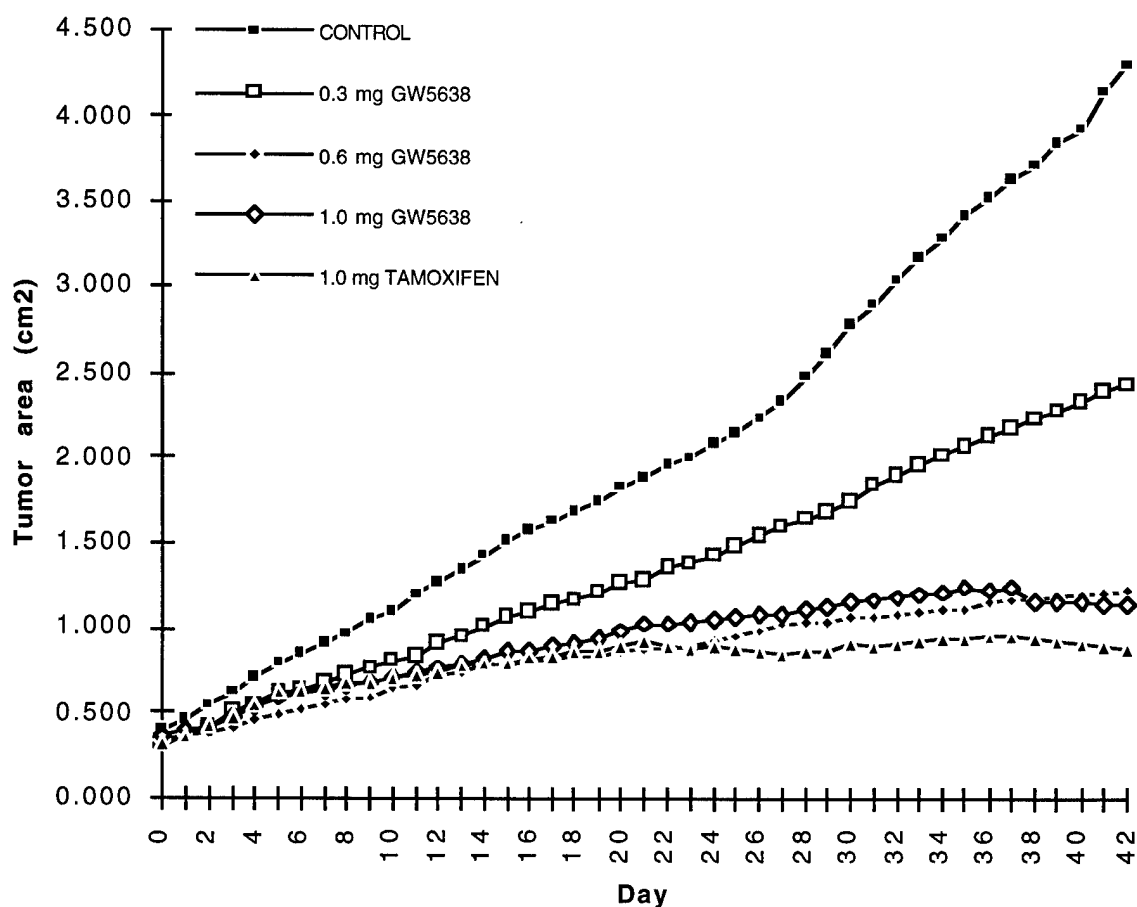


Figure 1.

GW5638 is able to inhibit MCF-7 tumor growth as well as tamoxifen in nude mice. Day 0 indicates the first day of treatment, 2 weeks after inoculation of tumors. Statistical analyses revealed that each treatment group had a significant effect over control (ANOVA, $p < .05$) and that there was no significant difference between the two highest doses of GW5638 and tamoxifen.

Characterization of Tamoxifen-Resistant Human Breast Cancer Xenografts in Athymic Mice

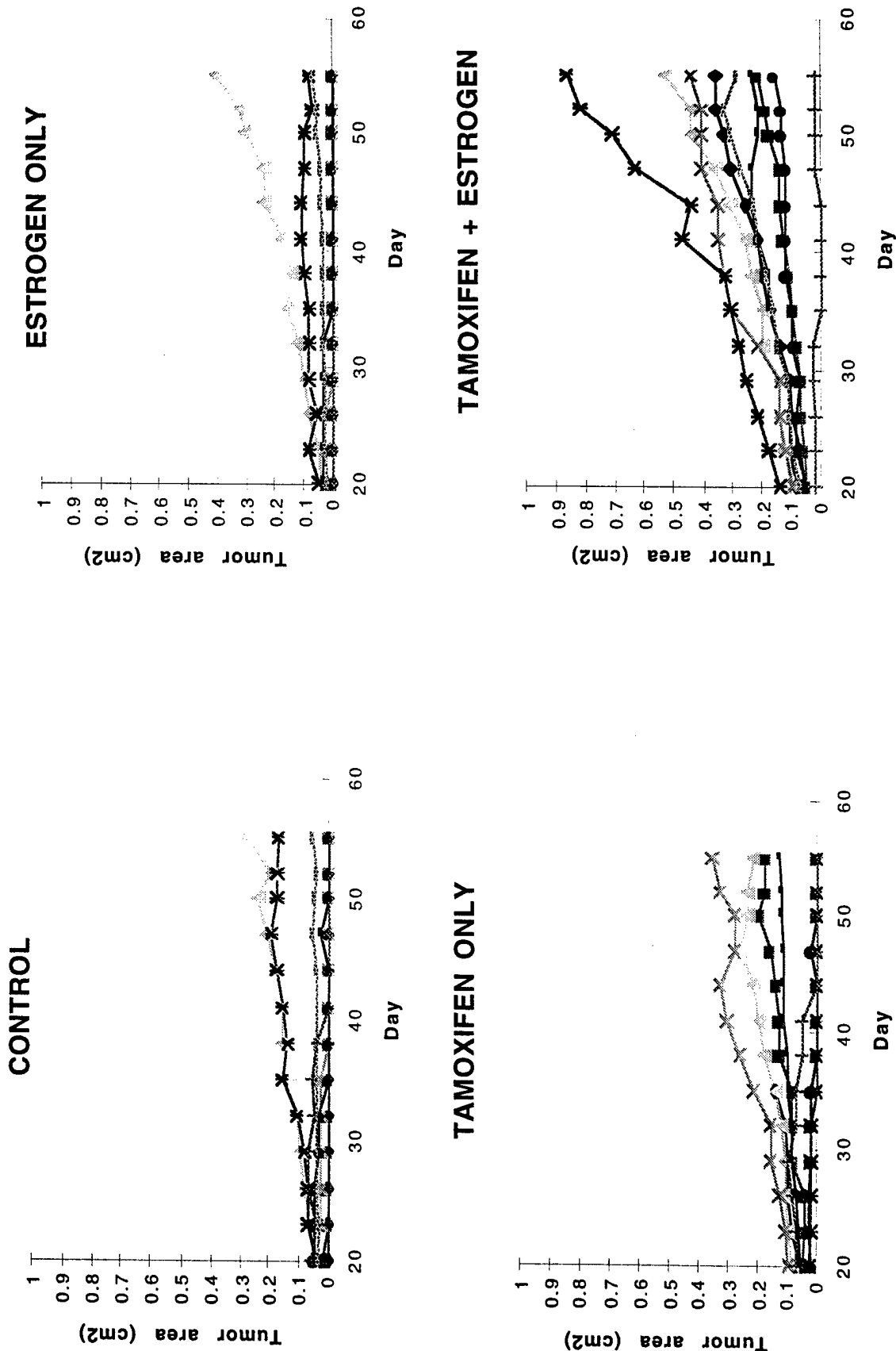


Figure 2. Evaluation of tamoxifen-resistant tumors in nude mice. Each line represents an individual tumor. Statistical analyses determined that the tumor was estrogen-independent and tamoxifen-dependent.

Effect of antiestrogen treatment on LCC2 breast cancer tumors in nude mice

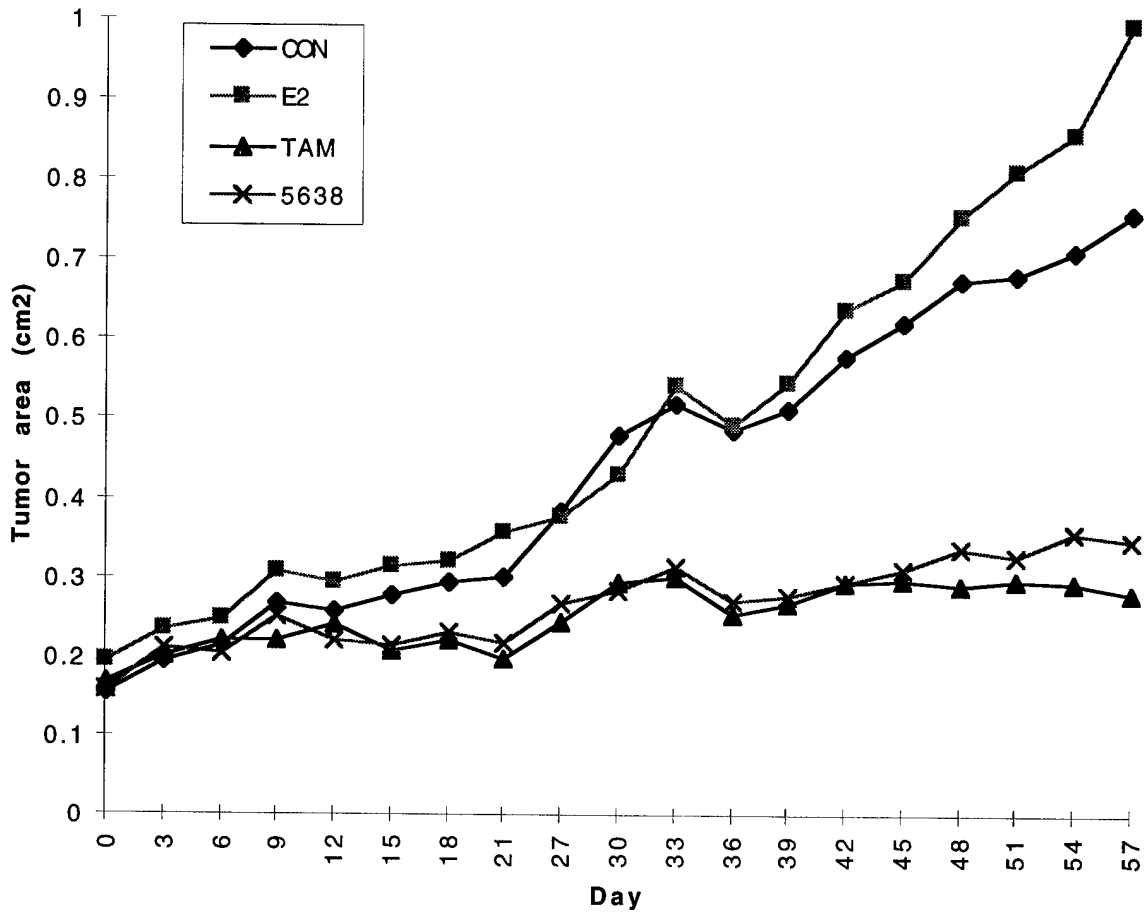


Figure 3. LCC2 tumors grow in the absence of estrogen in nude mice. Both tamoxifen and GW5638, given as 1.0 mg drug/0.1 ml corn oil every three days, inhibit the growth of this tumor. Day 0 indicates the first day of treatment.

(LCC2 cells provided by Dr. Robert Clarke at Lombardi Cancer Center)

Differential Display PCR

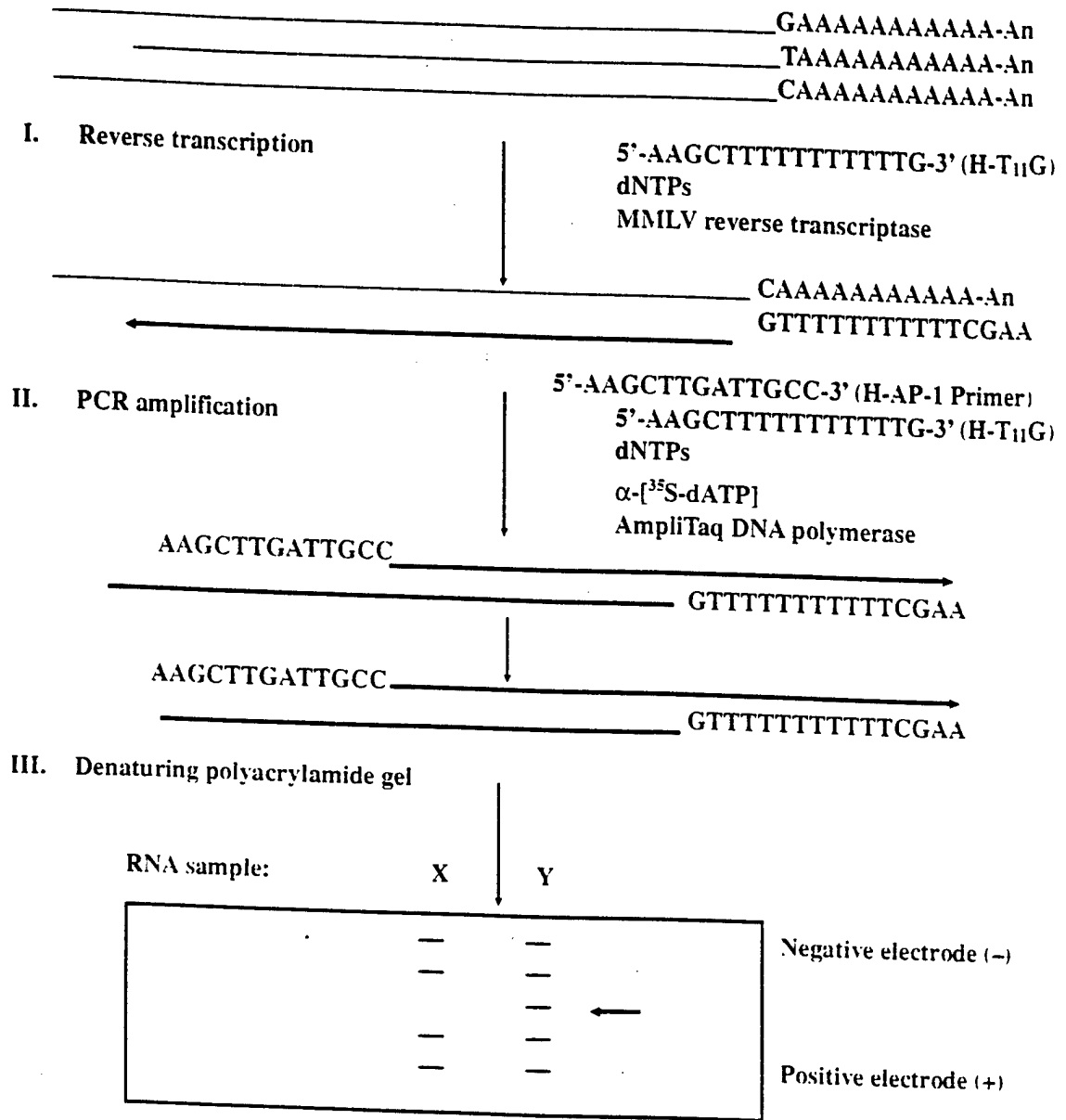


Figure 4. Schematic representation of the mRNA differential display technique as a tool for identifying and cloning differentially expressed genes. The two gel lanes represent cells treated with either compound X or compound Y. The band uniquely displayed in the right lane (depicted by the arrow) indicates an mRNA species induced only by treatment with compound Y.