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13. ABSTRACT (Maximum 200) Tamoxifen, the most widely used adjuvant therapy for the treatment of estrogen receptor-positive breast cancer, has been shown to inhibit the growth of MCF-7 breast cancer cells in athymic mice. However, in both the animal model and clinical setting, therapeutic utility is limited by the development of resistance to the drug. Our goal is to determine whether GW5638, a structurally similar yet mechanistically distinct compound, can inhibit the growth of tamoxifen-refractory tumors. We demonstrated that GW5638 was able to inhibit the growth of estrogen-dependent MCF-7 tumors in athymic mice. We subsequently developed tumor variants which are no longer sensitive to the inhibitory effects of tamoxifen; the tumors in fact require tamoxifen, but not estrogen, for growth. The resistant tumors were implanted into athymic mice treated with tamoxifen, GW5638, or both drugs. Two important findings emerged from these studies: 1) GW5638 alone cannot support the growth of tamoxifen-dependent tumors, and 2) GW5638 inhibits the growth of these tumors in the presence of tamoxifen. We conclude that tamoxifen-refractory tumors are not cross-resistant to GW5638, despite the structural similarities of the two compounds. These data support the therapeutic potential of GW5638 for the treatment of tamoxifen-resistant breast cancers.					
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FOREWORD

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Caroline E. Connor 6/23/99
PI - Signature Date

TABLE OF CONTENTS

I.	Front Cover	Page 1
II.	Report Documentation Page	Page 2
III.	Foreword	Page 3
IV.	Table of Contents	Page 4
V.	Introduction	Pages 5
VI.	Body	Pages 6-10
VII.	References	Page 11
VIII.	Appendix	Pages 12-15

INTRODUCTION

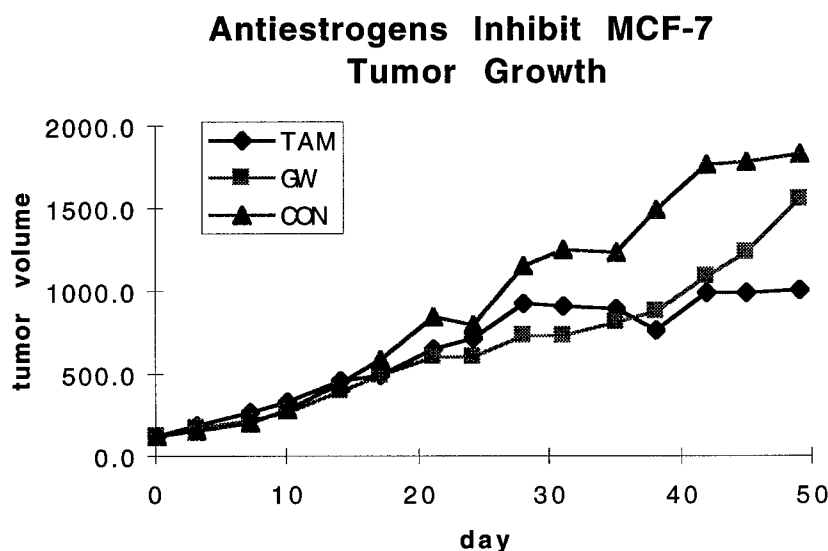
Estrogen mediates its biological responses through a specific interaction with the estrogen receptor (ER), an intracellular transcription factor which can bind specific DNA sequences and regulate gene expression (1). SERMs (selective estrogen receptor modulators) also bind to ER but induce unique conformations in the protein, allowing for tissue-selective effects (2, 3). One such compound, tamoxifen, has proven clinically useful in the treatment of ER-positive breast cancer. However, despite its bone-protective capabilities, tamoxifen has undesirable uterotrophic effects and, during treatment of breast cancer, resistance to the compound eventually develops (4). Therefore, there is a clinical need for improved SERMs in breast cancer therapy and prevention. GW5638, a compound which has been shown to be bone-protective and non-uterotrophic in animal models, appears to be mechanistically distinct from tamoxifen in a variety of *in vitro* and *in vivo* assays (5). This suggests that GW5638 may offer utility against tamoxifen-refractory breast cancers. The goal of this project was to determine if GW5638 could inhibit the growth of tamoxifen-resistant tumors in an athymic mouse model. Additionally, in an effort to understand the property common to estrogens and SERMs that permits their action as bone-protective agents, we sought to identify GW5638-responsive genes in bone.

BODY

A. Tumor Inhibition Studies

The MCF-7 breast cancer model is well-established in athymic mice. The cell line is estrogen-dependent and tumors are growth inhibited by tamoxifen (6, 7). For these experiments, ovariectomized (OVX) female mice are given slow-release estrogen pellets prior to tumor implantation. We first evaluated the potential of GW5638 to inhibit the growth of MCF-7 tumors. Experimental groups were as follows: 1) placebo injections, 2) tamoxifen injections, or 3) GW5638 injections. Similar to tamoxifen, GW5638 inhibited tumor growth (Figure 1). We are currently repeating this experiment to verify the results, confident that GW5638 has inhibitory effects.

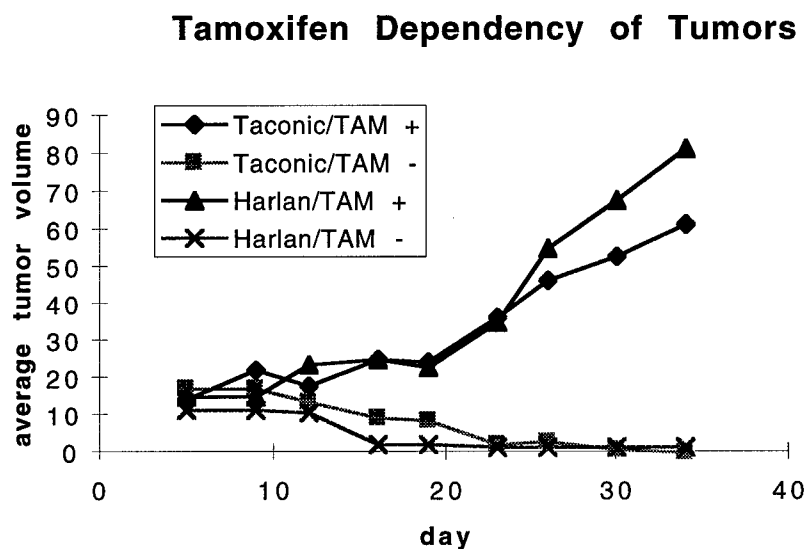
Figure 1.



Tamoxifen, the standard adjuvant therapy for estrogen receptor (ER)-positive breast cancer, offers initial success but eventually leads to resistance in many patients (8). This paradigm is also seen in the athymic mouse model, in which other laboratories have shown that continued treatment with tamoxifen leads to a tumor which becomes dependent upon rather than inhibited by the drug (9). One of the goals of this project was to assess whether GW5638 could inhibit the growth of tumors no longer sensitive to tamoxifen inhibition. In order to complete this aim, we first had to develop and characterize a series of tamoxifen-resistant tumors. This was accomplished over the course of several years through continuous dosing with tamoxifen and repeated tumor passaging from animal to animal.

Once tumors began to consistently grow in the presence of tamoxifen, they developed an estrogen-independent phenotype, unlike the parental MCF-7 tumor. This phenomenon is consistent with previous results from other laboratories (9). When analyzed in athymic mice supplied by two different companies, animals with putative tamoxifen-resistant tumors were treated with and without tamoxifen for 5 weeks, as seen in Figure 2. Tumors grew only in animals dosed with tamoxifen; we had created the expected tamoxifen-dependent tumor variant.

Figure 2.

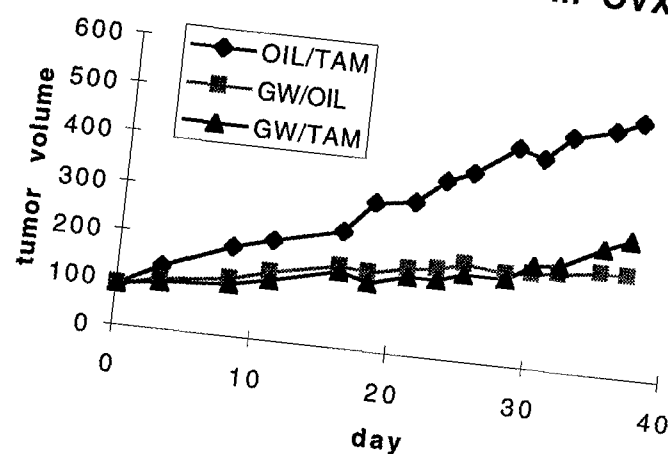


Once the tamoxifen-dependent tumors were characterized, we tested the ability of GW5638 to inhibit their growth. Initiation of experiments was difficult due to low tumor take rate and extremely slow growth rate of these tumors as compared to original MCF-7 tumors. Animals were treated with tamoxifen injections three times weekly to establish tumor growth, at which time animals were randomized by tumor volume into three groups. The first treatment group was given injections of both tamoxifen and oil to demonstrate the growth of tumors in the presence of tamoxifen. The second group was given injections of GW5638 and oil to determine whether the tumors could be stimulated with GW5638. We predicted that GW5638 would not stimulate tumor growth, as we believe this compound is mechanistically distinct from tamoxifen. The third group was given injections of both tamoxifen and GW5638 to test for the ability of GW5638 to inhibit the growth of tamoxifen-stimulated tumors. This experiment was performed initially in OVX athymic mice and the results demonstrate that a) GW5638 does not stimulate the growth of the

tamoxifen-dependent tumors and b) GW5638 can inhibit the growth of such tumors in the presence of tamoxifen (Figure 3).

Figure 3.

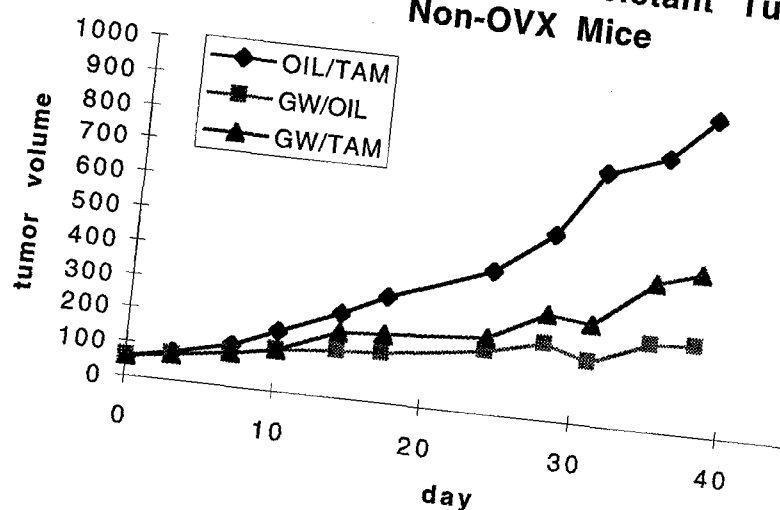
Inhibition of Tamoxifen-resistant Tumors by GW5638 in OVX mice



To verify this experiment, we repeated it in non-OVX mice, a more clinically relevant model, and obtained similar results, as shown in Figure 4.

Figure 4.

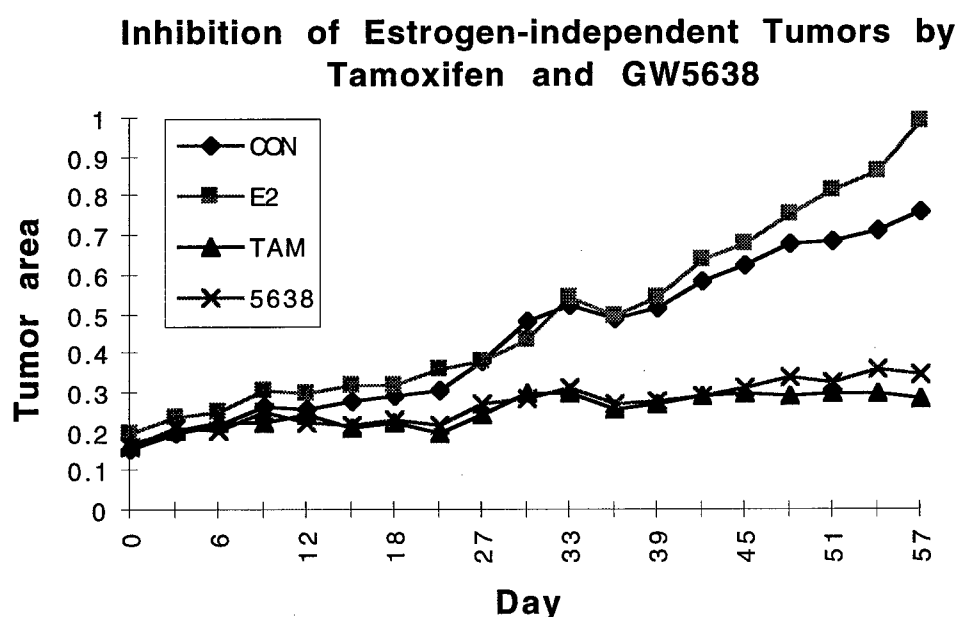
Inhibition of Tamoxifen-resistant Tumors in Non-OVX Mice



During the months of development of the tamoxifen-resistant tumors, we tested the effect of GW5638 on the MCF-7/LCC2 cell line *in vivo*. This cell line, derived *in vitro*

from the parental MCF-7 cell line at the Lombardi Cancer Center, is reported to be hormone-independent, sensitive to estrogen, and resistant to the growth inhibitory effects of tamoxifen when analyzed in the athymic mouse model (8). We performed a tumor inhibition experiment comparing treatments with placebo, estrogen, tamoxifen, and GW5638. As demonstrated in Figure 5, in our experiment, the tumor variant grew equally well in the absence or presence of estrogen and both SERMs tested were able to inhibit its growth. This suggests that GW5638 can inhibit the growth of an estrogen-independent tumor.

Figure 5.



B. Identification of GW5638-Responsive Genes

Despite the observation that all ER ligands, except pure antiestrogens, are protective against bone loss, specific genes which mediate this effect have not been identified (10). We attempted to identify GW5638-regulated genes in bone using differential display PCR (ddPCR) (11, 12). Female rats were OVX at 6 months of age, and animals were treated with either estrogen, tamoxifen, GW5638, or vehicle on day 15 post-OVX. Total RNA from pulverized bone was pooled for each treatment group. This RNA was reverse transcribed into partial cDNA sequences through PCR amplification, prior to being used in extensive ddPCR analysis. Bands which appeared to be uniquely regulated in several repeated

expression may have been hard to detect when using RNA blots composed of a variety of different bone cell types.

Due to our interest also in identifying genes regulated by estrogen itself in bone, we sent RNA samples from vehicle- or estrogen-treated animals to Genome Systems, Inc. An analysis was performed using this company's verified human microarray, consisting of approximately 7,000 clones, in hopes of finding regulated genes. Only two genes were shown to be regulated by estrogen treatment; however, the subsequent Northern analysis could not detect any changes in RNA levels.

Our difficulty in identifying estrogen- or GW5638-regulated genes in bone was disappointing. However, one possible explanation is that GW5638 may not, in fact, regulate a unique subset of genes. Additionally, the fact that many classes of SERMs are bone-protective may be due to non-genomic effects of these compounds, which would not have been seen in the aforementioned analyses.

Conclusions

We conclude that GW5638 is able to inhibit the growth of MCF-7 breast cancer tumors in mice. Additionally, we have created a tamoxifen-resistant tumor variant which is dependent upon tamoxifen for growth. GW5638, a mechanistically distinct compound, can inhibit the growth of such tumors and does not stimulate these tumors when given alone. Unfortunately, our efforts to identify GW5638-responsive genes were ineffective; this is not surprising as specific SERM-regulated genes have not yet been identified, perhaps indicating that non-genomic effects may play a role in mediating bone-protection.

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APPENDIX

1) Key Research Accomplishments

- Demonstrated the ability of GW5638 to inhibit the growth of MCF-7 breast cancer tumors in athymic mice.
- Developed tamoxifen-resistant tumors *in vivo*.
- Demonstrated the ability of GW5638 to inhibit the growth of tamoxifen-resistant breast cancer tumors in athymic mice.

2) Reportable Outcomes

Manuscripts/Abstracts/Presentations

1. **Connor, C.E.**, Norris, J.D., Gottardis, M.M., Willson, T.M., Dewhirst, M.W., and McDonnell, D.P. Inhibition of tamoxifen-refractory breast cancer by GW5638. (1999) *In preparation*.
2. **Connor, C.E.**, Norris, J.D., Dewhirst, M.W., and McDonnell, D.P. Therapeutic Potential of GW5638 for the Treatment of Tamoxifen-Resistant Breast Cancer. (1999) SPORE Investigators Workshop, Rockville, MD *Selected talk*.
3. **Connor, C.E.**, Buxton, J., Dewhirst, M.W., and McDonnell, D.P. Evaluation of GW5638, a novel antiestrogen, as an antitumor agent in nude mice. (1997) 20th Annual San Antonio Breast Cancer Symposium, San Antonio, TX *Poster presentation*.
4. **Connor, C.E.**, Buxton, J., Dewhirst, M.W., and McDonnell, D.P. Evaluation of GW5638, a novel antiestrogen, as an antitumor agent in nude mice. (1997) SPORE Investigators Workshop, Rockville, MD *Poster presentation*.

Patents

1. McDonnell, D.P., Norris, J.D., **Connor, C.**, and Wijayaratne, A. (1998) A method of preventing or treating estrogen-dependent diseases and disorders. (pct. app.)

Development of tumor line

1. Development of tamoxifen-dependent MCF-7 tumor variants in athymic mice.

3) Cited Abstracts (attached)

SPORE Investigators Workshop, 1999

Therapeutic Potential of GW5638 for the Treatment of Tamoxifen-Resistant Breast Cancer

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SERMs (selective estrogen receptor modulators) are believed to regulate estrogen receptor (ER) transcriptional activity by inducing distinct conformational changes in the ligand-binding domain of the receptor, each of which has a unique biological consequence. Tamoxifen is the most widely used SERM in the treatment of breast cancer; however, its therapeutic utility is limited by the eventual development of resistance. We believe that this may result from the ability of tamoxifen to switch from an antagonist to a partial agonist in breast cancer cells. We hypothesize that compounds which are mechanistically distinct from tamoxifen may be able to inhibit the growth of tamoxifen-resistant tumors. Toward this goal, our laboratory has identified GW5638, a SERM which lacks partial agonist activity. Additionally, using phage display technology, we identified peptides which bind to ER specifically in the presence of tamoxifen and not GW5638. This demonstrates that the two compounds expose unique surfaces on ER, attesting to their mechanistic differences. These data provide a rationale for the examination of GW5638 as a treatment for tamoxifen-refractory breast cancer tumors.

For our pre-clinical evaluation of GW5638, we developed several MCF-7 cell breast cancer tumor variants in mice which are resistant to the tumoristatic effects of tamoxifen. Unlike the parent tumor line, these tumors actually require tamoxifen, but not estrogen, for growth. The resistant tumors were implanted into ovariectomized (OVX), athymic mice and animals were treated with tamoxifen alone, GW5638 alone, and a combination of tamoxifen and GW5638 at equal doses. Two important findings emerged from these studies: 1) GW5638 alone cannot support the growth of tamoxifen-dependent tumors, and 2) GW5638 inhibits the growth of these tumors in the presence of tamoxifen. These results were confirmed in a similar experiment conducted in non-OVX mice, a more clinically relevant paradigm. We conclude that tamoxifen-refractory tumors are not resistant to GW5638, despite the structural similarities of the two compounds. Cumulatively, these data support the introduction of GW5638 into the clinic for the treatment of tamoxifen-resistant breast cancers.

20th Annual San Antonio Breast Cancer Symposium, 1997

EVALUATION OF GW5638, A NOVEL ANTIESTROGEN, AS AN ANTITUMOR AGENT IN NUDE MICE. Connor CE*, Buxton J, Dewhirst, MW, and McDonnell DP, Duke University Medical Center, Durham, North Carolina 27710.

Using the athymic nude mouse model, it has been previously demonstrated that tamoxifen is able to inhibit the growth of MCF-7 breast cancer tumors. Continued treatment with the drug resulted in resistance which actually manifests as tamoxifen dependence. GW5638 is a recently developed tamoxifen analog which offers bone and cardiovascular protection. Interestingly, this compound not only fails to demonstrate uterotrophic activity in ovariectomized rats but also opposes the partial agonist activity of tamoxifen in all cell and promoter contexts examined, suggesting that it is mechanistically distinct from previously studied antiestrogens. These unique characteristics make GW5638 a likely candidate for the treatment of tamoxifen-failed breast cancer patients. *In vivo* experiments were conducted to determine whether this compound would behave as tamoxifen in the nude mouse breast cancer model. A dose-response experiment revealed that at equivalent doses, GW5638 suppressed tumor growth as well as tamoxifen. To determine whether this compound can rescue tamoxifen-failed tumors, efforts are underway to provoke failure through long-term tamoxifen treatment. An alternative approach has been to use MCF-7/LCC2, a cell line variant which no longer responds to tamoxifen treatment. Previous studies have indicated that only ICI, the pure steroidal antagonist, can inhibit the growth of this cell line *in vitro*. Our current studies will allow us to ascertain if GW5638 can inhibit growth of the tumor *in vivo*.

We conclude that GW5638 is as efficacious as tamoxifen in its ability to inhibit the growth of MCF-7 breast cancer tumors in nude mice and may be able to rescue tamoxifen-failed tumors.

SPORE Investigators Workshop, 1997

EVALUATION OF GW5638, A NOVEL ANTIESTROGEN, AS AN ANTITUMOR AGENT IN NUDE MICE

Caroline E. Connor, Jake Buxton, Mark W. Dewhirst, and Donald P. McDonnell
Duke University Medical Center, Durham, NC

Estrogen mediates its biological responses through a specific interaction with the estrogen receptor (ER), an intracellular protein located within target cell nuclei. Following binding to latent receptor, hormone promotes conversion to a transcriptionally active form which can bind specific DNA sequences and regulate gene expression. Antiestrogens, despite their ability to deliver ER to DNA, freeze the receptor in different conformational states, all of which demonstrate reduced transcriptional activity in classical ER response systems. Recent evaluations of the biological activity of ER antagonists *in vitro* have led to the classification of antiestrogens into three distinct groups represented by tamoxifen, keoxifene, and ICI. Thus, a link was established between receptor structure and biological activity.

The partial agonist 4-OH tamoxifen is widely used as adjuvant therapy for the treatment of breast cancer. Tamoxifen enigmatically exhibits desirable antagonist activity in breast, partial agonist activity in the cardiovascular system and bone, but is unfortunately associated with uterine hypertrophy. GW5638 is a recently developed tamoxifen analog which offers bone and cardiovascular protection. Interestingly, this compound not only fails to demonstrate uterotrophic activity in ovariectomized rats but also opposes the partial agonist activity of tamoxifen in all cell and promoter contexts examined, suggesting that it is mechanistically distinct from previously studied antiestrogens. These unique characteristics make GW5638 a likely candidate for the treatment of tamoxifen-failed breast cancer patients.

Using the athymic nude mouse as a model, we have demonstrated that GW5638 is able to potently inhibit the growth of MCF-7 breast cancer tumors. To determine whether this compound can rescue tamoxifen-failed tumors, efforts are underway to provoke failure through long-term tamoxifen treatment. An alternative approach has been to use MCF-7/LCC2, a cell line variant which no longer responds to tamoxifen treatment. Previous studies have indicated that only ICI, the pure steroidal antagonist, can inhibit the growth of this cell line *in vitro*. Our current studies will allow us to ascertain if GW5638 can inhibit growth of the tumor *in vivo*. Future work involves the identification of genes regulated by this compound as a means of understanding its tissue-selective action.



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