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TAMOXIFEN: AN EMERGING PREVENTIVE

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1. ABSTRACT

Tamoxifen is well known for its actions as an antagonist of estrogen receptor-mediated signaling and is one of the most extensively used endocrine agents both in the clinic and in the research setting. Tamoxifen has emerged from recent Breast Cancer Prevention Trials, conducted to evaluate risk reduction, as an effective preventive agent. Specifically, comparing tamoxifen to placebo (for 5 years) has shown that tamoxifen: (a) significantly reduced the risk of breast cancer recurrence, in those with a history of the disease; (b) reduced or delayed

breast cancer progression, from an noninvasive to invasive breast cancer; (c) prevented or substantially reduced the risk of getting breast cancer (risk of occurrence) in healthy women with risk factors. The extraordinary outcomes offer support for the use of tamoxifen in multilevel preventive approaches and predict that it will continue to be vital in facilitating mechanistic studies. Information produced by mechanistic studies is needed to understand how to prevent cancer and how to confront treatment problems; such as resistance. Molecular determinants of the "resistant phenotype" to tamoxifen are currently being identified. The next major effort will be to link these determinants to readily detectable biological changes that could be used to indicate the development of resistance before clinical manifestations develop.

2. INTRODUCTION

Breast cancer is a hormone dependent disease and mammary tumor cells often express ER. Although there are at least two forms of the receptor, ER alpha and beta, it is ER alpha that is routinely measured in patient samples. The ER plays a central role at many stages of this disease. Furthermore, ER alpha is critical target for the actions of endocrine anti-cancer agents and tamoxifen-mediated breast cancer prevention.

The observation of a connection between breast cancer and ovarian estrogens was made long before it was recognized that a receptor even existed. This observation was described by Dr. Beatson[1] in 1896, when he reported the stunning discovery that an advanced breast cancer patient went into remission after the removal of her ovaries. The problem of estrogen dependent tumor growth has been confronted ever since.

There is often a positive correlation between ER levels, age, and progesterone receptor (PR) status. ER-driven changes in proliferation and cell survival can be inhibited by tamoxifen, one of several Selective Estrogen Receptor Modulators or SERMS. Tamoxifen is perhaps the most widely used endocrine agent for breast cancer and its clinical success relates, in part, to its effectiveness in binding to the ER and opposing many but not all of its actions.

Our understanding of the mechanisms responsible for breast cancer, and the effectiveness of current treatments in delaying cancer development, progression and recurrence or preventing it entirely has been advanced tremendously by findings from clinical trials.

3. CLINICAL TRIALS

3.1. Prevention-based clinical trials involving tamoxifen

3.1.1. National Surgical Adjuvant Breast and Bowel Project (NSABP P-1), Breast Cancer Prevention Trial (1)

The Breast Cancer Prevention Trial (BCPT), protocol 1 of the NSABP, compared the effectiveness of tamoxifen (1) in preventing breast cancer in women with a higher than average risk of the disease due either to age (healthy women 60 years and older with a 5 year, age-associated risk of 1.66% or greater), or a personal history of breast cancer (35 to 59 years old who have had lobular carcinoma *in situ* or LCIS).

The outcome of this study showed that tamoxifen was able to reduce the *overall* risk of developing invasive breast cancer by 49% (see Table 1). The reduction in risk was 44% for premenopausal women under the age of 44, and 55% for postmenopausal women 60 years old and older in the tamoxifen arm of the study. The risk of noninvasive breast cancer was reduced by 50%. Interestingly, the risk of ER positive invasive tumors was reduced by 69%. The risk of developing invasive breast tumors that were ER negative didn't change (1-3).

When tamoxifen was administered to women with a history of LCIS breast cancer (which also tends to be ER positive), the risk of developing invasive disease was reduced by 56 to 86%. This study also showed that the longer a women remained on tamoxifen, the greater the risk reduction. For example, after 1 year of therapy with tamoxifen the risk of invasive breast cancer was reduced by 35%, after 5 years of tamoxifen, it was reduced by 69%. Beyond 5 years, no further benefit could be detected in association with tamoxifen.

Based on these findings, the use of tamoxifen to reduce the risk of breast cancer was recommended for pre- and postmenopausal women. In the U.S. tamoxifen is licensed to reduce the incidence of early breast cancer for use in healthy women who have a greater than average risk of developing breast cancer (1.66% or greater – 5 year risk) either because of age or a family history (1, 2, 5).

Concerns previously raised about tamoxifen-associated increases in colon cancer, liver cancer, and retinal toxicity, were not substantiated by the P-1 trial findings (1, 2). However, there were increases in the number of cataracts, thromboembolic events and endometrial cancers in the tamoxifen group, suggesting that the risk:benefit ratio must be carefully considered, especially in the presence of additional risk factors (1, 5, 6). Tamoxifen correlated positively with improvements in the lipid profiles, bone mineral density measurements, and in the incidence of fracture among those in the tamoxifen arm of the trial.

3.1.2. Italian and Royal Marsden Breast Cancer Prevention Trials (7, 8)

The Italian trial (7) compared the effectiveness of tamoxifen to placebo in reducing the incidence of breast cancer in women, ages 35-70, who have previously had a hysterectomy. Those who had undergone ovariectomy or were on hormone or estrogen replacement therapy (H/ERT) were also accepted into the trial.

In the British (Royal Marsden) trial (8), the effectiveness of tamoxifen in lowering the incidence of breast cancer was compared to placebo in women at a higher

Table 1. Breast Cancer Prevention Trials Evaluating Tamoxifen vs. Placebo

Breast Cancer Prevention Trials	Accrual No.	Med. F-U	% HRT Use	Selection Criteria	Yrs	Findings	Comments
NSABP P-1 **BCPT** Breast Cancer Prevention Trial (1, 3, 5)	13,388	54.6 mos.	0	2 H.Risk Grps: 60 yrs & over, risk = 1.67% or LCIS 35-59 yrs old	5	Tamoxifen lowered the risk of developing breast cancer by: 49%	Resistant (?) ER+ Invasive breast. cancer in: tamox vs. placebo placebo
ITALIAN European ------- Institute of Oncology (3, 7, 9)	5,408	46 mos.	14	Hysterectomy---------- ages 35 to 70 ---------- Ovariectomy in 48%	5	No. Br. Cancers: HRT/Tamox. = 1, HRT/Placebo = 8; Overall difference: *n.s.d*	Poor compliance; variable HRT use; low statistical power; early age at ovariectomy
ROYAL MARSDEN Royal Marsden Hospital Group (3, 7)	2,494	70 mos.	41	H. Risk = family history: breast cancer in 1 or more 1st degree relatives ages 30 to 70 yrs.	8	Risk increased for those on ERT at entry or decreased if started during trial; Overall difference: *n.s.d.*	61% were under 50; ---------- HRT use: variable; -------- more BRCA1 or 2 mut. carriers in tamox. group with ER+ tumors was possible
EBCTCG Early Breast Cancer Trialists Collaborative Group	37,000	10 yrs total*	- -	Reduction in risk of recurrence = 1, 2, 5 yrs. tamoxifen	10	Tamoxifen (5 yrs) Reduction in risk at 10 years was: 42% reduction in breast cancer recurrence; 47% reduction in contralateral breast cancer recurrence	Metanalysis of 55 different adjuvant breast cancer trials worldwide; node negative or positive early breast cancer, 4 arms: tamoxifen for: 1-, 2-, or 5-yrs vs.placebo; followed for 10yrs*

Accrual No., total enrollment; Med. F-U, median follow up, % HRT use, use of hormone replacement therapy at the time of enrollment (shown as a percent of the total); Yrs., trial duration in years; H. Risk Grps., high risk groups; tam or tamox, tamoxifen; mut, mutant; criteria, criteria used to select study participants, may reflect risk. Note: In all trials, tamoxifen was compared with placebo. The NSABP P-1 was led by Bernard Fisher; the Italian trial was led by Umberto Veronissi; and the Royal Marsden Trial was led by Trevor Powles (1, 3, 5, 7 9)

than average risk due to their family history (one or more first degree relatives diagnosed with breast cancer under the age of 50, or in both breasts, or after multiple abnormal biopsies).

Findings from the Italian and British trials did not yield results that were consistent with those of the P-1 study. The Italian trial found no significant difference in the breast cancer risk reduction between the two groups: tamoxifen vs. placebo, and only marginal differences were detected in the British trial (8). Issues involving of protocol compliance and trial participant selection criteria were suggested to account these differences. Trial participants in the Italian study was considered to be a low risk group selected on the basis of prior hysterectomy. British trial participants were selected on the basis of a strong positive family history, suggesting that a greater than average number of BRCA-1 or -2 mutation carriers, who are likely to be insensitive to tamoxifen, could have been included in the group (8). In the Italian and British studies, hormones were used (mainly ERT) by 14- and 41% of the study populations, respectively. In that the ultimate goal of prevention is long term health, a truer gauge of tamoxifen's role in breast cancer prevention might be based post-trial duration. This would address the question of how long the preventive/therapeutic effects of tamoxifen would be sustained. Would the health benefits persist over time or be limited in duration?

3.1.3. National Surgical Adjuvant Breast and Bowel Project NSABP (B-24) (9-10)

The B-24 (9-10) trial tested the effectiveness of tamoxifen for 5 years versus placebo as adjuvant therapy for patients with ductal carcinoma *in situ* (DCIS). There were 1,894 participants; all had undergone surgical resection and treatment with radiation before receiving tamoxifen. An analysis of this trial demonstrated positive benefits in association with tamoxifen (9). A summary of the NSABP B-24 trial is given in Table 2.

3.1.4. Gruppo Universitario Natoletano (GUN) (11)

The Italian GUN (11) trial was initiated in order to identify predictive markers of adjuvant tamoxifen efficacy in early breast cancer. In this trial, 433 breast cancer patients were randomized to receive tamoxifen or placebo (including a subgroup to receive concurrent chemotherapy) for two years following local-regional treatment. Tumor samples were taken for the evaluation of 8 biological markers (11). In an analysis of the findings a significant association was revealed for HER2 and

Table 2. National Surgical Adjuvant Breast and Bowel Project (B-24) Trial

Category	Invasive	Nonivasive	Ipsilateral	Contralateral
% Reduction in Risk	43	31	30	52

This trial compares tamoxifen to placebo in women with DCIS following resection and radiation. DCIS is a non-invasive form of breast cancer. However, it is associated with a substantial risk of developing invasive breast cancer. The 1,894 participants in this trial were evaluated for the prevention of relapse or occurrence of invasive disease while on tamoxifen for 5 years or placebo. A clear benefit was found in each of the categories tested, shown as the % risk reduction associated with tamoxifen, compared to placebo (9-10)

tamoxifen. There were no predictive associations between the other biological markers tested (microvessel counts, prolactin receptor, DNA ploidy, S-phase fraction, and EGFR) and the effectiveness of tamoxifen. Adjuvant tamoxifen therapy was found to be effective in reducing the hazard of death for patients whose tumors were HER2 negative. Strikingly, tamoxifen therapy of those patients with HER2-overexpressing tumors was found to be detrimental in the absence of chemotherapy or ineffective when given concurrently (11). The results of this study indicate that *HER2 is a negative predictor of tamoxifen efficacy without chemotherapy*. If confirmed, tamoxifen may no longer be a valid treatment option for those with HER2 overexpressing breast cancers.

3.1.5. Adjuvant Tamoxifen Offer More (aTTom) (2)

This trial compares adjuvant tamoxifen therapy for 3 years with that of placebo in women that had been treated with tamoxifen for at least 2 years in order to determine the optimal duration of tamoxifen therapy in the adjuvant setting (2).

3.1.6. Adjuvant Tamoxifen Longer Against Shorter (ATLAS) (2)

In the ATLAS (2) trial adjuvant tamoxifen therapy for 5 years is compared with that of placebo in women who have been treated with tamoxifen for at least 2 years in order to determine the optimal duration of tamoxifen therapy in the adjuvant setting. Note that both the aTTom and ATLAS trials are designed to determine the optimal duration of tamoxifen therapy in ER positive breast cancers (2).

3.1.7. International Breast Cancer Intervention Study (IBIS) (12)

In the IBIS (12) trial, over 7,000 women, ages 45-70, with an elevated risk of breast cancer (estimated to be 4-times greater than that of the general population), were randomized to compare the effectiveness of tamoxifen to placebo in reducing the risk of developing breast cancer (over 40% of the participants were taking HRT at the time of enrollment).

3.1.8. Study of Tamoxifen and Raloxifene (STAR) (4)

Findings from NSABP P-1 and *MORE* (*MORE* = *Multiple Outcomes of Raloxifene Evaluation,* not included here*)* trials, provided a rationale for the STAR (1, 4) trial which is designed to compare the effectiveness of Raloxifene to Tamoxifen in preventing invasive breast cancer in healthy and postmenopausal women and in those at high risk, ages 35 and older (the final results of this trial are projected for the year 2007). In addition to breast cancer risk reduction benefits, this study will also compare the incidence in side effects (deep vein thrombosis, pulmonary embolism) associated with Raloxifene vs. Tamoxifen, relative to placebo.

3.1.9. Hormone Replacement Therapy and Tamoxifen (HOT) (13)

Based on findings from the Italian breast cancer prevention trial, the HOT trial is a phase III prevention trial designed to evaluate the impact of postmenopausal HRT on the effectiveness of low dose tamoxifen in reducing invasive breast cancer risk over 5 years (13). 8,500 healthy postmenopausal women are being recruited into this trial and they must all be *de novo* HRT users. During a period of 5 years the participants will be given 5 mg of tamoxifen per day (or placebo) instead of the usual dose of 20 mg (10 mg twice daily). The primary endpoint will be the incidence of invasive and intraductal breast cancer (13), with several secondary endpoints related to cancer (including endometrial cancer, cardiovascular and embolic events, bone fracture and cataract incidence).

3.1.10. Tamoxifen Alone vs. Adjuvant Tamoxifen (for the prevention of breast cancer in the elderly (GRETA) (2)

The GRETA trial compared the effects of surgery plus adjuvant tamoxifen to tamoxifen alone (over a period of 5 years) on overall survival (OS) in 474 patients 70 years and older with early breast cancer. There were no differences in OS between the treatment groups, minimal surgery followed by tamoxifen was recommended.

3.1.11. Arimidex (Anastrozole) versus Tamoxifen, Alone or in Combination (ATAC) (14)

Data analysis from the ATAC trial, which compared the efficacy of anastrozole to that of tamoxifen, has revealed favorable findings (14). ATAC findings show that anastrozole is more effective than tamoxifen in reducing the incidence of breast cancer. A dramatic 70% reduction in contralateral breast cancer was observed in the anastrozole arm of this trial. In addition to the reduction of contralateral breast cancers, disease free survival (DFS) and time to recurrence were also found to be significantly decreased by anastrozole. The greatest benefit derived from this trial was for those in the hormone receptor positive group, relative to the overall study population (14). Interestingly, the improvements described for anastrozole were not observed among those taking the combination.

In addition to separate groups for anastrole and tamoxifen, there was a combination arm in this trial. However, this arm of the trial had to be discontinued early (14). This was because the effectiveness of coadministering tamoxifen and anastrozole together was found to be inferior

Table 3. Classification of Endocrine Agents

Androgens	Progestins	Estrogens	[a]SERMS	Aromatase Inhibitors	ERDs
Fluoxy-mestrone	Megestrol Acetate *(Megace)*	Estradiol *(E2)*	Raloxifene *(keoxifene, Evista, LY139481, LY156,758)*	4-hydroxyandrostenedione (*4-OH-A, formestane 1st generation, SAI[b])*	Faslodex *(ICI 182, 780, fulvestrant)*
	Medroxy-Progesterone Acetate	Diethyl-stilbesterol *(DES)*	Fareston *(toremifene)*	Aromasin *(exemestane, FCE 24,304 2nd generation, SAI)*	
			Nolvadex (*tamoxifen ICI 46,474)*	Aminogluthemimide (*Orimethen, 1st gen., NSAI[c])*	
			Idoxifene *(CB 7432)*	Fadrozole *(CGS 16,949A, 3rd generation, NSAI)*	
			Droloxifene *(3-hydroxy-tamoxifen)*	Arimidex *(anastrozole, ZD 1033, 3rd generation, NSAI)*	
			Ospemifene (*FC-1271a*)	Letrozole *(Femara, CGS 20,267, 3rd generation NSAI)*	
			Arzoxifnene (*LY353381*)		
			Lasifoxifene		
			MDL 103,323		

Shown are some of the endocrine agents used or investigated for the treatment and prevention of breast cancer, and/or conditions associated with menopause. [a] SERMS, Selective Estrogen Receptor Modulators; [b] SAI, steroidal aromatase inhibitor; [c] NSAI, nonsteroidal aromatase inhibitor; [d] ERDs, Estrogen Receptor Downregulators. [e]ICI 182,780 is a steroidal compound that acts as a pure antiestrogen of ER signaling, causing neither vasomotor symptoms nor endometrial stimulation as seen with tamoxifen

to monotherapy with either agent alone (ATAC Trialists' Group). This finding raises the possibility of antagonism between the two agents, despite the utility of anastrozole, which was well demonstrated in this clinical trial.

3.1.12. Intermediate Marker Project: Anastrozole, Combination or Tamoxifen (IMPACT) (15)

The IMPACT trial (15) has a design similar to that of ATAC. However, in this case, the neoadjuvant setting is used as a platform for biomarker discovery. The efficacy of anastrazole, tamoxifen, and the combination will be compared in the traditional way and by molecular marker sampling in order to identify and define early *changes on the way to a clinical response*. Ki-67 and other proliferation markers will be used to compare the relative effects of each agent on proliferation (details are given in ref.15).

3.2. Summary of Trial Findings and Implications

Findings from several recent trials confirm the known favorable effects of tamoxifen on *blood lipid profiles* (LDL and total cholesterol decrease) and on *bone mineral density* (BMD, where tamoxifen diminished the rate of BMD loss) as well as the association between tamoxifen and *endometrial cancer or thromboembolic events,* which were found to increase (1-2, 9). Each of these outcomes arises from the estrogenicity associated with tamoxifen, and represents ER-mediated agonism.

With respect to the primary endpoints measured in the NSABP (P1), (B24), and EBCTCG trials (see Tables 1. and 2.), comparisons between tamoxifen and placebo reveal several striking findings. First of all, tamoxifen significantly *reduced the risk of breast cancer recurrence* in those with a history of the disease. Secondly, five years of tamoxifen afforded protection against *breast cancer progression* (reducing and possibly preventing the changes required to go from an noninvasive to invasive). And, third, in healthy women (with risk factors, like having a positive family history or being 60 years of age or older) tamoxifen treatment *diminished breast cancer occurrence* altogether. These extraordinary outcomes clearly show that a net preventive effect can be achieved by tamoxifen in health and at different levels and stages of cancer progression and development.

4. AROMATASE INHIBITORS (AIs)

Arimidex (also called anastrozole) is a third generation aromatase inhibitor (see Table 3.), which is available for use in the treatment of postmenopausal women with advanced breast cancer (15, 17-18). Recent clinical trial findings indicate that AIs could be superior to tamoxifen in some breast cancers (14-16), and anastrozole is currently being evaluated in clinical trials for adjuvant therapy of early breast cancer. Whether overall survival (OS) in early breast cancer is greater with AIs than it is with tamoxifen has not been established.

4.1. Mode of action

Anastrozole and other AIs act in a way that is entirely distinct from that of tamoxifen. AIs do not bind to the ER but rather, they bind to the cytochrome P450 (CYP) enzyme, aromatase. This enzyme is responsible for catalyzing the conversion of adrenal androgens to estrogens. When anastrozole binds to the CYP P450 for aromatase, it becomes inactivated. The inactivation of aromatase *disables* peripheral pathways that are responsible for converting androgens into estrogens.

Although the aromatase enzyme is best known for its ability to catalyze the conversion of testosterone to

estradiol (E2), the main substrate for aromatase is androstenedione which becomes aromatized to *estrone* (E1) in the peripheral tissues like fat, liver, breast tissue, muscle, and in mammary tumor cells, where this enzyme is most active (17-19). In postmenopausal women the concentration of *androstenedione is 4-times greater* than that of testosterone (17) and the major circulating estrogen is estrone.

Furthermore, AIs are devoid of intrinsic estrogenic activity and as potent inhibitors of aromatase they effectively reduce circulating estrogen levels in postmenopausal women (17). Notably, these interactions, between AIs and aromatase, are reversible (17-19).

Currently there are 3 main AIs approved by the FDA for use in breast cancer. These are anastrozole (Arimidex), exemestane (Aromasin), and letrozole (Femara), as shown in Table 3. In addition to their use for advanced or metastatic breast cancers, AIs may also be potent alternatives to tamoxifen for adjuvant therapy in the early breast cancers of postmenopausal women (20-23). Anastrozole, for example, has been approved for the adjuvant treatment of early, ER positive breast cancers in postmenopausal women.

4.2. Limitations Associated with AIs

There are three main limiting factors associated with AI use for the treatment of breast cancer.

4.2.1. Menopausal Status

AI use is generally limited to *postmenopausal women*, or women whose ovaries are no longer functional where estrogen levels are not subject to feedback controls. In premenopausal women, ovarian steroid synthesis is responsible for the high circulating levels of estradiol (20-22).

4.2.2. Estrogen Deprivation

AIs *impose a state of estrogen deprivation*, because they have a complete lack of estrogenicity themselves and can enforce a dramatic reduction in estrogen levels, they also deprive tissues that are in critical need these estrogens (i.e.: in the skeletal and cardiovascular system). There is evidence that the concern for potential bone loss, elevated cholesterol levels (LDLs) and other complications (20-23) during prolonged AI therapy, may be warranted. In the bone substudy of the ATAC trial, participants in the anastrozole (Arimidex) group showed decreases in lumbar spine and total hip BMDs, relative to the starting values. Just the opposite was found, however, for study participants in the tamoxifen arm of the trial, who showed mean increases in each of these values.

4.2.3. ER Positivity

AIs *require the presence of functional ER for optimal antitumor activity*.

4.3. Limitations Associated with Antiestrogens (AEs)

There are also limitations associated with the use of AEs.

4.3.1. Menopausal Status

Although AEs, like tamoxifen, can be used by both pre- and postmenopausal women, antitumor activity also *requires the presence of functional ER*.

4.3.2. Estrogenicity

AEs vary in the estrogenicity they contribute from none (for ICI 182,780, also called Faslodex or fulvestrant) to a small but significant degree of agonism (for tamoxifen). Although can tamoxifen act as an antagonist in mammary tissue, it exerts agonism in other parts of the body. The same estrogenic or partial agonistic activity that is responsible tamoxifen's positive effects on serum lipids and bone mineral density in postmenopausal women is also responsible for increasing in the likelihood of endometrial cancer, thromboembolism, and cardiovascular incidents during prolonged tamoxifen therapy. Therefore, the usefulness of tamoxifen is limited in those with additional risk factors for these conditions.

4.3.3. Resistance

Tamoxifen, and other AEs, are *limited in the effective duration and quality of treatment* due to the notable development of resistance (discussed in Section 7).

- AEs or AIs?

The use of AIs is limited to those who have undergone oviarectomy or do not have functioning ovaries (i.e.: postmenopausal women). Tamoxifen is appropriate for both pre- and postmenopausal women (14, 16, 20, 23).

Tamoxifen has been shown to have long term benefits. A remaining question to be answered for anastrozole is the optimal duration of therapy. There have been some reports of continued benefit for several years (up to 9 years, in some cases) after tamoxifen therapy had been completed (EBCTCG, 24). The duration of benefit associated with AIs, unlike tamoxifen, has not yet been determined. AIs have no estrogenic actions of their own and they highly effective in preventing the formation of estrogens. The extent of estrogen deprivation resulting from therapy with AIs has raised concerns regarding the loss of estrogen sparing actions during therapy with these agents.

5. TAMOXIFEN METABOLITES

The antiestrogenic activity associated with tamoxifen is attributed to the formation of 4-hydroxytamoxifen (4OHT). 4OHT is a primary antiestrogenic metabolite of tamoxifen (25-28). It is regarded as the biologically active form of tamoxifen and the better antitumor agent. The conversion of tamoxifen to its 4-hydroxylated metabolite readily occurs *in vivo* (27). There is another metabolite, however, that also demonstrates high affinity ER binding and potent antiestrogenic activity, similar to 4OHT, called 4-hydroxydesmethyltamoxifen (4OH-DMT). This metabolite has received considerably *less* attention during the 30 years of research on tamoxifen and 4OHT, and so much less is known regarding the extent of its activities (25-26). A schematic representation of the overall metabolic pathways leading to the formation of tamoxifen metabolites is shown in Figure 1. There are several other metabolites of tamoxifen as well, and many have estrogenic activities. Note that all

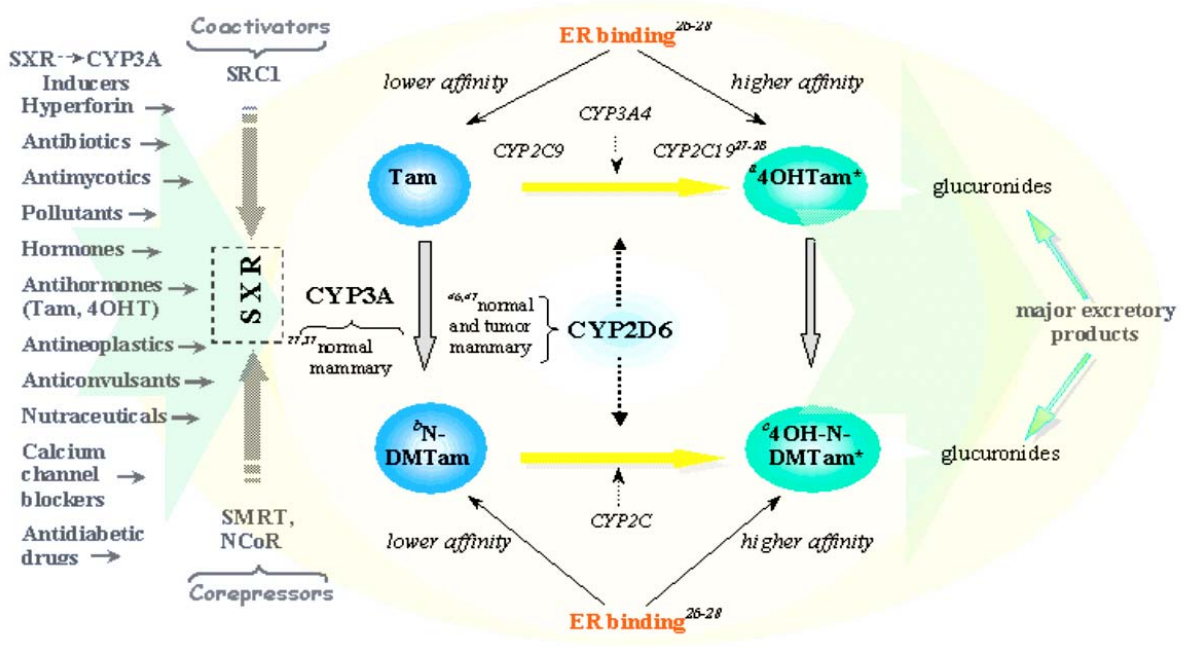

Figure 1. Major Metabolic Pathways for Tamoxifen. Shown are the main metabolic pathways used for the conversion of tamoxifen to N-desmethyl- and 4-hydroxylated metabolites. Cytochrome p450 (*CYPs*) enzymes shown in **bold** indicate the main enzymatic activities, the *CYPs* shown in italics indicate contributions that are variable; Tam, tamoxifen; ER, estrogen receptor; [a]4OHTam, 4-hydroxy-tamoxifen; [b]N-DMTam, N-desmethyltamoxifen; [c]4OH-N-DMTam, 4-hydroxy-N-desmethyltamoxifen; metabolites with asterisks* demonstrate higher affinity binding to the ER and greater estrogen antagonist activities than the parent compound tamoxifen (25-28).

Table 4. Metabolites of Tamoxifen

Major metabolites	**Minor metabolites**
N-desmethyl-tamoxifen	*Alpha* -hydroxy-tamoxifen
4-hydroxy-tamoxifen	*Alpha* -hydroxy-tamoxifen-*N*-oxide
4-hydroxy-desmethyl-tamoxifen	*Alpha* -hydroxy-*N*-desmethyl-tamoxifen
N-oxide tamoxifen	4-hydroxy-tamoxifen-*N*-oxide
	3,4-dihydroxy-tamoxifen
	3',4'-dihydroxy-tamoxifen
	tamoxifen-1,2-epoxide

of the metabolites along with the parent compound, contribute to the final biologic activity and clinical response to tamoxifen administration (a partial list is shown in Table 4).

5.1. The Significance and Biologic Activity of 4-Hydroxytamoxifen

The superior antitumor activity associated with 4OHT, relative to the parent compound, tamoxifen, is based on its stronger relative binding affinity for the ER and on the resulting potency of the 4OHT-ER complex as an antagonist. The following biologic characteristics have been described for 4OHT and metabolites of tamoxifen.

5.1.1. Relative Binding Affinity (RBA)

The binding affinity of 4OHT to the ER, relative to that of the natural ER-binding ligand, 17beta-estradiol (E2), is 10x times greater than tamoxifen and nearly equal to E2, although estimates vary according to the method of testing (26).

5.1.2. Antitumor Activity

4OHT exerts more potent antitumor activity then the parent compound, tamoxifen, both in the mouse and rat (27-28).

5.1.3. Cis-Trans Isomerization

4OHT undergoes spontaneous cis-trans isomerization, each isomer differs in estrogenicity (29-31). Cis-trans isomerization takes place in cell culture, media, stock solution, and *in vivo*. Recently, Malet *et al.* 2002 (31) confirmed that E2-induced growth could adequately be inhibited by a mixture of cis:trans isomers of 4OHT.

5.1.4. ER Antagonism

4OHT is a stronger antagonist of ER signaling than tamoxifen. It inhibits several estrogen-stimulated activities mediated by the ER, including cell growth, target gene expression, and cell cycle progression, which are reduced (experimentally) to or below the level of controls (25, 28, 32a, 32b).

5.1.5. Reactive Intermediates

4OHT may be further metabolized into a number of other molecular species including reactive intermediates. Reactive intermediates have a tendency to enter into covalent binding interactions that can cause damage to cellular proteins and DNA (33-35). The ratios of these metabolites can be influenced by a wide variety of drugs that use of the same metabolic pathways as tamoxifen.

5.2. Inter-individual Differences in the Uptake, Metabolism, and Activities

At the currently recommended dose of tamoxifen (20 mg daily), it takes approximately 4 weeks before steady state levels can be reached (26, 36). Most will be bound to plasma proteins with the free concentration ranging from 1 to 10% of the total. The terminal half-life of 4OHT also varies, ranging from 4 to 11 days. The variability in these values has been attributed to inter-individual differences. In some individuals, 4OHT and tamoxifen have been detected in various tissues months after treatment had been completed. These areas are considered 'deep pools' with 'half-lifes' that are unknown (28, refs. within). The metabolic pathways used by tamoxifen and the final metabolites produced, have also been shown to differ among individuals (28-29, 36-41). The presence of polymorphisms contributes to this variation. For example, polymorphisms have been identified for some of the cytochrome p450 enzymes involved in tamoxifen metabolism. As a result, affected individuals can be 'fast' or 'slow' metabolizers of agents or drugs utilizing the affected enzymatic pathway. In some cases these differences lead to changes in the concentrations and activities of tamoxifen metabolites with the potential to impact clinical efficacy (see Section 5.3).

The main metabolic pathway for tamoxifen inactivation is the N-demethylation pathway mediated by CYP3A4 (Figure 1, refs. 26, 28). CYP3A4 is also responsible for the metabolism of a vast number of drugs (antidiabetic agents, anticonvulsants, antineoplastics, antihormones, antibiotics, antimycotics, HIV protease inhibitors, calcium channel blockers), hormones (androgens, estrogens, glucocorticoids, pregnanes), xenobiotics (including endocrine disruptors, pesticides, pollutants), and even some dietary agents, vitamins or supplements (hyperforin in St. John's Wort, vitamin E, refs. 40-43). There is evidence that several of these agents, including tamoxifen, can activate the SXR (steroid and xenobiotic receptor, also called PXR or pregnane X receptor), which is a key transcriptional regulator of CYP3A gene expression (see below). CYP3A4 induction results in the metabolic conversion of various compounds into more water soluble forms for easy elimination or into reactive intermediates capable of inducing DNA damage (38-39, 41).

The simultaneous induction of CYP3A4 by compounds that are co-ingested can have profound effects on drug levels and interactions. Many drug-drug interactions involving tamoxifen have already been documented (28, 41, 44). Certain AIs, like 4-hydroxy-androstenedione (4-OH-A) can inhibit N-demethylation of tamoxifen without impairing the formation of 4OHT (45). Tamoxifen can also accelerate the elimination of other drugs when given concurrently. For example, the blood levels of both letrozole and anastrozole were shown to be lower when administered along with tamoxifen than during monotherapy with either each agent alone (16). Long term therapy with tamoxifen can also increase its own elimination (39) with the potential to reduce patient benefit and impact survival. Plant extracts, all forms of vitamin E, and a variety of enivironmental chemicals also have the potential to activate SXR and regulate CYP3A4 gene expression. Therefore, metabolic food-drug interactions have the potential to influence the potency and outcome of therapy with tamoxifen (38, 40, 42-43).

5.3. 4-Hydroxylation

There is evidence for the involvement of hepatic cytochrome p450 enzymes CYP2D6, CYP2C9 and CYP3A4 in the 4-hydroxylation reaction which converts tamoxifen into the antiestrogenic metabolite 4OHT (28, 46-49, see Figure 1). CYP2D6 is likely to be the primary catalyst of tamoxifen 4-hydroxylation (49). This enzyme has gained significant attention recently. CYP2D6 has been detected in normal and in breast tumor tissue, it is involved in the metabolism of many well known compounds, including chemotherapeutics and common pharmaceuticals, and several polymorphisms have been identified in the general population (28, 41). Over 70 variant alleles have been described on the CYP2D6 locus (41). The associated polymorphisms can cause the complete loss of enzymatic activity (i.e.: two null alleles), increase, decrease, or alter its substrate specificity. It is estimated that 10% of all Caucasians and less than 2% of Asians have polymorphic CYP2D6 forms. People with these polymorphisms are therefore known as 'poor metabolizers', the consequences of which are a slower than normal rate of conversion from tamoxifen to the more potent 4OHT metabolite form, suboptimal drug levels, and the potential for therapeutic failure. CYP2D6 gene amplifications also occur. These are associated with ultra-rapid metabolic reactions and potentially toxic responses or adverse drug reactions (28, 38, 41, 43, 46).

The impact of drug combinations on the contribution of CYP2D6 polymorphisms to the formation of 4OHT, the active metabolite of tamoxifen, has been examined in patients receiving both tamoxifen and antidepressants. A recent study was conducted on 12 women with breast cancer who were receiving tamoxifen and paroxetine (50). Paroxetine is an antidepressant used to control hot flashes that can be caused by therapy with tamoxifen. Paroxetine is of interest because it is metabolized through the same P450 CYP2D6 enzyme pathway as tamoxifen (Figure 1). The possibility that patients with CYP2D6 polymorphisms who were co-perscribed paroxetine and tamoxifen would have lower concentrations of 4OHT than those with the wild type CYP2D6 form was evaluated by correlating the levels and types of metabolites formed with the presence or absence of CYP2D6 polymorphisms (50). Suprisingly, coadministering paroxetine and tamoxifen was found to decrease the levels of 4OH-DMT (but not 4OHT). Furthermore, the decrease in 4OH-DMT observed occurred to a greater extent in patients with the wild type CYP2D6

gene then it did in those with polymorphic CYP2D6 forms (50).

5.4. Metabolic Activation

4OHT itself can also undergo further metabolism to form the catechol, 3, 4-dihydroxytamoxifen (3,4-di-OH-Tam). This reaction is catalyzed by CYP3A (33, 37, 39). The 3,4-di-OH-tam intermediate is a candidate for covalent binding interactions to DNA and proteins (33-35). The 3,4-di-OH-tam intermediate can either accumulate, with potentially damaging consequences, or it can be diminished by subsequent reactions. As 4OHT is the parent compound for this intermediate, the rate of 4OHT formation can influence the amount of catechol that will accumulate (35, 37-41).

The activity of the CYP3A4 enzyme can also influence the formation of intermediates. Several agents have the capacity to influence CYP3A enzyme activity without compromising 4OHT formation (via CYP2D6). It has been shown, for example that the steroidal aromatase inhibitor: 4-hydroxyandrostendione (4-OH-A, shown in Table 3) can interfere with CYP3A-mediated metabolism if co-administered with tamoxifen (45). As a result of this interference, 4-OH-A is able to inhibit the formation of N-desmethyl-tamoxifen without perturbing the biologically active metabolite, 4OHT (Table 4). Moreover, kinetic studies have demonstrated that the half-life of tamoxifen may be actually be increased by the concurrent administration of 4-OH-A. If confirmed, it would mean that lower doses of tamoxifen could be used to achieve the same therapeutic potential (45).

5.5. Therapeutic Potency of 4OHT

There are several factors that can influence the therapeutic potency of 4OHT. Drugs, xenobiotics, as well as hormones and other endogenous agents can influence the formation of 4OHT and other metabolites as well as the activities of pathways leading to them (28, 36, 38, 41, 51-52). The metabolic response to concurrent exposures (xenobiotics and medication) will ultimately be determined by the cell context and the preexisting molecular and genetic changes within it.

There are many ways that the therapeutic potency of tamoxifen can become altered (Figure 1). The expression of the CYP3A gene itself can be induced by natural compounds, endogenous substances, xenobiotics and many dietary agents. CYP3A is responsible for the metabolism of compounds such as steroids, procarcinogens, drugs and other agents that can trigger drug-drug interactions, and potentially influence the metabolism of tamoxifen. Many CYP3A inducers are also ligands for the SXR (or PXR) orphan nuclear receptor. This particular orphan receptor recognizes binding sites on the CYP3A gene (which are called xenobiotic response elements), thus allowing it to regulate CYP3A transcription (for review see ref. 38).

Takeshita and coworkers (51) have found that some common CYP3A inducers, like rifampicin or ketoconazole, are also ligands for the SXR, and can thereby influence CYP3A transcription. Furthermore, when these ligands bind to the SXR, the ligand-bound receptor will, in turn, form a complex on the CYP3A gene promoter and recruit needed coregulator proteins to it. The entire complex (the ligand-receptor-coregulator complex) becomes stabilized and functions to enhance or repress CYP3A gene expression. The levels of gene product can change several-fold in the presence of coregulators. The clinical implication from these studies is that the identity of the prevailing xenobiotic can have serious consequences for the clearance of tamoxifen and the formation of metabolites resulting from it (38-41, 50-52).

6. MOLECULAR BASIS FOR CHEMOPREVENTION WITH TAMOXIFEN

Tamoxifen can oppose estrogen-stimulated effects on proliferation and survival, DNA synthesis, target gene expression, coregulator binding, cell cycle progression, heat shock protein levels, cellular oxidant status, and the activities of growth factors, proteases, oncogenes, and tumor suppressors. In doing so, tamoxifen causes treatment-induced modifications in tumor growth and activity. Many of these modifications are accompanied by changes in molecular mediators or biological markers of tamoxifen's actions. In some cases changes in the levels of ER target gene products occur during the course of therapy with tamoxifen. It is known, for example, that tamoxifen down regulates ER alpha (53-54) and causes detectable changes in the expression of several ER-regulated genes. The progesterone receptor (PR), bcl-2, cyclin D1, pS2, c-fos, c-Jun, p21, c-myc, transforming growth factor beta (TGF beta), and heat shock proteins can be affected by tamoxifen (53-64). In other instances, the effects of tamoxifen on E2- ER stimulated activities are gauged by detecting changes in markers of biologic activity such as Ki-67 or MIB-1, whose staining intensity correlates with cell proliferation. Collectively, these biomarkers act as indicators of the tumor's growth state, apoptosis level, and/or metastatic potential. Molecular information gained from the analysis of tumor samples, taken at biopsy and again at surgery or during the course of treatment, can be correlated with histopathologic parameters and patient characteristics to estimate tamoxifen's efficacy or predict disease free survival.

6.1. Targets for the Preventive Effects of Tamoxifen

Tamoxifen and its metabolites exert multiple molecular effects on cancer cells that are consistent with cellular protection and cancer prevention. And many, but not all, of tamoxifen's actions are dependent upon the presence of functional ER. For some of tamoxifen's actions, there is no ER involvement, for others the involvement of the ER uncertain. Factors which contribute to tamoxifen's ER dependence and to its overall efficacy as a chemopreventive include (a) *ambient ligand concentrations* (tamoxifen and tamoxifen metabolites, pharmaceutical-, xeno-, or phytoestrogens), (b) *the presence of less readily detectable* forms of the ER, as well as ER subtypes and related proteins (membrane, cytosolic, nuclear, or mitochondrial ER; mutated, truncated or variant ER; ER alpha and/or ER beta; and the estrogen receptor related proteins, ERR alpha,

Table 5. Cellular Changes Associated with Tamoxifen

Alkalinization of acidic organelles[a] (84)
Protein kinase C (PKC) binding and non selective PKC inhibition (61)
Calmodulin binding and inhibition of cAMP-diesterase activation (81-83)
Alterations to plasma membrane fluidity (63-64)
Resensitization to certain chemotherapeutics (63-65)
Modulation of secreted protein activities (e.g.: cathepsin D, collagenase, urokinase plasminogen activator (82-84))
Changes in cell adhesion (68, 75-76)
Growth factor responsiveness (59, 62-63, 72)
Inhibition of membrane channels (79-81)
[a] *ER- and P-glycoprotein independent*

ERR beta, and ERR gamma), and (c) *elements of cellular context*.

6.2. Favorable Effects of 4OHT are both ER-Dependent and -Independent

In addition to the ER-driven actions responsible for the favorable effects of tamoxifen on blood lipid profiles and markers of bone mineral density, many of the molecular activities identified for tamoxifen can contribute to disease prevention. Some of these are listed. Tamoxifen and its metabolites can cause decreases in:

- Cell proliferative activity (53-54)
- Antiapoptotic cell survival programs (55-58)
- GLUT1 transporter and glucose efflux activities (59)
- ER alpha expression levels (53-54, 60)
- Protein kinase C (PKC) activity (61)
- Expression of vascular endothelial- and basic fibroblast growth factors (VEGF, bFGF), involved in the promotion of angiogenesis (62)
- Glucosylceramide synthase activity leading to intracellular ceramide accumulation (63-64)
- Tumor cell repopulation after chemotherapy (65)
- Heat shock protein 27 (hsp 27) expression (66)
- Plasma homocysteine levels in healthy women (67)

Tamoxifen and its metabolites can increase:

- Apoptosis induction (63, 68)
- Expression and recruitment of inhibitory regulators of ER action, i.e. corepressors, to target gene promoters (69-71)
- G0/G1 cell cycle fraction (72)
- TGF beta accumulation and activity (leading to apoptosis) (63)
- Tumor suppressor gene (maspin) expression and activity (73-74)
- Protein tyrosine phosphatase (PTPase) activity (leading to diminished cellular responsiveness to EGF stimulation, ref. 75)
- Arachadonic acid release and cyclooxygenase-2 (COX-2) production, leading to apoptosis (76)
- Antioxidant enzyme expression and damage protection (77-80)
- Intracellular calcium influx pathways (81-83)

Potentially favorable effects of 4OHT that may also be ER-independent include PKC inhibition, altered membrane fluidity, and cell adhesion changes (see Table 5.). Tamoxifen has also been shown to interfere with transport across membrane channels including those for calcium and chloride, bind to calmodulin, inhibit cAMP phosphodiesterase activity, promote apoptosis, increase agonist-driven calcium elevation, induce spatial expansion of calcium waves, and exert antioxidant and fluidizing effects on cell membranes (81, 83 and refs within). Zhang and colleagues have postulated that the antitumor activities associated with tamoxifen and 4OHT are based in part on the ability to deregulate and promote the spreading of local calcium signals (81-83).

There is evidence that tamoxifen is able to modulate the protease activities of cathepsin D, collagenase, and urokinase plasminogen activator proteins (84, refs within). Tamoxifen has also been shown to resensitize cancer cells to the effects of certain chemotherapeutics (84). 4OHT can reverse multidrug resistance, increase the sensitivity of drug resistant breast cancer cells to adiamycin, and cause alkalinization of acidic organelles without affecting cytoplasmic or nuclear pH (84). At sufficiently high, loading dose concentrations, some of the ER-dependent activities of tamoxifen can be replicated in cells that do not express receptor. The changes induced by tamoxifen play a complex role in prevention, which are in some cases able to increase chemotherpeutic agent sensitivity and avert cancer progression.

Changes in COX-2 and PTPase activities have been identified in tamoxifen-treated cells (75-76). Tamoxifen has been shown to increase arachadonic acidic release, elevate prostaglandin I_2 (PGI_2) production, and amplify COX-2 - activities proposed to have a protective function by promoting cancer cell apoptosis (76). Using ER positive breast cancer cells Freiss and coworkers have shown that 4OHT treatment causes PTPase activity to increase and cellular responsiveness to mitogenic growth factors, like EGF, to decrease (75). By increasing PTPase expression, 4OHT is proposed to function as a negative regulator of the growth factor pathway (75). Although the major mode of growth inhibition associated with tamoxifen treatment includes G1 arrest, cytostasis and cytoreduction, tamoxifen is capable of inducing apoptosis as well. Some of the mechanisms identified in association with tamoxifen-induced apoptosis include the activation of MAPK, c-myc, caspase-3, collapse of the mitochondrial transmembrane potential, and ER beta-mediated increases in inducible nitric oxide synthase (iNOS) and nitric oxide (NO) production (74) thus effectively eliminating cancer cells (63).

Recent *in vivo* experiments have demonstrated that tamoxifen, unlike E2, decreases the secretion of VEGF, a

potent mediator of tumor angiogenesis and neovascularization (62). *In vitro* and *in vivo* evidence has also identified a role for tamoxifen in the ER-dependent upregulation of maspin tumor suppressor gene expression in breast tumor and non-tumor breast tissue (73, 74). Furthermore, this activity is in direct opposition to that of E2, which was found to downregulate maspin expression. Taken together, these findings are consistent with the chemopreventive actions of tamoxifen and provide a molecular explanation for the increased OS and metastasis protection seen in breast cancer patients being treated with tamoxifen (62, 73-74). Tamoxifen also counteracts the effects of E2 on cell cycle progression, tumor cell survival, proliferation, and aggressiveness by modifying the activities of ER-regulated events affecting cyclin D1, bcl-2, IGF1(R), TGF alpha, EGFR, pp90rsk, MAPK, c-myc, hsp27 and other mediators (36, 40, 55-58, 60, 62, 66, 72) with the potential to influence tumor growth and endocrine sensitivity.

Tamoxifen also plays a role in preventing oxidative DNA damage caused by metabolites of E2. Tamoxifen is able to counteract the effects of potentially damaging metabolites by upregulating cellular enzymes involved in detoxification. These activities have been explored in experiments designed to test tamoxifen's effects on DNA damage induction by H_2O_2 in ER positive cells that had been treated with E2 (77). In these experiments Mobley (77) showed that E2 exposure increased the susceptibility of ER positive cells to oxidative DNA damage and that treatment with tamoxifen or 4OHT prevented it. These same investigators also demonstrated that E2 treatment caused catalase enzyme activity, peroxide metabolism, and cellular glutathione levels to decrease in ER positive breast cancer cell lines. The negative impact of E2 metabolites on the intracellular redox signaling and antioxidant enzyme activities was confirmed by studies in which increases in the formation of reactive oxygen species and sensitivity to oxidative DNA damage could be detected after treatment with E2. Importantly, these effects could be opposed by tamoxifen (78). These findings led to the reasoning that by down regulating antioxidant enzyme activities, E2 enhances oxidative DNA damage responses and contributes to the development and progression of breast cancer (78).

4OHT has recently been reported to up-regulate the expression of quinone reductase (QR), a phase II enzyme (79). This occurs by binding mainly to the beta form of the ER. 4OHT-ER complexes were shown to activate electrophilic or antioxidant response elements (EpRE or ARE) within the QR gene promoter, whereas E2-bound ER complexes were shown to cause *re*pression. The metabolic activities associated with phase II enzymes like QR are responsible for protecting cells against harmful reactive oxygen species (ROS). These findings support a role for tamoxifen (4OHT) as a protective agent (79).

Montano and colleagues have demonstrated that 4OHT is also involved in the regulation of several phase II enzymes and that this regulation is mediated by ER beta (80). In addition to QR gene expression, 4OHT was shown to stimulate the expression of glutathione-S-transferase Pi (GST-Pi), and gamma glutamylcysteine synthase heavy subunit (GCSh), phase II enzymes responsible for the detoxification of electrophilic compounds (that might otherwise inflict damage upon cellular DNA), free radical scavenging activities, protection against reactive oxygen species (ROS), and the maintainence of intracellular redox balance (79-80). Thus the ability of 4OHT to exert ER beta subtype-selective effects on the transactivation of phase II gene promoters is directly linked to its role in cancer prevention. The chemopreventive activities associated with tamoxifen and 4OHT are shown schematically in the context of other chemopreventives in Figure 2. The ability to control a battery of antioxidant enzymes involved in the prevention of cellular and DNA damage, as well as its role in tumor growth suppression and estrogen antagonism, suggests that 4OHT functions as a *bona fide* preventive agent (80). These findings lend support to the recent clinical trial results that led to the first time ever government approval of an anticancer drug – tamoxifen – for use as a breast cancer risk reducer and chemopreventive in healthy, higher than average risk women.

7. CELLULAR MEDIATORS OF TAMOXIFEN SENSITIVITY AND RESISTANCE

Two categories are used to classify the resistance to tamoxifen, these are: *de novo* and acquired resistance. Tamoxifen sensitive tumors can acquire resistance after a term of continued exposure that usually occurs over a number of years. In these tumors, the word *resistance* is used to indicate that the desired response, which is that of estrogen antagonism, can no longer be elicited. Based on the parameters of growth and gene expression, for example, tumors that were originally sensitive to tamoxifen demonstrate resistance by becoming *insensitive* or refractory to the effects of tamoxifen (i.e.: tamoxifen-independent tumor growth) or by becoming *growth-stimulated* in the presence of tamoxifen (which occurs in most cases of acquired resistance). There are two real concerns associated with the phenomenon of acquired resistance. The first concern is that it places limits on the duration of effective therapy, and the second is that the molecular changes probably take place well in advance of the clinical manifestations associated with resistance (i.e.: disease progression).

7.1. Predictors and Modifiers of Tamoxifen Resistance

The molecular basis for predicting sensitivity to tamoxifen therapy is founded largely upon the detection of ER alpha in tumor samples. And indeed, most tumors with high ER expression levels will respond to tamoxifen therapy. However, a portion of these will be unresponsive from the start. Among those that do respond to tamoxifen, the presence of ER alpha reveals little about the duration or nature of the response to treatment. Nevertheless, ER alpha (the ER form that is tested for) is one of the best-studied predictors of sensitivity to endocrine treatment and one of the few molecular markers that is in routine use today.

Understanding the molecular changes responsible for acquired resistance to tamoxifen and the subsequent

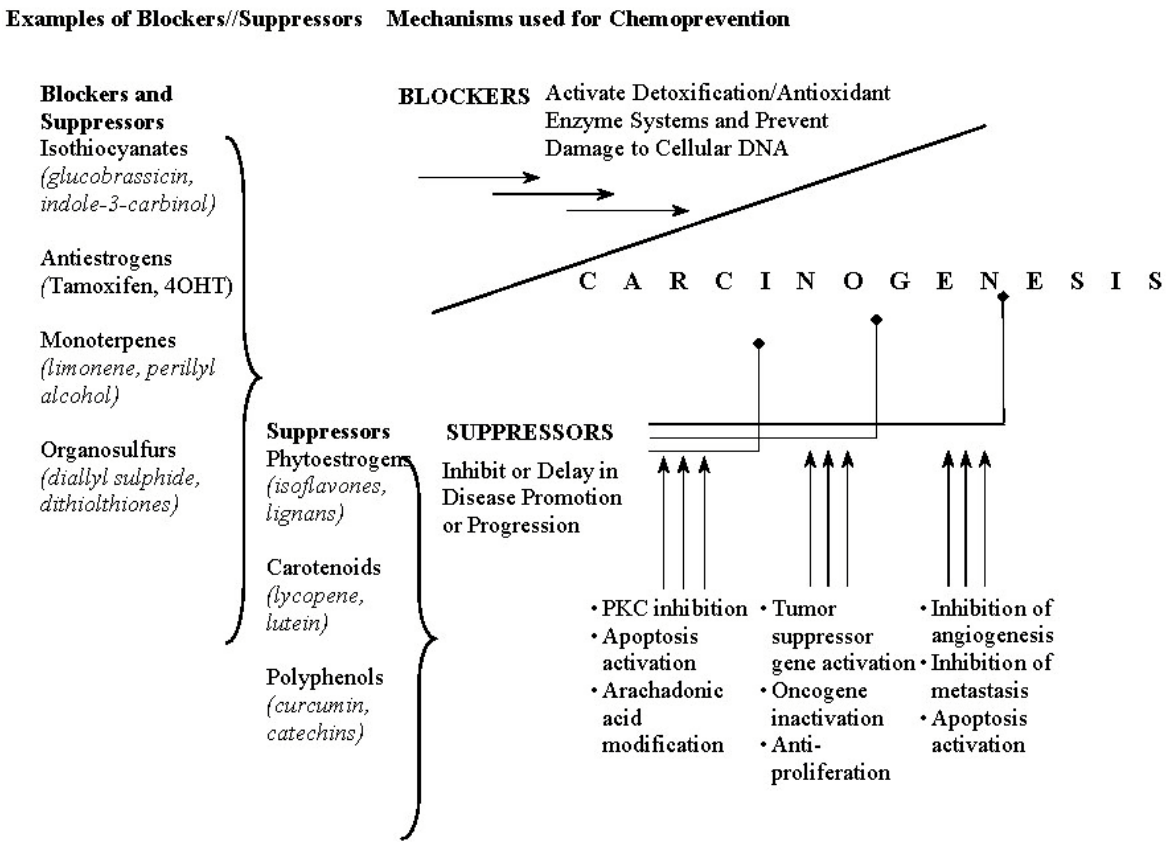

Figure 2. Simplified Schematic Illustrating the Role of Tamoxifen and 4OHT in Chemoprevention.

occurrence of disease progression, which often occur while treatment is still ongoing, has become an urgent goal in chemoprevention. *Factors with the potential to modify tamoxifen resistance include cell signaling mediators, like the ER and ER-regulated genes, coregulators, growth factors, cell cycle regulators, and tumor suppressors, (52-60, 68-72, 85-121) as well as exogenous agents including endocrine active, chemopreventive, and bioactive dietary compounds.*

Information derived from cell line models of resistance generated by prolonged exposure to tamoxifen or by genetic manipulation, from animal studies, and from the tumors of patients with breast cancer, indicate that tamoxifen resistance is pleiotropic in nature. Furthermore, the refractory or growth stimulatory tamoxifen responses observed in resistant tumors can also be accompanied by altered E2 responses (including E2 hypersensitivity and insensitivity) and changes in ER expression. In tamoxifen resistant cell lines ER expression changes can range from highly elevated levels to the loss of ER expression altogether. In the tumors of tamoxifen resistant breast cancer patients, ER expression is maintained and often increases above pretreatment levels (89). Notably, there is no single molecular alteration that can account for hormonal resistance, and several cellular phenotypes have been described.

Recent studies show that that the prognostic value of ER detection can be improved by the simultaneous identification of ER target proteins, like the progesterone receptor, PR (24, 86-88, 91, 96) or bcl-2 (55-56, 58, 60, 85-86, 95-96, 110). In an analysis of 214 breast cancer patients undergoing tamoxifen therapy, Castagnetta and coworkers (87) found that although ER was a good discriminator of tamoxifen sensitivity it was not sufficient to predict disease free survival (DFS) or OS. The predictive ability of ER positivity improved, however, if PR detection was also included. These studies identified PR negativity as an indicator of increased risk factor for early relapse and suggested that the inclusion of other biologic variables could further improve the utility of these predictions (87). Other investigators have examined the levels of ER and ER-regulated proteins, like PR, pS2, bcl-2, p21, cyclin D1, or hsp27, in relation to *molecular-biologic factors* (such as coregulator protein levels, ER subtype ratios, indices of proliferation, apoptosis, DNA ploidy, cell cycle distribution), *tumor histopathology* (tumor grade, lymph node involvement), and/or *patient characteristics* like patient age, menopausal status. In one study, antiestrogen sensitive, ER positive breast cancers were shown to express high levels of ER and various ER-regulated proteins (PR, pS2, bcl-2), at the time of diagnosis (60). Early in the treatment regimen (several weeks after tamoxifen therapy was initiated) a decline in the expression levels (initially in the level of ER followed by PR, pS2, and bcl-2) was shown to be associated with favorable treatment responses (53-54, 60, 95). Much later during the course of therapy with

Table 6. Anti-Estrogen Resistance: Manifested by Altered Growth Inhibitory Responses to Tamoxifen

Experimental Findings	Relationship to Tamoxifen Resistance	References
Coactivator overexpression or amplification	(see text)	71, 105, 106
Low corepressor (NCoR-1) expression	Associated with shorter relapse-free survival	69
ER positivity and HER2/neu overexpression are inversely correlated	Consistent with resistance to tamoxifen	106
AIB1 and HER2 expressing tumors from patients that had adjuvant tamoxifen therapy after surgery	High levels of AIB1 and HER2 in tamoxifen-treated Tumors were associated with poor DFS (indicative of tamoxifen resistance)	107
AIB1/ HER2/neu overexpression correlate inversely with ER / PR levels, and predict resistance to tamoxifen. AIB1 overexpression correlates with p53 (at high levels and inactivated) and HER2/neu overexpression.	Perturbance of cellular growth controls resulting from coactivator (AIB1) dysregulation (along with other oncogenic events) may lead to E2-independent growth and damage endocrine agent (tamoxifen) sensitivity.	108
Overexpression of AIB1delta 3, an exon 3-deleted splice variant of AIB1 found in MCF7 cells and in breast tumor tissues	AIB1delta 3 overexpression potentiates ER, PR, EGF, increases proliferative potential of ER ligands, contributes to hormone dependent tumor growth and antiestrogen resistance	109
Regulation of ER alpha levels may be essential for restriction of AF1 activity and for preservation of hormone-dependent receptor behavior	Elevated ER alpha levels can result in activation of receptor transactivation indepenent of phosphorylation and result in a proliferative advantage	89
Bcl2 was found to be upregulated by tamoxifen in breast cancer cells with mutant Ras	4OHT stimulated bcl2 upregulation may play a role in tamoxifen-induced resistance in certain cells	110
Constitutive cyclin D1 (over) expression	Cyclin D1 acts as a gatekeeper of proliferation, could contribute to the development of resistance	103
Cyclin D1 stimulation of ER alpha expression	Cyclin D1overexpression and elevated ER alpha levels correlate with antiestrogen resistance	92
Tamoxifen caused increased IGFI binding to IGF-R in tamoxifen resistant cells	Tamoxifen can sensitize cells to the proliferative effects of IGFI by raising IGFI receptor levels	94
Multiple mechanisms coexist with growth regulatory pathways that can free the cell cycle from steroid controls	Coexistence of multiple mechanisms override controls exerted by ER-dependent signaling to contribute to tamoxifen resistant phenotype	112
In 10 out of 11 resistant cell lines, CYP1A1 and CYP1B1 transcripts were elevated relative to the levels found in antiestrogen sensitive cells	Suggested that CYP1A1, CYP1B1 genes may be involved in ability of resistant cells to abolish growth-inhibitory effects of antiestrogens	113
Constitutively active Akt3	Tumor growth is inhibited by E2, stimulated by tamoxifen	98
Inappropriate receptor interacting protein (RIPs) expression in resistant tumor samples: lower SUG1	Resistant MCF7 cells were found to contain lower RIP140 levels	14

tamoxifen, ER,PR, bcl-2 and pS2 returned to or surpassed pretreatment levels in patients that had acquired resistance to tamoxifen (60). This was demonstrated by a loss in the growth inhibitory responses to therapy. Fowler and colleagues (89) found a positive correlation between chronically upregulated ER levels and increases in growth factor stimulation, promoter occupancy, and ligand-independent transcriptional activation, all of which have consequences for tumor progression and antiestrogen resistance. In addition to the loss of tamoxifen sensitivity, inappropriate increases in ER alpha levels may also be accompanied ligand independent growth stimulation (89, 91). Findings from these studies demonstrated that the limitations associated with ER detection as an independent prognosticator could be overcome by identifying additional ER- and tumor-related markers.

Although there is no unifying theory to explain the driving forces that underlie tamoxifen resistance, numerous candidates have been proposed as mediators of this phenomenon. A few examples and the mechanisms put forth to explain their roles in resistance, are described here or shown in Table 6. Of particular interest are recent studies showing that changes in the activities of coactivators (SRC1, SRC3/AIB1), corepressors (NCoR, SMRT), growth factors (IGFI-R, TGFbeta I, II-R, HER-2), cell cycle regulators (cyclin D1), and kinases (Akt1, PKC, MAPK), can impair antiestrogen sensitivity and hasten the development of resistance (69-71, 92, 97, 107-109, 114-115, 117-122). The potency of tamoxifen and its metabolites as ER antagonists can be diminished under conditions of coactivator overexpression or corepressor deficiency (69-71, 97, 107-109). For example, increases in coactivator levels have been shown to disrupt the corepressor-bound 4OHT-ER alpha complex and promote coactivator binding to the 4OHT-occupied ER instead (for review see 71). Corepressor levels that are too low for efficient ER binding can also lessen the effectiveness of repression (69, 115, 117). In either case, the loss of 4OHT-mediated antagonism, would be expected to result.

ER alpha corepressors have been investigated as

mediators of tamoxifen's inhibitory actions and potential sources of resistance. Girault and colleagues (69) assessed changes in the expression of 27 different coregulator genes taken from the ER alpha positive tumor specimens of 99 breast cancer patients receiving adjuvant tamoxifen therapy. Their findings revealed a strong positive correlation between low NCoR1 expression levels and poor outcome, predictive of tamoxifen resistance (69). The presence of mutations in either the corepressor or ER can affect the inhibitory function of the corepressor complex. Experiments identifying mutations in ER alpha that influence corepressor binding have provided insights into the mechanisms underlying formation of complexes and the consequences that changes may have for ER signaling (115-117). One such change involving an ER alpha point mutant, D351Y, identified in tamoxifen resistant tumor cells (116), has been shown to weaken corepressor interactions without impairing coactivator responses (115). The stability of the antiestrogen-bound receptor complex has been shown to be essential to corepressor signaling (118). In order to act as a repressor of ER signaling, the complex that forms when 4OHT binds to the ER must be conformationally stable, competent for cell-specific binding interactions, and capable of recruiting ciritical signaling mediators to the promoters of estrogen-regulated genes (69-71, 115, 117-118). Anything that interferes with this process could lessen or even eliminate the potency of 4OHT as an antagonist of ER signaling and leave the 4OHT-ER complex vulnerable to binding by coactivators in the ambient environment. Under these conditions, the 4OHT-bound ER could be converted from an antagonist into an agonist, capable of stimulating tumor growth and gene expression (115, 117).

AIB1 (amplified in breast cancer, also known as SRC3) is a coactivator that generally recognizes the E2- or agonist- (but not antagonist-) bound conformation of the ER. Once the coactivator complex forms, it stabilizes the DNA-bound E2-ER and becomes part of a platform onto which other coactivators and preinitiation factors assemble. When coactivators are amplified or overexpressed the potential to form aberrant complexes resulting from coactivator binding to the tamoxifen-occupied receptor, increases greatly. As a consequence of abnormal coactivator binding, ER-dependent transactivation and growth stimulation become *intensified* instead of repressed. There is sufficient evidence to support a model in which the levels and types of coregulators expressed within a particular cell line or tumor function to restrict conformations available to the receptor and to prevent the appropriate ligand-induced response from occurring. In the current example, AIB1 overexpression would overcome corepressor binding to the 4-OHT-bound ER. The novel complex that forms 'AIB1:4OHT:ER' with the AIB1 coactivator in place of a corepressor, like NCoR, disallows the inhibitory ER form, and instead drives the antagonist-(4OHT)-ER complex into a functional coactivator form, despite the presence of 4OHT. However, coregulator overexpression alone may not be sufficient to sustain the complexes that need to form and critical phophorylation events, which are capable of activating growth factor pathways, may be needed to reinforce coregulator binding interactions to the receptor. Fleming (118) tested the hypothesis that tamoxifen resistance may be attributable to shifts in the balance of tumor cell coregulators. By determining the protein levels for SRC1 (coactivator) and SMRT (corepressor) in the breast tumor specimens from a cohort of tamoxifen-treated patients, they were able to show a positive correlation between elevated SRC1 coactivator, nodal positivity, and disease recurrence, consistent with acquired tamoxifen resistance (118). *In vitro* studies uncovered differences in the pattern of coregulator recruitment induced by the 4OHT-bound ER alpha vs. ER beta DNA complexes that reinforced these findings (40, 118).

In an elegant study conducted by Lonard and colleagues, 4OHT was shown to increase the stability and concentration of SRC1 and SRC3 coactivator proteins, in a manner that was both cell-specific and ER-dependent (119). Coactivators and nuclear receptors undergo continuous proteasomal degradation followed by resynthesis (120), and this maintains transcriptional competence. These studies indicate that transcriptionally inactive 4OHT-ER can protect against coactivator degradation and increase steady state protein levels (119).

Many growth factors can induce estrogenic responses in the absence of E2 or amplify them in the presence of E2, and, to some extent, these responses are inhibitible by antiestrogens. The regulatory interactions that occur between growth factors and ER are complex and bi-directional (72, 92-94, 97, 105-107, 121-122). Insulin-like growth factor-I (IGF-I) and its receptor (IGFI-R) as well as other members of this family, can upregulate the expression of ER alpha and its target genes in the absence of E2 by Akt activation, cross-talk interactions, and ER phosphorylation (93-94). EGFR and HER-2 accomplish similar types of ER activation involving a number of mediators (40, 89-90, 90-92, 122). Both raloxifene and pure antiestrogens can activate IGFI through ER alpha, while 4OHT does not (72, 93). Akt activity can impair tumor cell responsiveness to tamoxifen. Constitutive overexpression of Akt can reverse the hormonal responses to E2 and tamoxifen where E2 exposure inhibits tumor growth and tamoxifen treatment stimulates proliferation (98). Tamoxifen sensitivity can be restored to Akt-overexpressing cells with the use of a dominant negative Akt mutant or by n-3 (eicosapentaenoic) fatty acid treatment (98, 123).

Growth regulators, like transforming growth factor beta, whose activation leads to growth inhibition, and their receptors (TGF beta RI, II), can be upregulated by 4OHT (105). These effects of tamoxifen can be lost in the context of inactivating TGF beta-R mutations, and may give rise to resistance. Note, tamoxifen and 4OHT can also induce cell death by activating apoptosis programs (63). This latter may occur in the presence or absence of ER and requires micromolar concentrations of tamoxifen to do so (68). Tamoxifen and 4OHT can also induce cell death via ER at nanomolar levels by causing necrosis or by activating mediators whose downstream targets enable apoptosis (63, 68).

Schiff and coinvestigators used a xenograft tumor model to identify changes associated with acquired tamoxifen resistance (102). The resistant phenotype they described remained estrogen dependent, ER alpha positive, and sensitive to inhibition by pure steroidal antiestrogens, yet it had become growth-stimulated by tamoxifen. Conversion to the tamoxifen resistant phenotype was associated with oxidative stress and subsequent activation of JNK, c-jun, and AP1 (102).

In antiestrogen sensitive cells, tamoxifen causes the G0/G1 cell cycle phase fraction to increase and reduces the number of cells the S and G2/M phases, consistent with its ability to promote cytostasis (36, 40, 72). By contrast, cyclin D1 induces G1/S phase progression and promotes proliferation. Furthermore, cyclin D1 can interact with the ER and bind to the same coactivators (i.e.: SRC1). Not surprisingly, cyclin D1 overexpression has been linked to tumor cell insensitivity to tamoxifen (103). Cyclin D1 amplification/overexpression is thought to be an early event in breast cancer development and has been shown to correlate with ER positivity in premalignant tumors, such as DCIS (104). Less often, however, cyclin D1 amplification can be seen in intermediate, poorly differentiated, or high-grade ER positive DCIS tumors (104, 122).

HER-2 or epidermal growth factor receptor-2 is overexpressed in up to 30% of all breast cancers. The effect of HER-2 on ER alpha levels and cell signaling activity is, however, somewhat controversial (92, 97, 105-106, 122). Some studies have shown an inverse correlation between HER-2 expression, ER positivity, and prognosis (92). In these studies, E2-stimulation was shown to repress HER-2 gene expression. This due, in part, to the conformation assumed by E2-activated ER, which can bind and sequester critical coactivators (like SRC1) needed for HER-2 transcription (92). Thus, by interacting with SRC1, the E2-ER was suggested to enforce repression of the HER-2 enhancer and downregulate HER-2 levels (92). Since tamoxifen is incapable of inducing the coactivator-binding conformation of the ER, it would leave SRC1 free to transactivate HER-2.

The simultaneous presence of ER positivity and HER-2 overexpression has been detected in tumors that are growth-stimulated by tamoxifen (90, 105, 122). It is known that the E2-ER can down-regulate HER-2 expression, while tamoxifen cannot. Since the cells of most HER-2-overexpressing tumors are ER negative, the coexistence of both ER alpha and HER-2 in the same tumor cell may reflect a state of transition, possibly permitting cross talk temporarily needed to sustain survival. In *in vitro* studies, using human breast cancer cell lines that were HER-2 gene-amplified, the overexpression of HER-2 was shown to confer antiestrogen resistance and to induce estrogen-independent transactivation responses (90). When the HER-2-amplified cells were transfected with a dominant-negative form of Akt1, the ability of tamoxifen to inhibit E2-stimulated activity was partially restored (90). A recent review of clinical studies and *in vitro* models regarding the role of HER-2 in ER positive breast cancers (106) suggests the potential for incomplete endocrine resistance and the involvement of downstream signaling and phosphorylation events in tamoxifen responsiveness.

In a recent analysis of 93 breast carcinomas and matched normal breast tissues (108), AIB1 overexpression was found to correlate with ER and PR negativity, high tumor grade, p53 stabilization, and HER-2 overexpression (Table 6). Based on these results, AIB1 overexpression was suggested to dysregulate signal integration mechanisms between growth factor and steroid receptor pathways and lead to a loss of growth control (108). In a study involving 316 breast cancer patients (107), an inverse correlation between tumor AIB1 levels and DFS was detected in tamoxifen-treated patients, but absent in patients that had not undergone treatment with tamoxifen. Elevated levels of AIB1 and HER-2 in the same tumors correlated with poor patient outcomes. Signaling through the HER-2 receptor-activated MAPK pathway, which can phosphorylate both AIB1 and ER, was suggested to reduce tamoxifen sensitivity and treatment benefit in these patients (107).

There is also substantial evidence linking alterations in growth factor and kinase activities to adaptive cellular changes (like estrogen hypersensitivity) during prolonged therapy with tamoxifen and the subsequent development of resistance (121). Additional mechanisms suggested to underlie adaptive hypersensitivity include increased aromatase levels, activating modifications to the ER, and sustained cross talk signaling (121).

These findings underscore the need for molecular information that would identify signaling mediators and elements of cellular context that could be targeted by tamoxifen-containing combinations in order to restore antiestrogen sensitivity, enhance therapeutic benefit, or extend the duration of effective treatment. Chemopreventives and bioactive agents investigated for use in combination with tamoxifen include isoflavones, lignans, vitamins and vitamin anologs (A, D, and E), gamma linoleic acid, n-3 fatty acids, polyphenols, and many others (40). Potential for interference, reductions in treatment efficacy, and the chronic safety of these combinations have not been determined.

Numerous mechanisms may account for the role of tamoxifen as an emerging *preventive*. Antiangiogenic, antiproliferative, and antioxidant activities (36, 40, 56, 58, 77-80) and a number of favorable effects that can avert disease progression and metastasis have been identified in tumor cells treated with tamoxifen (61, 64-66, 75-76, 81-84).. Tamoxifen has also been shown to activate tumor suppressor genes like maspin (73-74), regulators of apoptosis (56, 58, 63), inhibitory growth factors such as TGF beta (72), and genes involved in free radical and oxidative DNA damage protection (79-80), consistent with its role as a breast cancer preventive.

8. OUTLOOK AND PERSPECTIVES

Our understanding of the mechanisms responsible for breast cancer, and the effectiveness of

current treatments aimed at delaying or reversing it, has increased greatly in the past decade. We have identified major regulatory pathways involved in tumor growth and dysregulation, not only can we detect critical coregulator proteins that are capable of intensifying these pathways or silencing them altogether, but we can also up- or down-regulate them with the application of existing endocrine agents.

9. ACKNOWLEDGEMENTS

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Footnote: [1] Beatson GT On the treatment of inoperable cases of carcinoma of the mamma. Suggestions for a new method of treatment with illustrative cases: *Lancet* ii:104 (1896)

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