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13. ABSTRACT (Maximum 200 Words)

Estrogens and antiestrogens are important in the development, treatment and possible chemoprevention of breast cancer. Rapid estrogen responses happen too quickly to involve transcription and there is much debate as to the mechanisms by which they operate and their relevance to breast cancer. This proposal aims to design and use selective chemical probes to answer those questions. Progress has been made in the synthesis of a screening panel to probe the role of rapid estrogen responses in breast cancer proliferation and resistance chemotherapy. We have generated new, potent compounds based on tamoxifen and estradiol core structures including endoxifen, a compound since discovered to be a major bioactive metabolite of tamoxifen and possibly a predictor of successful tamoxifen response in patients. We have shown for the first time that a number of SERMs like tamoxifen and raloxifene can activate ERK phosphorylation identically to estradiol. We have also generated the first tamoxifen-polymer conjugate that is capable to binding to estrogen receptor in vitro as well as activate rapid responses in breast cancer cell lines. Ongoing studies will use these compounds to better understand rapid estrogen signaling and make better therapeutic and chemopreventive agents for breast cancer.

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Introduction
Estrogens and antiestrogens are of utmost importance in the development, treatment and possible chemoprevention of breast cancer. Although much progress has been made in understanding the mechanisms by which estrogen and SERMs function, a class of mechanisms that is getting increased scrutiny is the rapid responses. These responses happen too quickly to involve gene transcription and there is much debate as to the receptors responsible for these responses and the mechanisms by which they operate. This proposal aims to design and use selective chemical probes to begin to answer those questions. In particular, estrogen responses related to breast cell proliferation and resistance to apoptosis will be studied using a variety of chemical probes including polymer-based drugs designed to test the potential role of cell surface estrogen receptors.

Body
This project has focused on developing and testing chemical probes of rapid responses to estrogen relevant to the treatment and chemoprevention of breast cancer. The proposed project was broken into 4 main tasks:

1. Determine the effects of the ligand structure on both rapid signaling and estrogen receptor-mediated transcription by testing a screening library of various known and novel estrogen response modulators in a number of assays.
2. Test the potential role of estrogen receptor alpha (ERα) or ERβ in rapid signaling, by performing assays with various N-terminal deletion and chimeric mutants of ERα and ERβ.
3. Test the role of cell surface receptors in rapid estrogen signaling, by developing cell-impermeable, non-proteinaceous estradiol conjugates.
4. Test the potential role of rapid estrogen signaling in breast cancer proliferation and survival, by treating various breast cell lines with selective compounds discovered above and measuring changes in cell growth, cytotoxicity and apoptosis

As will be described in the rest of this report, much significant progress has been made with task 1 and 3 while only initial progress in the other tasks has been made due to some difficulties encountered in assay development. Potential solutions have been found to circumvent those difficulties and it is expected that progress will be made in the coming months. As will be described later in the report, it is clear from our first year of work on this project that rapid responses have distinct but overlapping structure-activity relationships compared to the better-understood transcriptional responses. This has important implications in ligand design of hormone-based breast cancer treatments. An update on the research follows and is organized by the specific tasks of the statement of work.

Task 1. Determine the effects of the ligand structure on both rapid signaling and estrogen receptor-mediated transcription by testing a screening library of various known and novel estrogen response modulators in a number of assays.
<u>*Task 1a.*</u> *Generate the screening library by synthesizing a small number of estradiol and triphenylethylethylene analogs and combining it with commercially*

available and previously synthesized compounds. (Months 1-6)
The initial screening proposed is shown in **Figure 1** including a few compounds that were not in the initial panel that have since been synthesized.

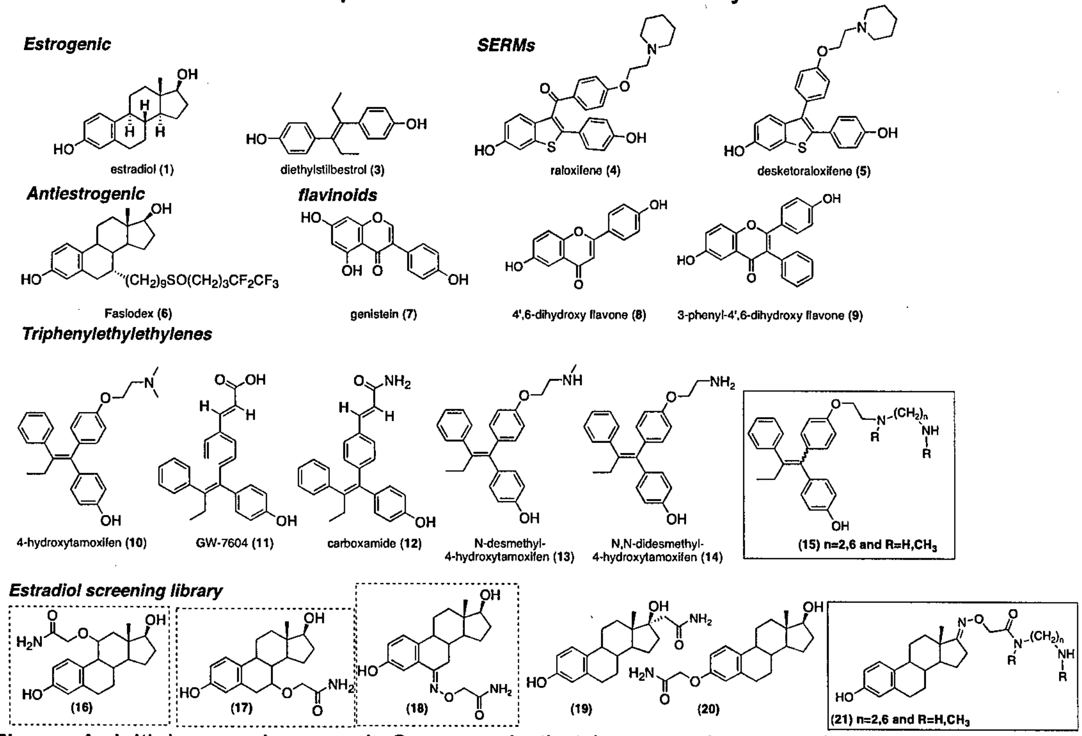


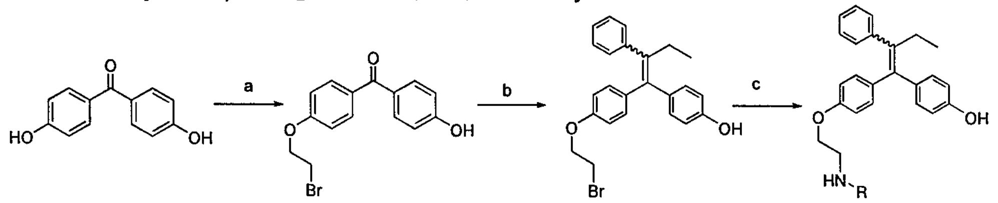
Figure 1. Initial screening panel. Compounds that have not been synthesized are boxed with dashed boxes. Compounds that have been synthesized that were not part of the proposed panel are boxed in solid boxes.

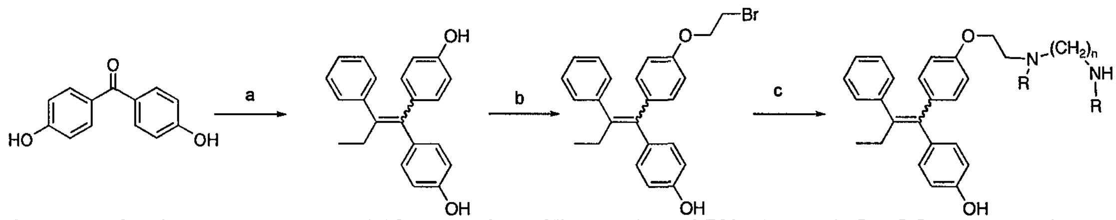
In generating this panel of compounds, a number of new synthetic approaches were developed. Below is a description of these new discoveries.

<u>Synthesis of triphenylethylethylenes</u>

In order to generate new side chain analogs of 4-hydroxytamoxifen, a new synthesis was developed that greatly simplified the approach compared to previous syntheses. The first approach was developed using a monoalkylation followed by McMurray coupling to generate analogs with different side chain moieties (**Scheme 1**). The final products could then be obtained by selective deprotection of the side chain using L-selectride. This approach was used to generate compounds **13**,**14** and **15** (R=H, n=2)

Scheme 1. a) KH, bromoethane, THF, DMF, reflux, 12% yield; b) propiophenone, $TiCl_4$, Zn dust, THF, reflux, 65% yield; c) RNH_2 in MeOH, 85°, 70-90% yield





Scheme 2. a) propiophenone, $TiCl_4$, Zn dust, THF, reflux, 95% yield; b) Cs_2CO_3, bromoethane, DMF, reflux, 70% yield; c) $RNH(CH_2)_nNHR$, THF, 80°, sealed tube, 100 % yield

Ultimately, the first step was too low yielding to generate large amounts of material; another synthesis was developed by modifying a previously reported synthesis of 4-hydroxytamoxifen (**Scheme 2**). [1]. This procedure allows for the facile generation of gram quantities of 4-hydroxytamoxifen analogs and has been used to make a number of different analogs of compound **15**.

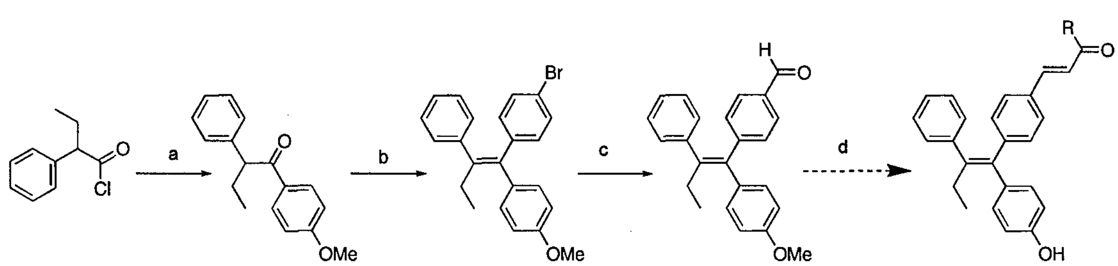
New synthesis of GW-7604 analogs

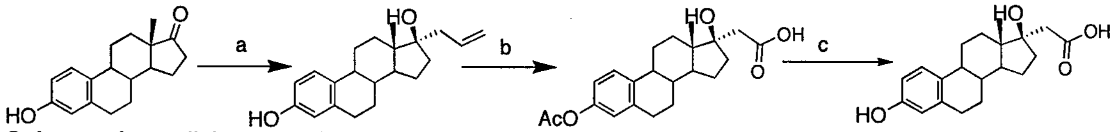
Another set of compounds in the library based on the triphenylethylethylene scaffold is the GW-7604 series (**11** and **12**). These compounds have been synthesized using a previous reported procedure, but an improved synthesis is needed [2]. These compounds are interesting due to recent reports that their ER-modulating properties are more like pure antiestrogens like fulvestrant than SERMs like tamoxifen [3]. A new synthesis has been designed and significant progress has been made (**Scheme 3**). This synthesis should allow for more facile investigation of the side chain requirements for this class of compounds.

Estradiol screening panel synthesis

The initial plan was to attach acetamide groups to 5 different positions on the estradiol steroid scaffold- 3, 6, 7, 11, and 17. Synthesis of the 3-substituted analog was straightforward after modifying a previously reported procedure [4], but as will be described later, generated a compound that was unable to bind to the estrogen receptor alpha . As a result, that substitution point is not being pursued at the current time.

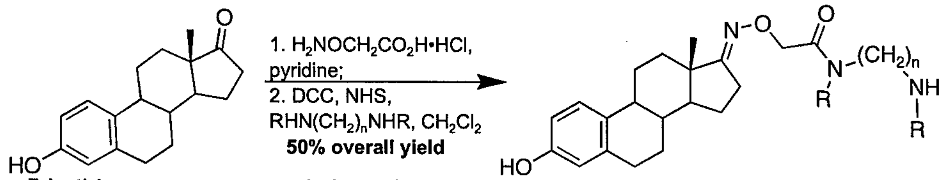
Scheme 3. a) anisole, montmorillonite K-10 clay, reflux, 70% yield; b) n-BuLi, 1,4-dibromobenzene, THF, -78°; HCl, EtOH, reflux, 50% yield; c) nBuLi, N-formylpiperidine, THF, -78, 30 % yield; d) (not yet performed) trimethylphosphonoacetate, KHMDS, THF, -78°; BBr_3, CH_2Cl_2.





Scheme 4. a. allyl magnesium bromide, THF, 75% yield. b. acetic anhydride, NEt_3, 100% yield; $RuCl_3$, $NaIO_4$, 30% yield. c. aqueous NaOH, 100% yield

Substitution at 17 has been accomplished through two different routes. The first involved Grignard alkylation of estrone to generate a 17-α alkyl group (**Scheme 4**). As will be described later, this compound only has moderate affinity for the receptor. As a result, another 17-substituted compound was made by forming the oxime at the 17-position starting from estrone (**Scheme 5**). Modifying a previously reported procedure [5], a number of analogs have been synthesized containing this substitution and they have been found to possess high affinity for the estrogen receptor.

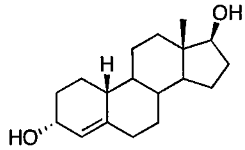


Scheme 5 In this case, compounds have been made with R=H or CH_3 and with n=2 or 6.

Difficulties have been encountered with synthesizing analogs derivatized at the 6, 7 or 11 position. A number of approaches have been attempted to oxidize any of those positions and then couple various reagents to them. They have all been unsuccessful. The decision has been made to focus on substitution at the 7 position because these analogs look most like fulvestrant (also known as Faslodex and ICI 182,780), a pure antiestrogen in rapid response assays. A new synthetic route based on the most recent literature report is being started and there is hope that the problems can be solved [6].

New compounds

Since the submission of this proposal, there have been reports of compounds with no reported activity in regulating estrogen receptor-mediated transcription, but still possessing the ability to stimulate rapid signaling. The first molecule in this class was the estren derivative, 4-estren-3α,17β-diol (**22**), which was shown



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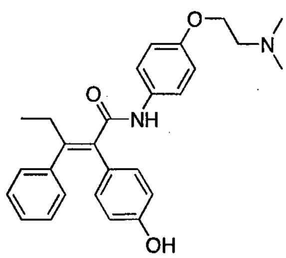

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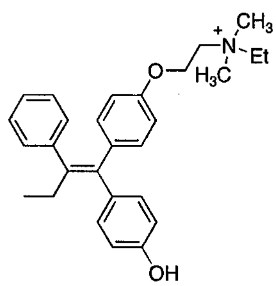

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to selectively activate rapid signaling in bone without much transcriptional modulating activity [7]. The utility of this compound in rapid responses in other tissues has yet to be explored. The other compound reported to be in this class is STX (**23**), a compound with the opposite alkene stereochemistry as tamoxifen. This compound mimicked estradiol's ability to rapidly reduce the potency of the $GABA_B$ receptor agonist baclofen to activate G-protein-coupled, inwardly rectifying K+ channels in hypothalamic neurons, a model of estrogen-induced prevention of hot flashes [8]. This compound has not been tested in any other models of integrated estrogen signaling and it could also be possible that it acts through a receptor other than the nuclear receptor– STX has no discernable affinity for the estrogen receptor alpha or beta in vitro

In addition, some people have focused on making analogs of known ligands of the estrogen receptor and somehow restricting their access across the plasma membrane. Every compound with transcriptional activity has good cell permeability by definition because the receptor is intracellular, but modifying the compound so that it is charged can restrict diffusion across the membrane. Q-Tam (**24**), a quaternary ammonium salt of tamoxifen, was found to induce apoptosis in damaged mammary epithelial cells through direct decrease in Akt phosphorylation[9]. All of these compounds have been either purchased or synthesized and will be used in later experiments.

<u>Task 1b</u> Test the ability of the compounds to modulate nuclear-initiated signaling by performing reporter gene assays at classic ERE promoters or nonclassical AP-1 promoters.
The overall goal of this proposal is to develop chemical tools to study rapid responses to steroid hormones. Key to accomplishing this goal is being able to correlate the ability of the compounds to bind to the nuclear receptor in vitro with the ability to directly activate the kinases and regulate the gene transcription by different transcription factors in cells. Therefore, assays for all three activities need to be developed and will be described below.

<u>Nuclear receptor binding</u>
There have been many assays reported to measure the binding of compounds to either the estrogen receptor. Most involve competition experiments using purified receptor or crude cell extracts and radiolabeled steroid hormone. We have used a commercially available assay kit based on fluorescence polarization with purified recombinant estrogen receptor alpha and beta and a fluorescent hormone analog. The assays were performed in 96 well plates and are fairly routine. A standard competition curve for estradiol is shown in **Figure 2**. **Table 1** lists the binding affinities of any compound that has not been reported previously in the literature. From the data, it is clear that the original plans for sites were conjugation were not going to result in compounds with enough affinity, so new conjugates were synthesized quickly and high affinity compounds were produced. It also appears that the length of the linker arm extending away from the compound is not crucial in obtaining high affinity compounds.

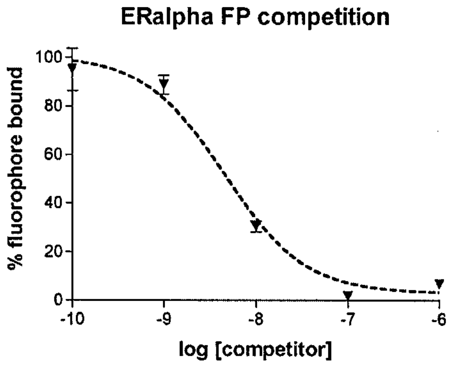


Figure 2. Estrogen receptor alpha competition binding experiment vs. 2 nM Fluormone™ with estradiol as the competing ligand. Each point represents three separate samples

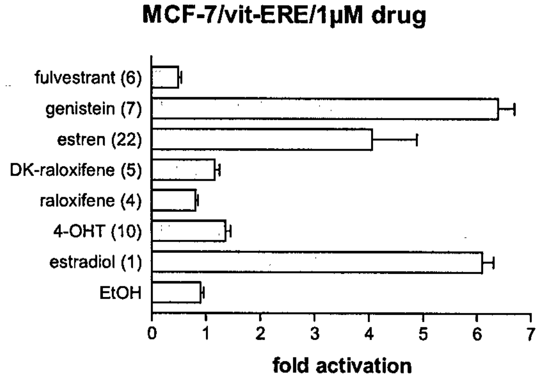


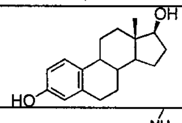
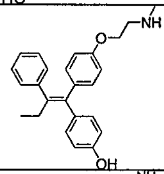
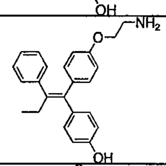
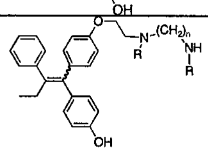
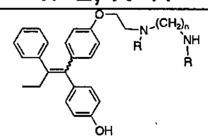
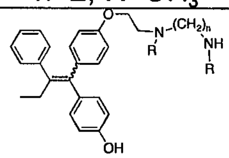
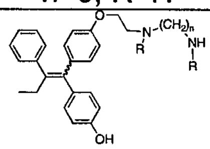
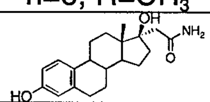
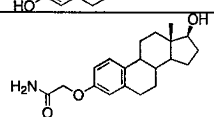
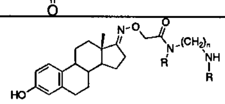
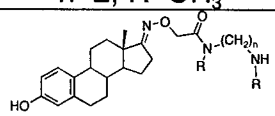
Figure 3. Luciferase reporter gene assay using vitellogenin-ERE promoter in transiently transfected MCF-7 cells. The number next to each compound refers to the structures in Figure 1.

Luciferase reporter gene assays

A key component of this project is measuring the estrogen receptor-mediated transcriptional activity of the compounds. For estrogen receptor, MCF-7 cells, which contain both ERα and ERβ, were transiently transfected with a luciferase reporter plasmid controlled by a simple estrogen response element (ERE)-containing promoter from the upstream region of the vitellogenin gene. The ER-negative HeLa cell line was also used for these experiments, but an expression plasmid for either ERα or ERβ was cotransfected with the reporter plasmid. A dual luciferase reporter gene system was used to normalize for transfection efficiency, meaning that an enzymatically orthogonal form of luciferase from a different species was cotransfected on a constitutively active expression plasmid. The DNA was transfected into the cells using Lipofectamine 2000 and standard protocols. After transfection, the cells were treated with drug for 1-2 days and the activities of the two luciferases were measured independently using a commercial kit. This assay is quite robust and reliable.

Figure 3 shows the fold activation of transcriptional activation at the ERE response element of a number of the compounds from the screening panel. As is to be expected, most of the SERMs and antiestrogens act as antagonists and estradiol and genistein act as agonists. The only real surprise was the activity of estren. This compound was reported to have no activity with estrogen receptor, but it is clear that there is some agonist activity. The reason behind this activation is still being explored. The activity of antagonists can be also be measured by performing a competition experiment with 10 nM estradiol. **Table 1** lists the inhibitory potencies of any compound that has not been reported previously in the literature. From the data, it appears that the inhibitory potency of the compounds at repressing ER-mediated transcription correlates with binding affinity.

Table 1.

compound		Ki (nM)	IC50 (nM)
estradiol (**1**)		6.3 ± 0.2	N.D.
13		8.5 ± 3.9	40 ± 10
14		48 ±5	800 ± 400
15	 n=2, R=H	32 ± 10	150 ± 50
15	 n=2, R=CH_3	3.4 ± 2.1	39 ± 12
15	 n=6, R=H	9.8 ± 6.2	85 ± 55
15	 n=6, R=CH_3	6.2 ± 4.6	126 ± 33
19		850 ± 75	3275 ± 200
20		1100 ± 100	> 10 μM
21	 n=2, R=CH_3	9 ± 4	13 ± 6 (weak agonist)
21	 n=6, R=CH_3	22 ± 8	32 ± 11

<u>Task 1c.</u> Test the ability of the compounds to mimic estrogen's ability to inhibit apoptosis in breast cancer by treating a breast cell line with the compounds in the presence of taxol and testing for both early and late apoptosis events.

One of the key aspects of this project is determining the effect of various compounds on the tolerance to apoptosis that estradiol confers to ER positive breast cancer cells. We started these assays early in this project but have had some difficulty in obtaining reproducible results. Early efforts focused on using a previously reported assay for caspase 9 activity to indicate early events in apoptosis [10]. This assay has not been successful in our laboratories. We have also performed fluorescence microscopy studies to look at annexin V binding to the cell surface- a marker for the late stages of apoptosis. While some data were generated with this approach, a flow cytometry based approach will be much more statistically significant and efforts to run those assays are underway. The development of another assay investigating early apoptotic events is also ongoing.

<u>Task 1d.</u> Test the ability of the compounds to mimic estrogen's ability to rapidly initiate kinase signaling cascades known to be important in cell proliferation by treating different cell lines with the compounds and testing for modulation of kinase pathways starting with MAP kinase.

While there are many different assays that can be run to measure direct activation of the three kinases, the goal of this proposal is to start with assays that are well established. For our initial studies, we have decided to focus on the direct activation of ERK1/2 in two cell lines– the ER-positive breast cancer cell line MCF-7 and the ER-negative cell line MDA-MB-231 with or without transfected ERα. For measuring direct activation of ERK1/2, the cells are serum-starved for 3 days to quiet any background MAPK signaling. Cells are then treated with drug for various time points, then the cells are lysed and the cell lysates are analyzed for total ERK and phosphorylated ERK (pERK) using previously reported Western blotting procedures. [11] A sample blot for MDA-MB-231 cells transfected with ERα is shown in **Figure 4**. Estradiol stimulates ERK phosphorylation about as strongly as epidermal growth factor (EGF), which is consistent with previous reports. [12]

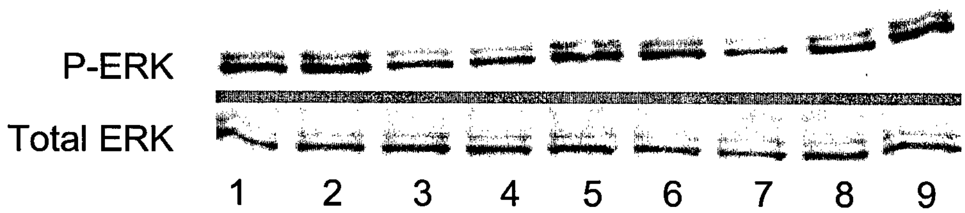


Figure 4. Immunoblot of ERK activation in ERα -transfected MDA-MB-231 cells. Phospho-ERK (top row) and total ERK was measured using two different blots. **Lane 1** DMSO, **Lane 2** EGF, **Lane 3** EGF+PD98059, **Lane 4** PD98059. **Lane 5** 1μM E_2, **Lane 6** 10 nM E_2 , **Lane 7** 1μM E_2,+PD98059 **Lane 9** 10 nM E_2+PD98059. **lane 10** fulvestrant

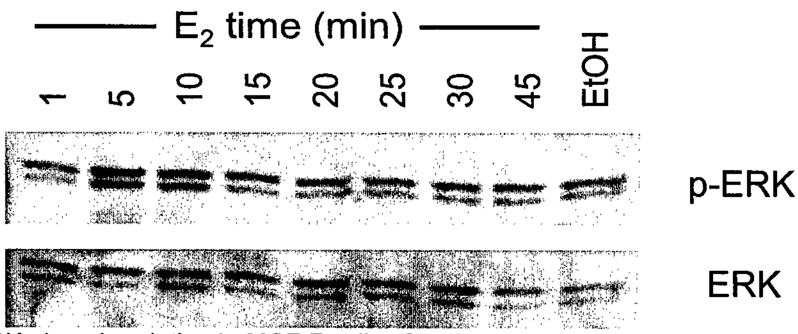


Figure 5. ERK phosphorylation in MCF-7 cells after doing with 10 nM estradiol.

Care is taken to not exceed an ethanol or DMSO concentration in the media over 0.01% since higher levels of either solvent can stimulate ERK phosphorylation. The specificity of the MAPK pathway for ERK phosphorylation is shown by the inhibition of estradiol stimulation by the MEK inhibitor PD98059. Specificity for an estrogen response is shown with the inhibition of estradiol stimulation using the antiestrogen fulvestrant. While the assay is not high-throughput, it can be performed using a good-sized number of ligands at once.

The time course of activation was also determined in MCF-7 cells and is shown in **Figure 5**. The ERK activation after dosing with estradiol was maximal at 5-10 minutes with most of the activation returning back to baseline after 15 minutes.

The effects on ERK activation of a number of other compounds in the screening library are shown in **Figure 6**. Tamoxifen, 4-hydroxytamoxifen, estren, raloxifene and desketoraloxifene all elicited ERK phosphorylation after 15 minutes in MCF-7 cells. This activation was MAPK specific as it was inhibited by PD98059. All of the responses were estrogen receptor specific in that they activation could be blocked by fulvestrant (also known as ICI 182,780) except for the tamoxifen compounds. It appears that ERK activity *increases* in the presence of fulvestrant. This experiment has been repeated and the same result is obtained. Work is currently underway to try to understand the origin of this effect with a focus on previously reported antiestrogen binding site on cells [13].

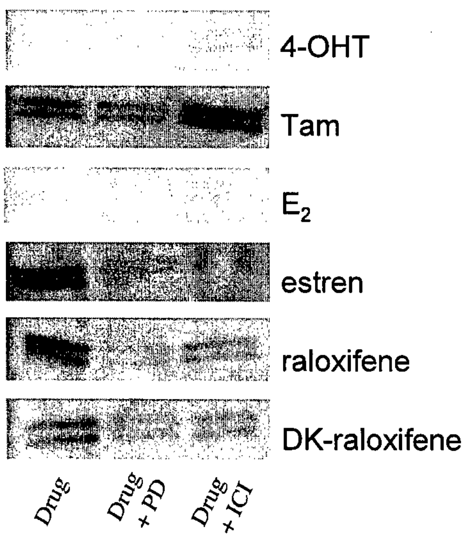


Figure 6. phospho-ERK levels in MCF-7 cells after 15 minute exposure to different estrogen receptor ligands. **column 1**-100 nM compound; **column 2**- compound + PD 98059; **column 3**- compound + fulvestrant (ICI 182,780)

Problems with activation assays and potential solutions

The major obstacle facing this project currently is the lack of consistent and vigorous activation of the MAPK pathway. The fold activation is usually 2-3 fold over baseline, but many times the baseline seems to be much higher than normal and no ER-induced activation is seen. Various types of serum starved and serum-free conditions have been tried as well as cell lines expressing high levels of Her2/neu. We have tried other antibodies as well as immunoprecipitating ERK and performing kinase enzymatic assays. Thus far, we have not found a technique that gives highly reproducible results.

There are a number of other possible solutions in the literature that we are currently pursuing. One involves making a form of the estrogen receptor that localizes to the membrane. These receptors lack the nuclear localization site and include an addition myristoylation and prenylation sites and have been reported to have strong ERK activation properties. We are also trying luciferase reporter gene assay for downstream transcription factors that are effectors of MAPK activation. We are currently using an Elk1 reporter plasmid as well as an SRF reporter plasmid with the hope that ERK activation can be measured in this manner[14]. We will also keep trying the immunoblotting techniques with different MCF-7 cell cultures from different labs with the hope of finding one that demonstrates strong ER-mediated activation.

<u>*The case of endoxifen*</u>
N-desmethyl-4-hydroxytamoxifen (compound **13**) was synthesized as part of the series of 4-hydroxytamoxifen analogs designed to test the feasibility of conjugating 4-hydroxytamoxifen to a polymer scaffold. During the course of the synthesis, we began collaborating with David Flockhart and colleagues at Indiana University School of Medicine. They had preliminary data that this compound, which they named endoxifen, was a metabolite of tamoxifen in women undergoing tamoxifen therapy, but was missing in women with deficient cytochrome P450 2D6 activity. We gave them some of our chemically synthesized material and they confirmed the metabolite structure with our material [15]. Together, our labs tested the activity of endoxifen in binding assays, luciferase assays, and rapid response assays. Thus far, endoxifen appears to be identical in activity and potency to 4-hydroxytamoxifen. Further studies by Flockhart and colleagues have shown that endoxifen is at much higher concentrations in normal patients than 4-hydroxytamoxifen and should be considered a major bioactive metabolite of tamoxifen. Furthermore, patients with deficient CYP2D6 activity, either through mutation of the gene or through the use of CYP2D6 inhibitors (such as the antidepressants fluoxetine and paroxitene) may be more likely to have a poor response to tamoxifen [16]. This work has resulted in a letter sent to the FDA warning of a potential drug interaction between tamoxifen and drugs that are known to inhibit CYP2D6. Work is ongoing to determine whether endoxifen has any unique activities distinct from 4-hydroxytamoxifen.

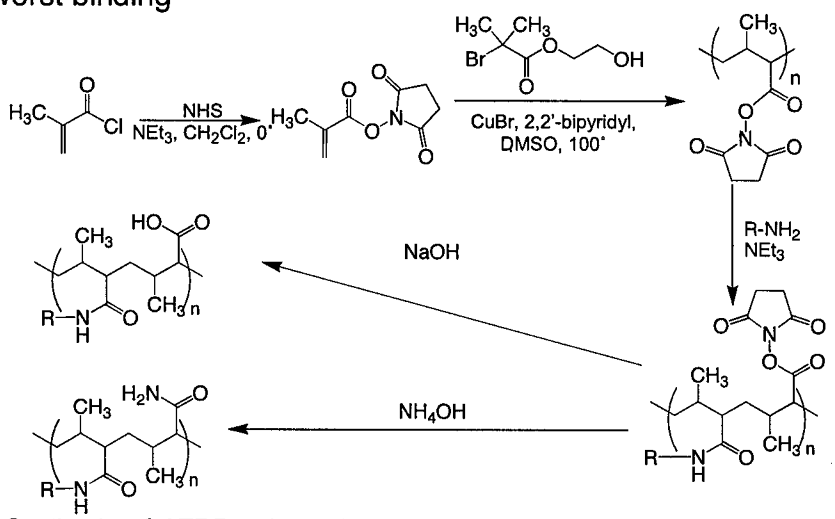
<u>*Task 2.*</u> *Test the potential role of estrogen receptor alpha (ERα) or ERβ in rapid signaling, by performing assays with various N-terminal deletion and chimeric*

mutants of ERα and ERβ. (Months 12-36)
All of the necessary mutants are prepared and active in luciferase reporter gene assays. This task will begin after a more robust rapid signaling assay is in place.

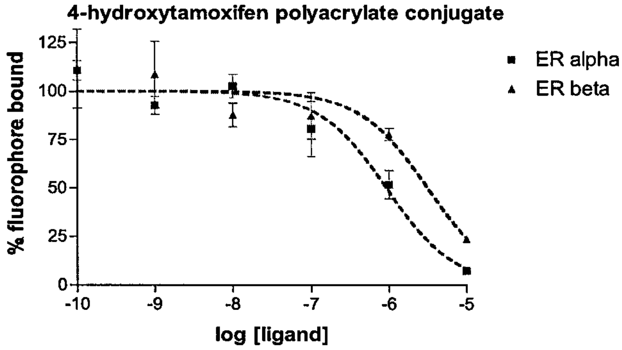
Task 3. Test the role of cell surface receptors in rapid estrogen signaling, by developing cell-impermeable, non-proteinaceous estradiol conjugates.

<u>Task 3a.</u> Determine the feasibility of using polymer-conjugated estrogen ligands as probes of ER function by conjugating active estradiol & tamoxifen analogs to polymers synthesized using atom transfer radical polymerization (ATRP) and testing for their ability to bind to ER in vitro (Months 6-12)

Polymer scaffolds derived with bioactive molecules have been used for quite some time, in large part due to the advantage of being able to easily manipulate the bulk properties of the polymer.[17, 18] A poly (methacrylate) polymer was synthesized as an N-hydroxysuccinimide activated ester using atom transfer radical polymerization (ATRP). ATRP allows for the generation of polymers with very narrow molecular weight ranges and the activated ester allows for conjugation of a number of different compounds through simple amide coupling chemistry- estrogenic compounds, antiestrogens and reporter groups like aminofluorescein and biotin. (**Scheme 6**) Activated polymers have been synthesized in two sizes, 10,000 and 50,000 weight average molecular weight with polydispersity index numbers 1.1 and 1.5 respectively, conjugated with 1% aminofluorescein and then the remaining activated esters were hydrolyzed either to the carboxylic acid or the carboxamide. The first generation polymer involved coupling the smaller polymer with the N,N-didesmethyl-4-hydroxytamoxifen (**14**). The conjugation occurred on approximately 80% of the available side chains as judged by NMR integration and the remaining side chains were converted either to the free acid or the amide. Unfortunately, compound **14** was later found to have the worst binding



Scheme 6. Synthesis of ATRP polymer-ER ligand conjugates. The R group represents the 4-hydroxytamoxifen analogs



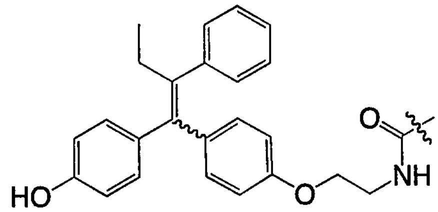


Figure 7. ER binding assay of first generation 4-hydroxytamoxifen-polymer conjugates. Curves generated using fluorescence polarization kit with purified ERα or ERβ vs. 10 nM Fluormone™

affinity of the tamoxifen analog panel, so we have now synthesized a second generation tamoxifen polymer using compound **15** (n=6, R=H) and incorporating the compound into about 20% of the side chains. All of these polymers are dialyzed extensively in water and have been shown to be water soluble at all the concentrations necessary for testing in the assays, but the second generation polymers are generally more soluble..

<u>ER binding</u>

A key test for the use of the conjugates is determining whether they can bind to the receptor in vitro. The BSA conjugates were reportedly unable to do this, but as seen in **Figure 7**, the first-generation tamoxifen containing polymers appear to bind to estrogen receptor with decent affinity. This represents a significant breakthrough in steroid hormone conjugate development. Also, we are hopeful that the second-generation tamoxifen polymer using the much more potent tamoxifen analog will be significantly more effective. The estrone analogs based on compound **21** are also currently being conjugated to the polymers and characterized.

<u>Task 3b.</u> Develop cell-impermeable polymer scaffolds suitable for cell-based assays by synthesizing well-defined polymers of different sizes and derivatizations from a single monomer unit using ATRP and testing for their general utility in biological screens. (Months 12-24)

The polymers are currently being tested for their chemical and enzymatic stability. The fluorescein-containing polymer has simplified these studies. Since these polymers are larger than free fluorescein, a significant difference is seen in the fluorescence polarization values for the polymer-fluorescein conjugates compared to free fluorescein. Hydrolysis of the fluorescein from the polymers using concentrated NaOH, followed by neutralization resulted in samples with significantly lower polarization values. Stability in serum and potential nonspecific binding should also be able to be detected using this assay.

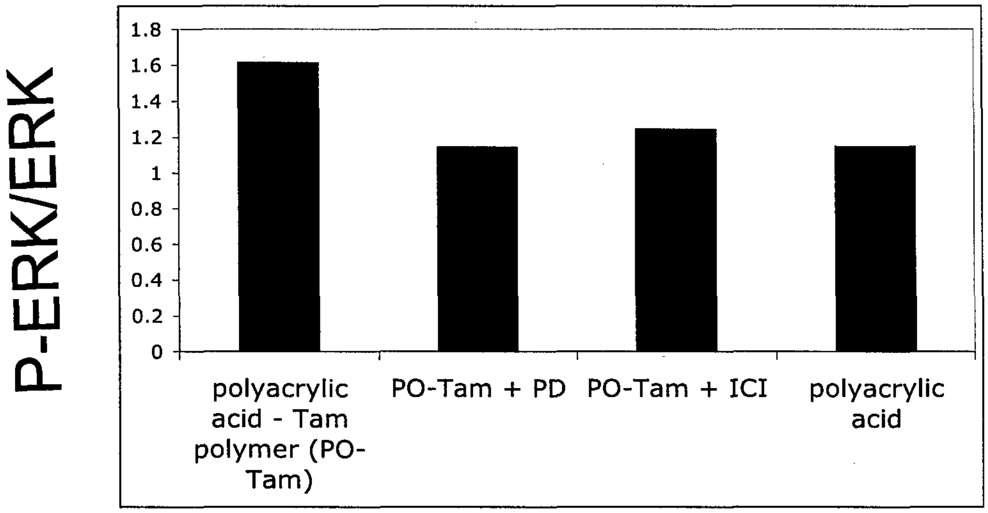


Figure 8. Normalized phospho-ERK levels in MCF-7 cells after 15 minute exposure to different 1st generation 4-hydroxytamoxifen-polymer conjugate. All concentrations of polymer tested were the equivalent of 100 nM tamoxifen-containing side chains. **bar 1**- tamoxifen-polymer conjugate; **bar 2**- conjugate + PD 98059; **bar 3**- conjugate + fulvestrant (ICI 182,780); **bar 4**- polyacrylate scaffold with no tamoxifen conjugated to it.

<u>Task 3c.</u> After establishing the ideal polymer scaffold, active compounds will be coupled to the polymers and tested for their ability to elicit rapid steroid hormone responses in the different assays. (Months 24-36)

The first generation polymer was tested for activity in the ERK activation assay. Although the data (shown in **Figure 8**) are preliminary, there does seem to be some activation of ERK that is not seen with the polymer scaffold alone. The second-generation tamoxifen polymer and the estrone-conjugated polymer will be tested as soon as it characterization is complete

<u>Task 4</u>. Test the potential role of rapid estrogen signaling in breast cancer proliferation and survival, by treating various breast cell lines with selective compounds discovered above and measuring changes in cell growth, cytotoxicity and apoptosis (Months 24-36)

Assays are in development, but no progress has been made on this task.

Key Research Accomplishments (thus far)

- New synthesis of 4-hydroxytamoxifen analogs results in best method reported to date to make these series of important compounds.
- New synthesis of GW-7604 and analogs that is much higher yielding than previously reported synthesis
- New 4-hydroxytamoxifen analogs with high affinity for estrogen receptor and potency in cell-based assays
- First reported synthesis and testing of endoxifen, a major, bioactive metabolite of tamoxifen that may be an important indicator of tamoxifen response in breast cancer patients.
- First report of raloxifene and desketoraloxifene acting as agonists of rapid, estrogen-induced ERK phosphorylation.
- First use of ATRP to generate polymer conjugates capable of binding to the estrogen receptor.
- First report of macromolecular tamoxifen conjugates capable of stimulating rapid ERK phosphorylation.

Reportable Outcomes

Manuscripts/abstracts (included in appendix)

1. Trebley, J.P.; Rickert, E.L.; Reyes, P.T.; Weatherman, R.V.; "Tamoxifen-based Probes for the Study of Estrogen Receptor-Mediated Transcription." *Ernst Schering Research Foundation Workshop*. **2005**, in press

2. Johnson, M.D.; Zuo H.; Lee. K-H; Trebley. J.P.;Rae, J.M.; Weatherman. R.V.; Zeruesanay, D.; Flockhart. D.A.; Skaar, T.A.; "Pharmacological characterization of 4-hydroxy-N-desmethyl tamoxifen, a novel active metabolite of tamoxifen." *Breast Cancer Research and Treatment* **2004**, *85*, 151-159.

Presentations

1. Speaker, Nuclear Receptor Drug Discovery Session, American Chemical Society Central Region meeting, Indianapolis, May 2004.

2. Speaker, Department of Basic Medical Sciences, Indiana University-Bloomington, April 2005.

3. Speaker, Schering Foundation Workshop on Chemical Genomics, Berlin, Germany, April 2005.

4. Poster presenter, Gordon Research Conference on Bioorganic Chemistry, June 2004.

Patents and licenses applied for

"Novel Triphenylethylethylene Analogs." Pre-disclosure form submitted to Purdue University Office of Technology Transfer

Degrees obtained that are supported by this award

Melinda Morrell, M.S. in Medicinal Chemistry and Molecular Pharmacology, 2004

Funding applied for based on work supported by this award

Received

Purdue Cancer Center and Indiana Elks Charities Cancer Pilot Grant Award

Applied for

1. Kimmel Cancer Foundation (Principal Investigator)
2. National Institutes of Health, R01 (Principal Investigator)
3. Breast Cancer SPORE. NIH (with Indiana University), member

Employment or research opportunities applied for and/or received

1. Mindy Morrell, employment with Med Institute, West Lafayette, IN
2. Joe Trebley, NSF Predoctoral Fellowship for Innovation Realization
3. New collaborations with Ken Nephew, Indiana University and Craig Crews, Yale University.

Conclusions

Currently, we have made significant progress in exploring the role of rapid nongenomic signaling in breast cancer prevention and treatment. We are on schedule with the proposed timeline and have developed all the necessary reagents to do the biological studies in the next year that will answer many of our questions about rapid estrogen signaling in breast. Only one set of compounds remain to be made and it is anticipated that those obstacles will be overcome. Most of the effort is currently focused on finding robust assays to measure these rapid responses and it is anticipated that there will be major improvement made to the original plan in the next year.

In terms of the new knowledge we have obtained thus far and its importance to breast cancer, we have been the first to synthesize a newly identified novel, bioactive metabolite of tamoxifen that may play a major role in determining the success of tamoxifen therapy. We have also shown that SERMs like tamoxifen and raloxifene can act similarly to estrogen in activating rapid responses. This agonist activity mimics the effects seen in some tissues and in tamoxifen resistant tumors; understanding the molecular determinants of this agonist activity could help produce better treatments and chemopreventive agents for breast cancer.

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Breast Cancer Research and Treatment **85:** 151–159, 2004.

Report

Pharmacological characterization of 4-hydroxy-*N*-desmethyl tamoxifen, a novel active metabolite of tamoxifen

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Key words: endoxifen, estrogen receptor, gene expression, metabolite, proliferation, receptor binding, tamoxifen

Summary

The antiestrogen tamoxifen is extensively metabolized in patients to form a series of compounds with altered affinity for estrogen receptors (ERs), the primary target of this drug. Furthermore, these metabolites exhibit a range of partial agonist and antagonist activities for ER mediated effects that do not depend directly on their absolute affinity for ERs. Thus, clinical response to tamoxifen therapy is likely to depend on the aggregate effect of these different metabolites resulting from their abundance in the patient, their affinity for the receptors, and their agonist/antagonist profile. A recent study has shown that plasma concentrations of the tamoxifen metabolite 4-hydroxy-*N*-desmethyl tamoxifen (endoxifen), in patents undergoing tamoxifen therapy, are dependent on the cytochrome P450 (CYP) 206 genotype of the patient and that medications commonly prescribed to patients on tamoxifen therapy can also inhibit endoxifen production. In this study we characterized the properties of this metabolite with respect to binding to ERs, ability to inhibit estrogen stimulated breast cancer cell proliferation and the regulation of estrogen responsive genes. We demonstrate that endoxifen has essentially equivalent activity to the potent metabolite 4-hydroxy tamoxifen (4-OH-tam) often described as the active metabolite of this drug. Since plasma levels of endoxifen in patients with functional CYP2D6 frequently exceed the levels of 4-OH-tam, it seems likely that endoxifen is at least as important as 4-OH-tam to the overall activity of this drug and suggests that CYP2D6 status and concomitant administration of drugs that inhibit CYP2D6 activity have the potential to affect response to tamoxifen therapy.

Introduction

It is estimated that in 2002 there were 203,500 women newly diagnosed with breast cancer and that 39,600 woman died of the disease [1]. Roughly 60–65% of these women will have tumors that are estrogen receptor (ER) positive. Of ER positive tumors, approximately 60–65% will respond to some form of hormonal therapy. Since its introduction 30 years ago, tamoxifen has been the front line hormonal therapy of choice [2]. With the additional use of tamoxifen in the adjuvant setting, and now with evidence that it is an effective chemo-preventive agent, it is not surprising that tamoxifen is the most commonly prescribed drug in the management of breast cancer. Tamoxifen is a selective estrogen receptor modulator (SERM) – that is a compound which competes with estrogen for binding to the ER and exhibits a spectrum of estrogen antagonist and agonist activity depending on the site of action. In the breast it is primarily an antiestrogen and it is believed to inhibit tumor growth by starving the tumor cells of survival and proliferative signals provided by estrogen signaling.

Tamoxifen is sometimes described as a pro-drug, since it is extensively metabolized yielding several compounds that are more potent antiestrogens than the parent compound [3]. However, tamoxifen is quite active itself and due to the relatively low concentration of the more potent metabolites, it seems likely that the overall response of the tumor is the result of the aggregate effect of the drug and its metabolites. Interestingly, some minor tamoxifen metabolites are potent estrogens and many investigators have suggested that different patterns of metabolism of the compound among patients might go some way to explaining why only approximately 60% of ER positive tumors respond to tamoxifen therapy.

We recently reported the identification of a tamoxifen metabolite (4-hydroxy-*N*-desmethyl tamoxifen – endoxifen), the plasma concentration of which in patients receiving tamoxifen is altered by concomitant administration of paroxetine [4]. Furthermore, we showed that plasma concentrations were dependent on the genotype of the patient with respect to polymorphisms of an enzyme known to metabolize both paroxetine and tamoxifen (cytochrome P450 2D6 – CYP2D6) [5]. In this study we present a detailed characterization of the ER binding properties of this metabolite and its effects on the expression of estrogen responsive genes and on the proliferation of ER positive breast cancer cells.

Methods

All chemicals were from Sigma (St. Louis, MO) unless otherwise noted.

Synthesis and purification of endoxifen

Endoxifen for use in this study was synthesized using two separate methods. The first method is described in our previous paper [4]. The second method was developed to facilitate the synthesis of large amounts of the compound for *in vitro* and *in vivo* characterization and is summarized in Figure 1. The product of each reaction was purified using silica gel chromatography. Endoxifen was purified following the final reaction using silica gel chromatography with 90:9:1 $CHCl_3/CH_3OH/NH_4OH$ as the eluent. Using ^{1}H NMR, ^{13}C NMR and high resolution mass spectrometry, the identity of the final product was confirmed as a 1:1 mixture of *E* and *Z* regioisomers of endoxifen (spectra are available upon request). Endoxifen prepared by either method produced identical results in the assays described below.

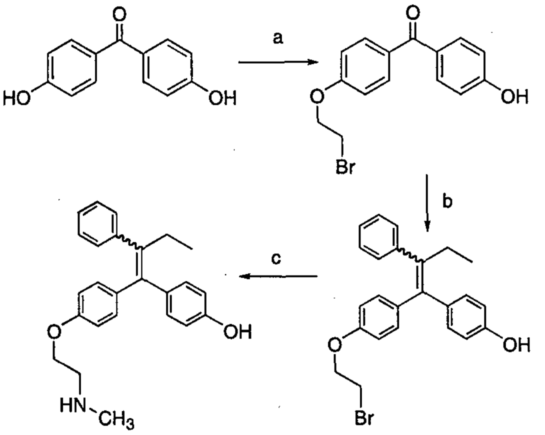


Figure 1. Synthetic scheme for the production of endoxifen. (a) KH, bromoethane, THF, DMF, reflux, 17% yield; (b) propiophenone, $TiCl_4$, Zn dust, THF, reflux, 65% yield; (c) CH_3NH_2 in MeOH, 85°, 70–90% yield.

Cell culture

MCF-7, T47D and BT474 cells were obtained from the tissue culture shared resource (TCSR) at the Lombardi Comprehensive Cancer Center and were routinely cultured in modified IMEM supplemented with 10% fetal calf serum (Life Sciences, Gaithersburg, MD).

Proliferation assays. Cell monolayers at about 70% confluence were withdrawn from estrogen by changing to an estrogen-depleted medium consisting of phenol red-free modified IMEM supplemented with charcoal stripped newborn calf serum. To remove residual estrogen the monolayer was washed every hour for 5 h with phenol red-free IMEM and cultured in fresh medium in between washes. The cells were then cultured overnight and trypsinized with phenol red-free trypsin. The cells were counted and 1000 cells plated into each well of several 96 well plates in 100 μl of the same medium. After allowing the cells to attach (5 h), 100 μl of medium containing 17-β-estradiol (E2), endoxifen, or 4-OH-tam (alone or in combination with E2), or the vehicle control (ethanol), were added to the wells to yield the indicated concentrations of the various compounds and a final ethanol concentration of 0.1%. Several duplicate plates were prepared and after allowing the cells to grow for various periods, the cells were stained with crystal violet (0.52% crystal violet in 25% methanol). After washing the plates to remove excess stain, the plates were dried and then the bound stain solubilized by addition of 100 μl of 100 mM sodium citrate in 50% ethanol. Staining intensity, which is proportional to cell number, was then determined by measuring absorbance at 570 nm using a 96 well plate reader [6].

ER binding assays

The binding of endoxifen to the ERs was investigated by competition binding assays using two different methods. The dextran coated charcoal method and a fluorescence polarization method were used to evaluate binding to ER-α, and the fluorescence polarization method was used to investigate binding to ER-β.

Dextran coated charcoal assay. Competitive binding assays were conducted using a modification of a standard method [7]. Briefly, MCF-7 cells were withdrawn from estrogen by washing and growth in depleted medium (as described above) for 3 days to maximize ER levels. The cells were harvested with phenol red-free trypsin (Biofluids, Rockville, MD) washed with TEG buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 10% glycerol) and re-suspended at approximately 30 million cells per ml in TEG buffer supplemented with 1 mM dithiothreitol, 0.5 M NaCl and protease inhibitors (1 mg/ml leupeptin, 77 μg/ml aprotinin, 1 μg/ml pepstatin A and 250 μg/ml pefabloc). A whole cell lysate was prepared by homogenization with a teflon/glass homogenizer (40 strokes on ice) followed by centrifugation at 105,000 × g for 30 min at 4°C. The supernatant was removed and the protein concentration measured using the method of Bradford [8]. The protein concentration in the lysate was adjusted to 2 mg/ml by dilution with the lysis buffer.

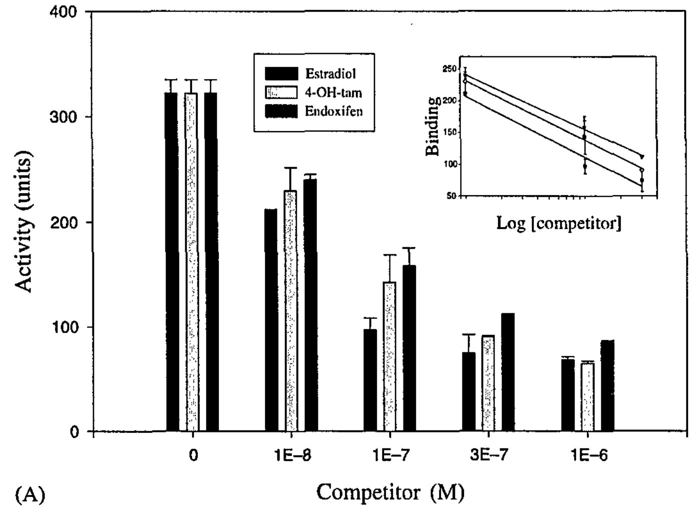
Binding reactions were set up on ice in disposable glass culture tubes (Fisher, Pittsburgh, PA). Whole cell lysate (100 μl) was mixed with 50 μl of TEG buffer containing drugs to yield a final concentration of 1 nM ^{3}H E2 (TRK 322, Amersham, Piscataway, NJ) in the absence or presence of various concentrations of unlabeled E2, endoxifen or 4-hydroxy tamoxifen (4-OH-tam). After an overnight incubation at 4°C the unbound steroids were removed by the addition of 500 μl of a 0.5% suspension of activated charcoal in a 0.05% solution of dextran (70,000 average molecular weight), 10 mM Tris, pH 8. The charcoal was pelleted by centrifugation and 500 μl of the supernatants from each tube was placed in scintillation vials with 5 ml of Ecoscint A (National Diagnostics, Atlanta GA) and relative levels of binding determined by scintillation counting.

Fluorescence polarization assay. Fluorescent polarization based competition binding assays were conducted to determine the relative affinity of 4-OH-tam and endoxifen for ER-α and ER-β using commercially available kits (P2698 and P2700 respectively, PanVera Corp., Madison, WI). Assays were conducted using a modification of the manufacturer's instructions. Briefly, serial dilutions of E2, endoxifen or 4-OH-tam were prepared in ES2 screening buffer (100 mM potassium phosphate, pH7.4, 100 μg/ml bovine gamma globulin) and 10 μl of each concentration was aliquoted into four wells of a black 384 well assay plate (3676 Corning, Corning, NY). Ten microliters of a solution containing 20 nM recombinant ER (α or β), and 2 nM of a proprietary fluorescent ER ligand (Fluormone^{TM}) was added to each well. The plate was shaken on a plate mixer and incubated for 5 h in the dark at room temperature. Fluorescence polarization signals were then measured using a Tecan Ultra fluorometer (Tecan, Durham, NC).

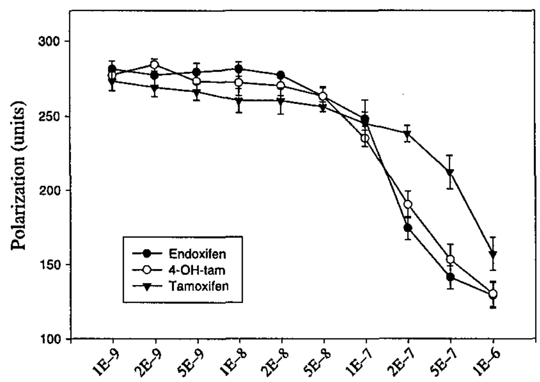
RNA preparation and analysis

MCF-7 cells were depleted of estrogen for 24 h as described above and then plated into 25 cm^2 flasks (Costar, Corning, NY). The medium was replaced every 24 h (phenol red free IMEM with 10% CCS) for 5 days after which the cells were treated with the indicated concentration of endoxifen or 4-OH-tam in the presence or absence of estradiol for 2 or 3 days. The monolayers were washed twice with phosphate buffered saline (PBS) and then solublized in RNAzol (Iso-Tex Diagnostics, Friendswood, TX), and total cellular RNA prepared according to the manufacturers instructions.

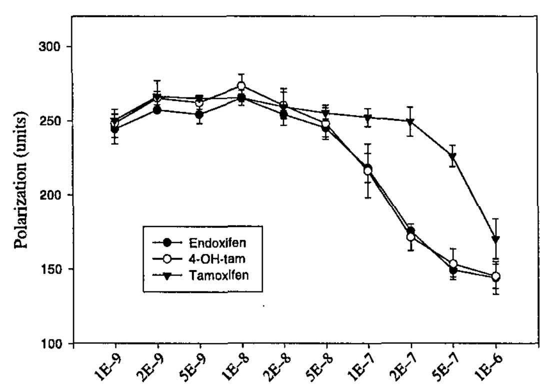
Northern blot analysis. Northern blot hybridization was conducted essentially as described previously [9]. Briefly 5 μg samples of RNA were fractionated on 1.2% agarose gels containing 2.2 M formaldehyde and transferred to nylon membranes (Hybond-N, Amersham, Arlington Hights, IL). The uniformity of RNA transfer was verified by examining the ethidium-stained ribosomal RNA bands on the filters under UV illumination. The filters were then hybridized using conditions previously described with riboprobes transcribed from plasmids containing cDNAs for the various genes being studied [9]. After washing, the



(A)



(B) Antiestrogen (M) plus 2 nM Fluormone™



(C) Antiestrogen (M) plus 2 nM Fluormone™

Figure 2. Relative ER binding affinity of endoxifen. The ability of various concentrations of endoxifen, 4-OH-tam and estradiol to displace ^3H estradiol (A) or a synthetic fluorescent estrogen (B and C) from MCF-7 nuclear extracts (A) or recombinant preparations of the α (B) or β (C) form of the ER were evaluated as described in Methods. The mean and standard deviation of at least 2 (A) or 4 (B and C) independent samples are presented for each point.

radioactive bands on the filters were imaged and quantitated with a phosphorimager (Molecular Dynamics model 445 SI, Sunnyvale, CA). Probes for pS2 and 36B4 were provided by Pierre Chambon (INSERM, Strasbourg, France) and a probe for Cathepsin D was provided by Dr Bruce Westley (University of Newcastle upon Tyne, UK).

Results

ER binding assays

To evaluate the ability of endoxifen to bind to the ER, classic radioligand receptor competition binding assays were conducted using cell extracts from ER positive breast cancer cells, as described in the methods section [7]. Figure 2(A) shows data from a representative experiment using MCF-7 cells. Each data point is the mean and standard deviation of two or three samples and each experiment was conducted at least twice. From these data the apparent relative affinity of 4-OH-tam and endoxifen was calculated to be approximately 35 and 25% that of estradiol, respectively under the experimental conditions used.

To further probe the ER binding characteristics of endoxifen, fluorescence polarization based receptor binding assays were conducted using recombinant human estrogen receptor alpha (ER-α). Comparison of the ability of 4-OH-tam and endoxifen to displace a proprietary fluorescent estrogen from the recombinant ER-α demonstrated that the compounds have an essentially identical affinity for the receptor under these conditions (Figure 2(B)). Similar experiments were conducted to evaluate binding to the beta form of the receptor (ER-β), which showed that both compounds had apparently identical affinity for this receptor also (Figure 2(C)).

Proliferation assays

Assays were conducted to characterize the ER agonist and antagonist properties of endoxifen with respect to the proliferation of several ER positive breast cancer cell lines (MCF-7, T47D and BT474). The proliferation of these cell lines is dependent on estrogenic stimuli and initial studies were conducted to determine the minimum concentration of estradiol required to maximally stimulate the growth of the cells (Figure 3(A)). The dose–response relationship exhibited by all three lines was very similar, with maximal proliferation being produced by stimulation with approximately 200 pM estradiol which was chosen as the dose for subsequent experiments (dotted line in

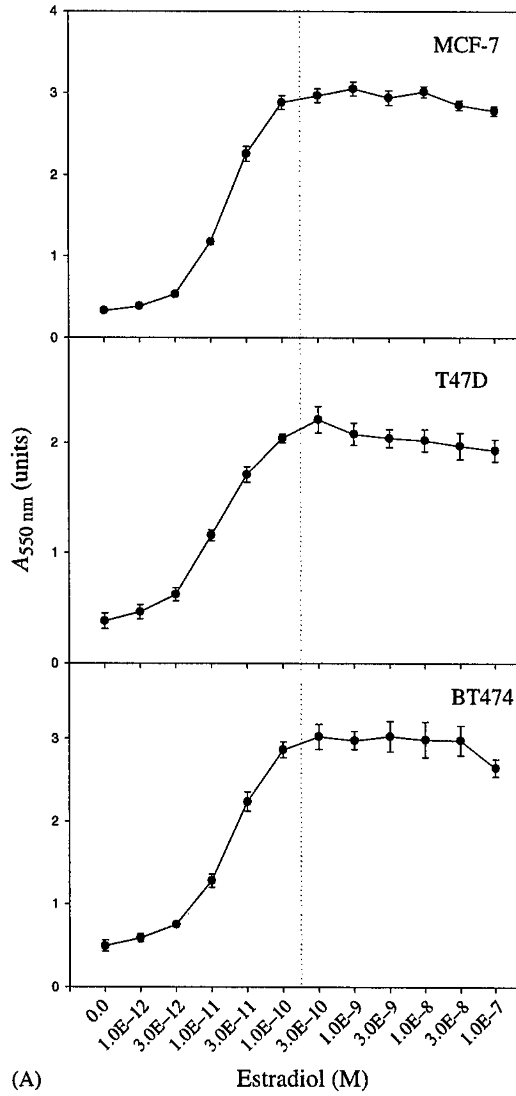
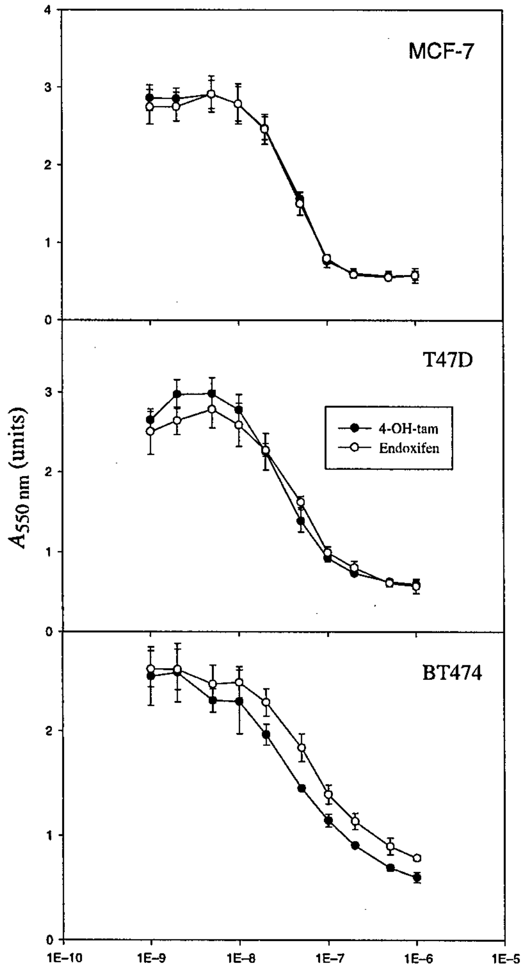
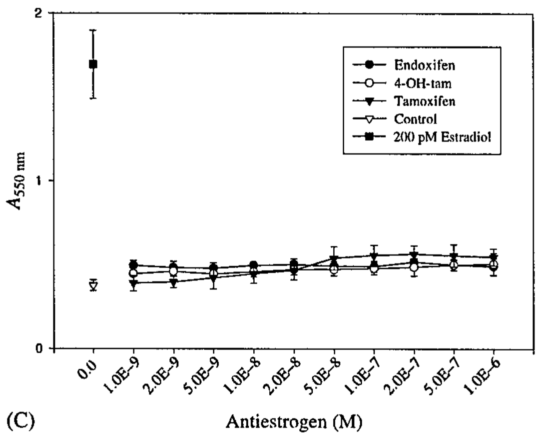


Figure 3. Inhibition of estradiol stimulated breast cancer cell proliferation by endoxifen and 4-OH-tam. MCF-7, T47D and BT474 cells were withdrawn from estrogen stimulation and treated with the indicated concentration of estradiol for 7–9 days (A). The ability of endoxifen or 4-OH-tam to inhibit the proliferation stimulated by 200 pM estradiol (B) or stimulate cell proliferation in the absence of estrogen (C) was then tested by growing the cells for 7–9 days in the indicated concentration of endoxifen or 4-OH-tam 200 pM of estradiol in the presence (B) or absence (C) of 200 pM estradiol.



(B)



(C)

Figure 3. Continued.

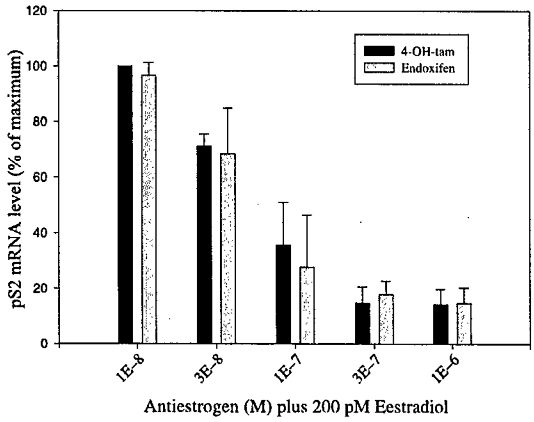


Figure 4. Inhibition of estrogen induced pS2 expression by endoxifen or 4-OH-tam. Cells were withdrawn from estradiol and treated with 200 pM estradiol in the presence of the indicated concentration of endoxifen or 4-OH-tam for 3 days. pS2 mRNA levels were determined as described in Methods and are expressed as a percentage of maximal pS2 levels normalized to total RNA. The experiment was repeated three times – representative data are presented.

Figure 3(A)). The ability of 4-OH-tam and endoxifen to inhibit the estrogen stimulated growth of the breast cancer cells was examined and both compounds were found to be essentially equipotent, with half maximal inhibition being seen at approximately 50 nM (Figure 3(B)). Both 4-OH-tam and endoxifen were essentially devoid of estrogen agonist activity in this system (Figure 3(C)).

Gene expression experiments

The effects of endoxifen on the expression of estrogen responsive genes was compared to that of 4-OH-tam by northern blot analysis. The inhibition of pS2 expression by both compounds is shown in Figure 4. Half maximal inhibition of expression is produced by both compounds between 3×10^{-8} and 10^{-7} M, consistent with the proliferation data.

Discussion

In advanced breast cancer, roughly 35% of patients with ER positive tumors will not respond to tamoxifen therapy, and ultimately all tumors that do respond will become resistant to the treatment, typically after a year to 18 months. Thus, much work has been conducted trying to understand the lack of response in tumors that exhibit primary resistance to tamoxifen and the mechanisms by which tumors acquire resistance. One focus of this work has been the possibility that an altered pattern of metabolism might in part explain these effects. Tamoxifen is extensively metabolized, predominantly by the liver, generating a complex series of compounds that exhibit altered affinity for ERs and a variety of agonist and antagonist properties [3, 9]. These compounds range in their activity from strong estrogen antagonists to potent estrogens. The most abundant metabolite is *N*-desmethyl tamoxifen (NDT) which is believed to be predominantly generated by the cytochrome p450 enzyme (CYP) 3A4. Patients receiving the standard does of 20 mg of tamoxifen per day typically have plasma concentrations of NDT of approximately 400–800 nM, (compared to 250–650 nM tamoxifen) when they reach steady state, which takes 4–6 weeks since the half-life of NDT is roughly two weeks [10, 11]. NDT has similar potency to that of tamoxifen and similar partial agonist activity [9]. Another important metabolite is 4-OH-tam, which is sometimes referred to as the active metabolite of tamoxifen, since it is significantly more potent than the parent compound. Depending on the assay system used, the apparent affinity of 4-OH-tam for ER-α is similar to that of E2, whereas that of tamoxifen is approximately 30–100-fold less [3]. Plasma concentrations of 4-OH-tam are however quite low, approximately 1–12 nM, thus describing 4-OH-tam as the active metabolite is over simplistic, since it is likely that for most patients it is the aggregate activity of the parent compound and metabolites that will determine the response that is seen. Several of the CYPs are known to be able to make 4-OH-tam (CYP2D6, CYP2C9, CYP2C19, CYP3A4) [4, 12].

Tamoxifen is generally a very well tolerated drug, however one of the most commonly reported side effects, hot flashes, can be sufficiently severe to lead to patients stopping their therapy [13]. Since treatment with estrogen is not a logical option for treating hot flashes in the context of antiestrogen therapy, several other approaches have been tried and recent studies have shown that the selective serotonin reuptake inhibitors (SSRIs) can be useful in this context [14, 15].

The mechanism by which the SSRI's can reduce the incidence of hot flashes is not known, and so a clinical study was initiated to examine the hypothesis that the SSRI paroxetine might alter the metabolism of tamoxifen resulting in the production of less 4-OH-tam, and thereby reducing the incidence of hot flashes [4]. This hypothesis was based on the knowledge that paroxetine is metabolized by, and can inhibit the activity of CYP2D6, one of the enzymes believed to be responsible for the formation of 4-OH-tam. No link was found between the incidence of hot flashes and the levels of any of the tamoxifen metabolites, but the study did show that the level of one chromatographic peak in the tamoxifen assays of patient plasma was highly sensitive to co-administration of paroxetine. This peak was determined to be a previously described minor tamoxifen metabolite: 4-hydroxy *N*-desmethyl tamoxifen [4, 16, 17]. A small quantity of endoxifen was synthesized to verify the identity of the chromatographic peak and to evaluate the antiestrogenic activity of the metabolite. We further showed that endoxifen is almost exclusively generated by the action of CYP2D6 and that patients who carry a CYP2D6 genetic polymorphism that results in reduced enzyme activity, make very little endoxifen.

The antiestrogenic properties of endoxifen were not known, and so this study was initiated to characterize the ER binding properties and estrogen agonist/antagonist profile of the compound, as a first step toward determining the importance of this metabolite to the overall clinical response of patients undergoing tamoxifen therapy.

N-desmethyl tamoxifen has very similar properties to those of tamoxifen and the addition of the 4-hydroxyl group to tamoxifen dramatically increases the affinity of the compound for the ER. It is, therefore, not surprising that we find endoxifen to have essentially identical properties to 4-OH-tam with respect to receptor affinity and effects of cell proliferation and gene expression. The ER binding characteristics of endoxifen are indistinguishable from those of 4-OH-tam as are its estrogen antagonist properties as determined by the suppression of estrogen stimulated breast cancer cell proliferation. These findings are entirely consistent with what has recently been learned about the structural requirements for ER binding and antiestrogen properties based on the crystal structure of the ligand binding domain of the ER [18]. Although we have not conducted an exhaustive comparison of their actions in all organ systems where estrogen exerts some effect, based on these data is seems likely that 4-OH-tam and endoxifen are essentially functionally equivalent.

Data from our previous study [4], from earlier studies [16, 17], and from a study describing an assay optimized for measuring endoxifen in patients samples [19], all show that endoxifen concentrations are notably higher than those of 4-OH-tam. More extensive ongoing studies have shown that plasma endoxifen concentrations are typically greater than those of 4-OH-tam when both drug and metabolite concentrations have reached steady state after 4 months of treatment with endoxifen concentrations being a mean of 6.8-fold greater (unpublished observations).

4-OH-tam has frequently been referred to in the literature as the active metabolite of tamoxifen, however, based on our present findings, if there is any validity to the concept of the predominance of a single active metabolite in response to tamoxifen therapy, then it is most likely endoxifen and not 4-OH-tam that is that metabolite. In reality it is more likely that it is the balance of the concentrations of all of the metabolites of tamoxifen along with the parent compound which mediate the clinical effects of the drug, but the fact remains that endoxifen is likely to play a significant role in this overall response. This realization is particularly important in light of our previous findings that show that plasma concentrations of endoxifen in patients are notably influenced by CYP2D6 genotype. There are more than 50 known genetic polymorphisms of this enzyme and roughly 7% of Caucasian patients do not express functional enzyme [20, 21]. Thus, it is possible that CYP2D6 genotype may have an important impact on patient response to tamoxifen since patients with defective CYP2D6 alleles have much lower plasma concentrations of endoxifen. We have recently described a facile method for genotyping archival paraffin imbedded tumor blocks for germline polymorphisms in several clinically important CYPs including CYP2D6 and we propose to attempt to address the question of the role of CYP2D6 genotype in tamoxifen response by interrogating historical clinical trials of tamoxifen efficacy [22]. Certain drugs that are known to inhibit the activity of CYP2D6 can mimic the effect of inactivating polymorphisms of the enzyme and reduce plasma concentrations of endoxifen [4]. This raises the possibility that co-administration of these agents might also alter patient response to tamoxifen therapy. Prospective clinical studies in which patients are genotyped for all of the relevant phases 1 and 2 metabolic enzymes and detailed records of concomitant medications are maintained will, however, be required in order to thoroughly evaluate the impact of tamoxifen metabolism on therapeutic outcome.

Acknowledgements

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Tamoxifen-based Probes for the Study of Estrogen Receptor-Mediated Transcription

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Abstract. The nuclear receptors are ideal targets to control the expression of specific genes with small molecules. Estrogen receptor can activate or repress transcription though a number of different pathways. As part of an effort to develop reagents that selectively target specific transcriptional regulatory pathways, analogs of 4-hydroxytamoxifen were synthesized with variations in the basic side chain. In vitro binding assays and cell-based luciferase reporter gene assays confirm that all the derivatives have high affinity for the receptor and high potency at repressing direct estrogen receptor-mediated transcription.

Introduction

One of the ultimate goals of chemical genomics is to study the role of a specific protein by directly altering its activity with a small molecule. This could be performed either at the protein level by direct binding or at the transcriptional level by modulating the expression of its gene. Reagents such as small interfering RNA (siRNA) that block the production of protein have great utility, but small molecules that could either block or activate transcription of specific genes at specific time points would have a dramatic impact on discerning the role of a specific protein in cellular processes. [1] One necessary component for developing these tools is a better understanding of the molecular mechanisms of transcriptional regulation and how small molecules can affect this complex process. [2]

Nuclear receptors such as the estrogen receptor (ER) represent an ideal system in which to study the effect of small molecules on the modulation of gene expression. Most nuclear receptors are ligand-dependent modulators of transcription, thus providing a tool to study the molecular mechanisms by which gene transcription is regulated. Nuclear receptors can activate or repress transcription upon ligand binding depending on the structure of the ligand, the nature of the promoter and the cell type. [3] The estrogen receptor is a particularly interesting member of the nuclear receptor family because its effects on transcription can vary greatly depending on the ligand structure and the cellular context. For example, estradiol (**1**) has been shown to activate the expression of the *c-Myc* gene in breast cell lines and the breast cancer drug tamoxifen (**2**) antagonizes this activation (**Figure 1**). [4] In a uterine cell line, however, tamoxifen and estradiol both activate *c-Myc* expression. Other ER ligands with very similar structures to tamoxifen antagonize *c-Myc* expression in both types of cell lines. This tissue-dependent response profile of tamoxifen has therapeutic importance because the ER-agonist effects of tamoxifen in the uterus and in tamoxifen-resistant breast tumors are major obstacles to improving the success of tamoxifen therapy. These different response profiles allow for comparison of the different transcriptional states to help elucidate the molecular mechanisms underpinning the selective modulation of specific subsets of genes.

It is well known that estrogen receptor regulates gene transcription by binding to specific DNA sequences in the promoter region, but ER can also regulate gene transcription through indirect means. Estrogen receptor can directly interact with other transcription factors such as AP-1 and alter their activity, but it can also rapidly activate signal transduction proteins such as ERK and Akt which can then activate downstream transcription factors such as Elk-1 and serum response factor (SRF). [5, 6] The activation of some of these rapid signaling occurs more prominently in cells in which tamoxifen acts as an estrogen receptor agonist, suggesting that the overall response profile of tamoxifen is tied to its ability to stimulate estrogen receptor crosstalk with other signal transduction pathways. [7] Some evidence suggests that these rapid signaling events are initiated from the plasma membrane. [8] Molecules that could selectively target only these crosstalk pathways would be very useful in delineating their role in the overall responses to tamoxifen. The work detailed here describes the synthesis and testing of tamoxifen analogs suitable for conjugation to other molecules such as fluorophores, affinity tags and cell-impermeable polymer scaffolds in order to better understand the role of crosstalk signaling in the control of estrogen receptor-mediated transcription.

Results and Discussion

Synthesis of 4-hydroxytamoxifen analogs (Scheme 1)

The key issue in making tamoxifen analogs suitable for conjugation to other moieties is the placement of the attachment point. One obvious location for attachment is the amine on the basic side chain. Based on the structure of 4-hydroxytamoxifen, the most potent form of tamoxifen, bound to the ligand binding domain of estrogen receptor alpha (ERα), the basic side chain extends out away from the interior of the binding pocket. [9] It has also previously been shown that endoxifen (**5**), a primary, bioactive metabolite of tamoxifen, can bind to the estrogen receptor both *in vitro* and in cells with only small decreases in affinity compared to 4-hydroxytamoxifen. [10] Based on this evidence, a number of analogs of 4-hydroxytamoxifen with different lengths of alkylamine side chains were synthesized.

The compounds were synthesized by using a modification of a previously reported synthesis of 4-hydroxytamoxifen. [11] The triphenylethylethylene scaffold can be synthesized as the diphenol (**3**) in a single step from commercially available starting materials and then monoalkylated with dibromoethane. The resulting compound (and every compound hereafter) is generated as a mixture of *E* and *Z* isomers, but the two forms readily interconvert at room temperature. Previous work with 4-hydroxytamoxifen has shown that despite this interconversion, the Z isomer is almost exclusively bound by the receptor both *in vitro* and *in vivo*. [12]

Coupling to different amines provided the different compounds for testing. Since the optimal distance between the tamoxifen scaffold and any conjugate is not known, alkyldiamines with two and six methylene unit spacers were synthesized. Previous work has indicated that the methylation state of the amines could also be important in increasing the affinity of ligands for the estrogen receptor, so analogs with methylated amines were also synthesized.

In vitro binding assays

The binding affinity of the compounds for estrogen receptor alpha was measured using a fluorescence polarization-based competition assay using purified full-length human estrogen receptor alpha. Displacement of a fluorescent ER ligand from the receptor by the competitor results in a decrease in the fluorescence polarization of the fluorophore. As shown in **Figure 2** and summarized in **Table 1**, all of the analogs had sub-micromolar affinities for the receptor. The only two compounds showing significantly different affinity for the receptor were the compounds with short extensions from the side chain terminating in primary amines (**5** and **7**). This could perhaps be due to some somewhat unfavorable interaction between the polar amine group and some nonpolar residues at the outer boundary of the binding pocket. A comparison of compounds **5** and **7** to compound **9** seems to indicate that pushing the primary amine further out of the binding pocket appears to be sufficient to overcome this unfavorable interaction.

Cell-based reporter assays

The ability of the compounds to modulate estrogen receptor-mediated gene transcription was tested using a luciferase reporter gene assay. The ER-negative HeLa cervical cell line was transiently transfected with a plasmid expressing human ERα and a plasmid containing the luciferase gene under the control of the vitellogenin promoter. This promoter contains two consensus estrogen receptor binding sites and is activated strongly in the presence of ER and estradiol. None of the compounds showed any agonist activity (data not shown), so antagonist activity was determined by performing competition assays in the presence of 10 nM estradiol. As shown in **Figure 3** and summarized in **Table 1**, the compounds were all antagonists of estradiol-induced ER activation at the vitellogenin promoter at relatively low concentrations. Although the variability between assays is much greater with cell-based assays than with the in vitro binding assay, compound **5** showed significant decrease in antagonist potency compared to the other compounds.

Whether this decrease is due to weaker binding affinity for the receptor or diminished cell uptake is unknown. Overall, however, all of the tamoxifen analogs all inhibited ER-mediated transcriptions at concentrations that are low enough to allow for future derivatization studies.

Conclusion

In summary, a novel set of tamoxifen analogs has been made using a relatively simple synthetic scheme. Receptor affinity assays and reporter gene assays indicate that many of the analogs have potencies similar to tamoxifen and would make suitable analogs to conjugate to other moieties in order to study roles of the different pathways leading to estrogen receptor-mediated transcriptional regulation. These moieties will include fluorescent molecules that will allow for the visualization of binding either inside the cell or on the cell surface. The analogs will also be conjugated to cell-impermeable polyacrylate polymers that should allow for selective targeting of membrane-initiated responses of estrogen receptor. It is envisioned that these tools will help elucidate the pleiotropic behavior of tamoxifen and could be used in the future to help engineer novel transcription factors that could either activate or repress the transcription of specific genes.

Materials and Methods

General methods

All reagents were purchased from Sigma-Aldrich. The expression plasmids used in this study, pSG5-ERα and ERE-luciferase were generously provided by Thomas Scanlan (UCSF) and have been described elsewhere [13, 14]. The ERE-driven luciferase reporter gene consists of two repeats of the upstream region of the vitellogenin ERE promoter from –331 to –289, followed by region –109 to +45 of the thymilidate kinase upstream region and the luciferase gene. Proton and ^{13}C nuclear magnetic resonance spectra (1H NMR, 13C NMR) were obtained on a Bruker ARX300 (300 MHz) instrument; 1H NMR chemical shifts are reported as δ values in parts per million (ppm) downfield from internal tetramethylsilane. 13C NMR chemical shifts are reported as δ values with reference to the solvent peak. Mass spectrometry (MS) and NMR instruments were provided by the Shared Resource center of the Purdue Cancer Center.

Synthesis of tamoxifen analogs

***E* and *Z* 4-{1-[4-(2-Bromo-ethoxy)-phenyl]-2-phenyl-but-1-enyl}-phenol (4)** Diphenol (**3**) (0.5 g, 1.59 mmol) [11] was dissolved in DMF (10 mL) and then cesium carbonate (2.07 g, 6.4 mmol, 4 equiv.) was added and the solution was heated at 60 °C for 15 minutes. 1,2 dibromoethane (0.5 mL, 5.7 mmol, 4.5 equiv.) was then added all at once and the reaction was allowed to stir for 16 hr. at 60 °C. 30 mL of water was then added to the reaction mixture and the compounds were extracted with ethyl acetate twice. The organic layer was washed with brine, dried with magnesium sulfate and then the solvent was evaporated under reduced pressure. Purification by flash silica gel chromatography using 30% ethyl acetate in hexane as the eluent provided 0.25 g of desired product (0.59 mmol, 37% yield) as a mixture of interconverting E and Z isomers. 1H NMR (300 MHz) (CDCl3) δ 7.15 (7H, m) δ 6.94 (2H, d) δ 6.83 (1H, dd) δ 6.78 (1H, d) δ 6.62 (1H, d) δ 6.56 (1H, d) δ 4.69 (1H, t) δ 4.57 (1H, t) δ 4.12 (1H, t) δ 4.01 (1H, t) δ 3.10 (2H, q) δ 1.77 (3H, t); 13C NMR (300 MHz) (CDCl3) δ 157.18, δ 153.83, δ 142.95, δ 141.704, δ 138.01, δ 137.48, δ 136.29, δ 132,55, δ 131.15, δ 130.13, δ 128.24, δ 126.42, δ 115.43, δ 114.75, δ 114.00, δ 68.30, δ 29.68, δ 14.06. MS (CI) m/z 423/425 $(M + H)^+$;

General synthesis of amine analogs The bromide (**5**) (50 mg, 0.12 mmol) was dissolved in THF (2 mL) and 0.5 g of the appropriate diamine (as described below) was then added and the solution was heated at 60 °C for 12 hr. in a sealed tube. The solvent was then removed evaporated under reduced pressure and then purification by silica gel flash chromatography using 5.5/4/0.5 $CHCl_3/CH_3OH/NH_4OH$ as the eluent provided the product as a mixture of interconverting *E* and *Z* isomers. Below is information for each compound:

***E* and *Z* 4-{1-[4-(2-Aminoethoxy)-phenyl]-2-phenyl-but-1-enyl}-phenol (5)** NH_4OH was used as the amine and 43 mg of purified product was isolated (0.11 mmol, 92% yield). 1H NMR (300 MHz) (CDCl3) δ 7.15 (7H, m) δ 6.88 (1H, d) δ 6.81 (2H, dd) δ 6.72 (1H, d) δ 6.58 (1H, d) δ 6.52 (1H, d) δ 4.51 (1H, t) δ 4.37 (1H, t) δ 3.58 (1H, t) δ 3.49 (1H, t) δ 3.12 (5H, m) δ 2.02 (1H, s) δ 1.76 (3H, t);. MS (CI) m/z 360 (M+H);

***E* & *Z* 4-{1-[4-(2-Methylaminoethoxy)-phenyl]-2-phenyl-but-1-enyl}-phenol (6)** 2 M methylamine in THF was used as the amine and 35 mg of purified product was isolated (0.094 mmol, 78% yield). 1H NMR (300 MHz) (CDCl3) δ 7.15 (7H, m) δ 6.88 (1H, d) δ 6.81 (2H, dd) δ 6.72 (1H, d) δ 6.58 (1H, d) δ 6.52 (1H, d) δ 5.76 (2H, s) δ 4.51 (1H, t) δ 4.37 (1H, t) δ 3.58 (1H, t) δ 3.49 (1H, t) δ 3.12 (5H, m) δ 2.02 (1H, s) δ 1.76 (3H, t); 13C NMR (300 MHz) (CDCl3) δ 157.61, δ 156.77, δ 156.26, δ 155.34, δ 143.18, δ 141.20, δ 138.43, δ 137.28, δ 136.78, δ 135.47, δ 135.10, δ 132.43, δ 131.13, δ 130,16, δ 128.26, δ 126.27, δ 115.71, δ 115.04, δ 114.40, δ 113.65, δ 66.56, δ 50.81, δ 36.14, δ 29.50, δ 14.11. MS (CI) m/z 374 (M+H);

***E* and *Z* 4-(1-{4-[2-(2-Aminoethylamino)-ethoxy]-phenyl}-2-phenyl-but-1-enyl)-phenol (7)** Ethylenediamine was used as the amine and 32 mg of purified product was isolated (0.087 mmol, 73% yield). 1H NMR (300 MHz) (CD_3OD) δ 7.15 (7H, m) δ 6.88 (1H, d) δ 6.81 (2H, dd) δ 6.72 (1H, d) δ 6.58 (1H, d) δ 6.52 (1H, d) δ 4.51 (1H, t) δ 4.37 (1H, t) δ 3.58 (3H, t) δ 3.49 (3H, t) δ 3.12 (5H, m) δ 2.02 (1H, s) δ 1.76 (3H, t); 13C NMR (300 MHz) (CD_3OD) δ 159.4, δ 158.5, δ 157.9, δ 157.0, δ 144.6, δ 142.4, δ 142.2 δ 140.2, δ 138.43, δ 137.6, δ 136.3, δ 133.47, δ 132.43, δ 131.13, δ 130,16, δ 128.26, δ 126.27, δ 115.71, δ 115.04, δ 114.40, δ 113.65, δ 66.56, δ 42.13, δ 31.2, δ 29.50, δ 14.11. MS (CI) m/z 403 (M+H);

***E* and *Z* 4-[1-(4-{2-[Methyl-(2-methylaminoethyl)-amino]-ethoxy}-phenyl)-2-phenyl-but-1-enyl]-phenol (8)** N,N' dimethylethylenediamine was used as the amine and 15 mg of purified product was isolated (0.035 mmol, 29% yield). 1H NMR (300 MHz) ($CDCl_3$) δ 7.15 (7H, m) δ 6.88 (1H, d) δ 6.81 (2H, dd) δ 6.72 (1H, d) δ 6.58 (1H, d) δ 6.52 (1H, d) δ 4.37 (1H, t) δ 4.12 (3H, t) δ 3.95 (3H, t) δ 3.6 (5H, m) δ 2.58 (3H, s), δ 2.50 (3H,s), δ 2.02 (1H, s) δ 1.76 (3H, t)

E and Z 4-(1-{4-[2-(6-Amino-hexylamino)ethoxy]-phenyl}-2-phenyl-but-1-enyl)-phenol (9) 1,6-diaminohexane was used as the amine and 40 mg of purified product was isolated (0.092 mmol, 77% yield). 1H NMR (300 MHz) ($CDCl_3$) δ 7.15 (7H, m) δ 6.88 (1H, d) δ 6.81 (2H, dd) δ 6.72 (1H, d) δ 6.58 (1H, d) δ 6.52 (1H, d) δ 3.6 (5H, m) δ 2.58 (2H, t), δ 2.50 (2H, t), δ 2.02 (1H, s) δ 1.6 (3H, t), δ 1.3 (8H, m)

***E* and *Z* 4-[1-(4-{2-[Methyl-(6-methylaminohexyl)-amino]-ethoxy}-phenyl)-2-phenyl-but-1-enyl]-phenol (10)** N,N' dimethyl-1,6-diaminohexane was used as the amine and 18 mg of purified product was isolated (0.037 mmol, 31% yield). 1H NMR (300 MHz) ($CDCl_3$) δ 7.15 (7H, m) δ 6.88 (1H, d) δ 6.81 (2H, dd) δ 6.72 (1H, d) δ 6.58 (1H, d) δ 6.52 (1H, d) δ 3.2 (2H, t) δ 3.1 (2H, t), δ 2.55 (2H, t), δ 2.45 (6H, s), δ 2.22 (2H, t) δ 1.6 (3H, m), δ 1.3 (8H, m).

Fluorescence polarization assay.

Fluorescent polarization based competition binding assays were conducted to determine the relative affinity of the 4-hydroxytamoxifen analogs for ERα using a commercially available kit (PanVera Corp., Madison, WI). Briefly, serial dilutions of the different compounds were prepared in ES2 screening buffer (100mM potassium phosphate, pH7.4, 100μg/ml bovine gamma globulin) and 50μl of each concentration was aliquoted into three wells of a black 96 well assay plate. Fifty microliters of a solution containing 20nM recombinant ERα, and 2nM of a proprietary fluorescent ER ligand (Fluormone-ES2) was added to each well. The plate was shaken on a plate mixer and incubated for 2 h in the dark at room temperature. Fluorescence polarization signals were then measured using a Packard Fusion fluorimeter. The data were then fit to a single binding site competition curve by nonlinear regression analysis (Prism 3 software package). K_i values were determined from the average of 3 different experiments and calculated using a K_D=4 nM for Fluormone binding to ERα.

Cell culture and transient transfection experiments

Cell Culture HeLa cells were obtained from the American Type Culture Collection (ATCC). HeLa cells were maintained in DME media without phenol red (Sigma) supplemented with 4.5 g/L glucose, 0.876 g/L glutamine, 100 mg/L streptomycin sulfate, 100 units/mL of penicillin G and 10% FBS at 37 °C in a air/carbon dioxide (95:5) atmosphere. Transfection assays were run with the same media conditions except the FBS was treated for 24 hours with dextran-coated charcoal.

Transient transfection assays HeLa cells were plated in 24 well plates and grown to approximately 70-80% confluency. Transfections were performed according to the protocol for Lipofectamine 2000® (Invitrogen). In order to normalize for the transfection efficiency in each well, the dual luciferase system was used in which a constitutively expressed, chemically orthogonal luciferase expression vector was also transfected. The total amount of DNA/well for each plasmid was as follows: pSG5-ERα 0.25 μg/well, ERE-luciferase 0.5 μg/well, and *Renilla*-luciferase 0.25 μg/well. The ratio of total DNA/Lipofectamine 2000® ratio was 1:5. After transfection, the plates incubated at 37 °C for 6 hours before dosing with drug. All drugs were delivered in DMSO or ethanol and the total concentration of organic solvent in each was 0.1% For competition experiments, the drug was added to media already containing 10 nM estradiol. After 18-24 hours, the cells were lysed and assayed for dual luciferase activity in a TopCount luminometer according to the protocol provided by Promega. The relative light units (RLU) were then calculated by dividing the output of the ERE-driven luciferase in each well by the output of the *Renilla* luciferase. Each drug concentration was tested in triplicate.

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Captions

Figure 1. estradiol (**1**) and tamoxifen (**2**)

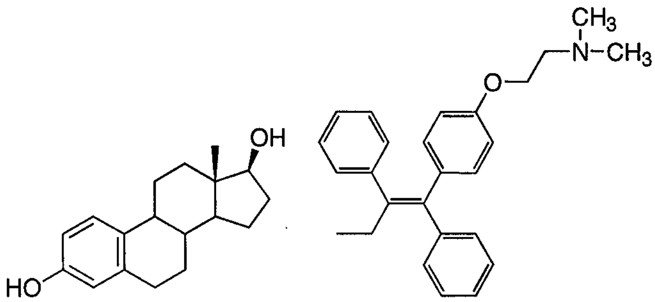
Scheme 1. a.) Cs_2CO_3, DMF, 60 °C; 1,2 dibromoethane, 16 hrs. b.) RNHR', THF, 60 °C, sealed tube, 12 hrs.

Figure 2. Relative ER binding affinity of tamoxifen analogs **7-10**. The ability of various concentrations of different compounds to displace a synthetic fluorescent estrogen from recombinant preparations of ERα was evaluated as described in the material and methods section. 100 represents no displacement of fluorescent ligand, zero represents total displacement. Each point represents the mean and standard error of the mean of 3 different samples. The lines represent the best fit to a single binding site competition model. Dashed lines represent the fit for the methylated compounds.

Figure 3. Competition of the compounds **7-10** versus 10 nM estradiol in transient transfection assay of HeLa cells with ERα and the vitellogenin A2 ERE-tk driven luciferase reporter gene. Curve represents the best fit to a single-site competition binding model. 100% activation represents the activation with 10 nM estradiol alone. Each point represents the mean and standard error of the mean of 3 different samples. Lines represent the best fit to a single binding site competition model. Dashed lines represent the fit for the methylated compounds.

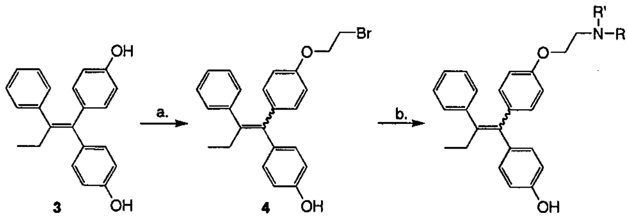
Table 1. Summary of Ki values for compounds calculated from the receptor competition experiments and IC50 values vs. 10 nM estradiol calculated from the reporter gene assays.

Figure 1



(1) (2)

Scheme 1.



	R	R'
5	H	H
6	CH_3	H
7	H	$(CH_2)_2NH_2$
8	CH_3	$(CH_2)_2NHCH_3$
9	H	$(CH_2)_6NH_2$
10	CH_3	$(CH_2)_6NHCH_3$

Figure 2

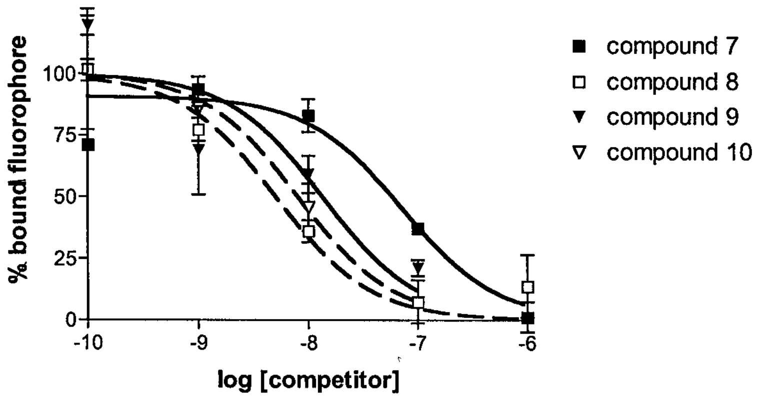
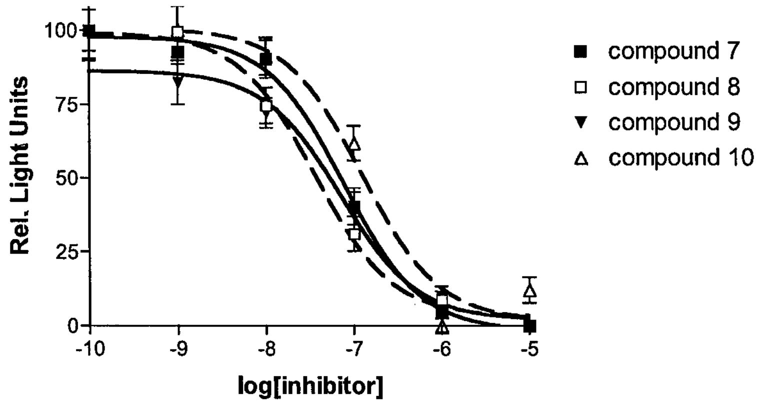

% bound fluorophore
100
75
50
25
0
-10
-9
-8
-7
-6
log [competitor]
compound 7
compound 8
compound 9
compound 10

Figure 3



100
75
50
25
0
-10
-9
-8
-7
-6
-5
Rel. Light Units
log[inhibitor]
compound 7
compound 8
compound 9
compound 10

Table 1

compound	Ki (nM)	IC50 (nM)
estradiol (**1**)	6.3 ± 0.2	N.D.
5	48 ±5	800 ± 400
6	8.5 ± 3.9	40 ± 10
7	32 ± 10	150 ± 50
8	3.4 ± 2.1	39 ± 12
9	9.8 ± 6.2	85 ± 55
10	6.2 ± 4.6	126 ± 33