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Review

Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer

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ABSTRACT

This article describes the origins and evolution of "antiestrogenic" medicines for the treatment and prevention of breast cancer. Developing drugs that target the estrogen receptor (ER) either directly (tamoxifen) or indirectly (aromatase inhibitors) has improved the prognosis of breast cancer and significantly advanced healthcare. The development of the principles for treatment and the success of the concept, in practice, has become a model for molecular medicine and presaged the current testing of numerous targeted therapies for all forms of cancer. The translational research with tamoxifen to target the ER with the appropriate duration (5 years) of adjuvant therapy has contributed to the falling national death rates from breast cancer. Additionally, exploration of the endocrine pharmacology of tamoxifen and related nonsteroidal antiestrogen (e.g. keoxifene now known as raloxifene) resulted in the laboratory recognition of selective ER modulation and the translation of the concept to use raloxifene for the prevention of osteoporosis and breast cancer.

However, the extensive evaluation of tamoxifen treatment revealed small but significant side effects such as endometrial cancer, blood clots and the development of acquired resistance. The solution was to develop drugs that targeted the aromatase enzyme specifically to prevent the conversion of androstenedione to estrone and subsequently estradiol. The successful translational research with the suicide inhibitor 4-hydroxyandrostenedione (known as formestane) pioneered the development of a range of oral aromatase inhibitors that are either suicide inhibitors (exemestane) or competitive inhibitors (letrozole and anastrozole) of the aromatase enzyme. Treatment with aromatase inhibitors is proving effective and is associated with reduction in the incidence of endometrial cancer and blood clots when compared with tamoxifen and there is also limited cross resistance so treatment can be sequential. Current clinical trials are addressing the value of aromatase inhibitors as chemopreventive agents for postmenopausal women.

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Contents

The enthusiasm with which the clinical community has embraced the use of antiestrogenic therapy to treat breast cancer is based upon the proven record of success that first the nonsteroidal antiestrogen tamoxifen and then the aromatase inhibitor have demonstrated in clinical trial. The reasons for the enthusiasm are obvious. Antihormonal therapy, particularly aromatase inhibition to create a "no estrogen state" in the postmenopausal breast cancer patient is effective, saves women's lives, is contributing successfully to reducing the national mortality from breast cancer, is relatively cheap and has fewer side effects and easy administration (oral) than any other anticancer strategy. However, the successful application of a therapeutic strategy to block the known growth stimulation property of estrogen in breast cancer was not greeted with such enthusiasm 40 years ago.

Estrogen is essential for life. Without the critical role of estrogenic steroids, reproduction would not be possible. Based on emerging knowledge from laboratory studies, the value of modulating the steroid environment during the menstrual cycle was advanced to clinical testing during the 1950s as a means of oral contraception. The results of these studies were to change society forever.

The Worcester Foundation for Experimental Biology is the place where Gregory Pincus established the scientific principles necessary to propose clinical testing of the oral contraceptive and M.C. Chang subsequently established the first protocols to perform *in vitro* fertilization. Simply stated, the Worcester Foundation was, at that time, the world center for steroid endocrinology and reproductive biology. Over the years, hundreds of scientists have trained at the Foundation and subsequently spread their knowledge throughout the world [1]. However, fashions in research change and new opportunities emerge.

In 1971, President Nixon made a national commitment to seek a cure for cancer by signing the National Cancer Act. Mahlon Hoagland, the President of the Worcester Foundation, responded to the initiative by appointing Professor Elwood V. Jensen, Director of the Ben May Cancer Research Laboratory at the University of Chicago, to be a member of the Foundation's Scientific Advisory Board. Jensen had discovered the estrogen receptor (ER) as the putative mechanism of estrogen action in its target tissues [2]. The known link between estrogen and breast cancer suggested that "antiestrogenic strategies" might have potential as therapeutic agents [3]. Jensen applied knowledge of ER action to breast cancer treatment by devising the ER assay to identify breast cancers that would respond to endocrine ablation [4] but not all breast cancers responded. Hoagland's plan was to encourage the exploitation of the rich resources in endocrinology at the Foundation to be used for cancer research. The scene was set for independent investigators to work in cancer endocrinology but it is fair to say no one in academic medical oncology was interested in development of new antihormone therapies. Combination cytotoxic chemotherapy was king. Industry and clinical trial groups were respectively convinced that (1) developing anticancer drugs was a very risky business and (2) the right combination of cytotoxic agents applied at the right time would cure cancer. The principle was working in childhood leukemia, why not breast cancer?

The authors first met at the Worcester Foundation during the closing months of 1972. By coincidence, we were both English and grew up in the same county of Cheshire. One of us (VCJ) had conducted a PhD (1968–1972) on the structure activity relationships of a group of failed contraceptives, the nonsteroidal antiestrogens, the other (AMHB) had worked on hormones and breast cancer at the Christie Hospital in Manchester where the first preliminary study of ICI 46,474 was subsequently completed [5]. This was before ICI 46,474 was renamed tamoxifen (Fig. 1).

We have started this review with an account of our individual experiences that led to the development of tamoxifen and the aromatase inhibitors. Our perspective is followed by a

description of the therapeutic target, the estrogen signal transduction system and we close with current clinical advances in antihormonal therapy.

General Motors Prize Awards Ceremony—Washington, DC, 2005

The Charles F. Kettering Prize from the General Motors Cancer Research Foundation is awarded annually for the most outstanding recent contributions to the diagnosis or treatment of cancer. V. Craig Jordan (VCJ) and Angela H. Brodie (AMHB) are recognized on separate occasions for their pioneering studies that defined the scientific principles used clinically for the targeted treatment and prevention of breast cancer. Their body of work using the selective estrogen receptor modulators, tamoxifen and raloxifene (VCJ) and the first suicide inhibitor of the aromatase enzyme 4-hydroxyandrstendione (AMHB) has been individually recognized by several of the worlds leading prizes including the Brinker International Award from the Susan G. Komen Breast Cancer Foundation (VCJ 1993, AMHB 2000), the Dorothy P. Landon/American Association for Cancer Research Prize for Translational Research (VCJ 2002, AMHB 2006) and the Charles F. Kettering Prize from the General Motors Cancer Research Foundation (VCJ 2003, AMHB 2005) (see photograph). Jordan and Brodie have been members and attended the Endocrine Society since 1981 and 1962, respectively.

1. V. Craig Jordan: ICI 46,474 to tamoxifen

In 1967 Arthur Walpole and Mike Harper at the Imperial Chemical Industries (ICI) Pharmaceutical Division in Alderley Park, Cheshire reported the antiestrogenic and antifertility properties of a substituted triphenylethylene ICI 46,464 [6,7]. The Alderley Park team had been tasked during the 1960s to discover compounds to modulate fertility. Although Walpole also had an interest in anticancer chemotherapy, [8] as head of the fertility control program, he did not conduct any laboratory investigations of ICI 46,474 as an anticancer agent. He did, however, ensure that ICI Pharmaceuticals Division patented the compound with the statement, "The alkene derivatives of the invention are useful for the modification of the endocrine status in man and animals and they may be useful for the control of hormone-dependent tumours or for the management of the sexual cycle and aberrations thereof. They also have useful hypocholesterolaemic activity." Nevertheless, there was no patent for ICI 46,474 in the United States in 1972.

Harper had moved to the Worcester Foundation in the late 1960s and was investigating the potential of prostaglandins to be used as a once a month contraceptive. Although it was clear that prostaglandins were too toxic for systemic use, it is perhaps relevant to point out that a prostaglandin is currently used with mifipristone (RU486) as an abortifacient.

In 1972 I had completed my PhD on the "Structure Activity Relations of Substituted Triphenylethylenes and Triphenylethanes" but the University of Leeds was having difficulty securing an appropriate external examiner for my thesis. Nobody cared about the topic and it was only after considerable negotiation that Arthur Walpole (from industry!) was permitted to undertake the task. This experience started a collaboration that only ended with his untimely death in 1977.

In that same year, I took a 2 year leave of absence from the Department of Pharmacology at the University of Leeds originally to work with Mike Harper on the contraceptive properties of prostaglandins but when I arrived, he had left to work in the World Health Organization in Geneva and I was told—do anything you like, as long as some of it involves prostaglandins! My passion was the application of chemistry to medicine and I had always wanted to develop targeted anticancer drugs. Two events occurred in 1972–1973 that permitted me to pursue my passion. A meeting between Jensen and me at the Worcester Foundation in November 1972 would solve the problem of how to conduct a systematic laboratory examination of the antitumor actions of ICI 46,474. Jensen offered to teach me the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model [9] and techniques to measure estrogen receptors (ERs) in animal and human tumors. These techniques were essential to reinvent ICI 46,474 (tamoxifen) as an antitumor agent targeted to the ER. A phone call to Arthur Walpole in the United Kingdom secured funding to support the work and introduced me to Lois Trench, the newly appointed drug monitor for ICI 46,474 at the recently acquired Stuart Pharmaceuticals in Wilmington, Delaware. The company quickly evolved into ICI Americas and now 30 years later is known as AstraZeneca. Lois Trench provided human breast cancers for me to establish that tamoxifen blocked, the binding of estradiol to the ER [10], and I was also asked to introduce ICI 46,474 first to the Eastern Cooperative Oncology Group (ECOG), [11] and subsequently to the National Surgical and Bowel Project (NSABP) [12]. The NSABP particularly would propel ICI 46,474 from obscurity in the 1960/1970s to center stage

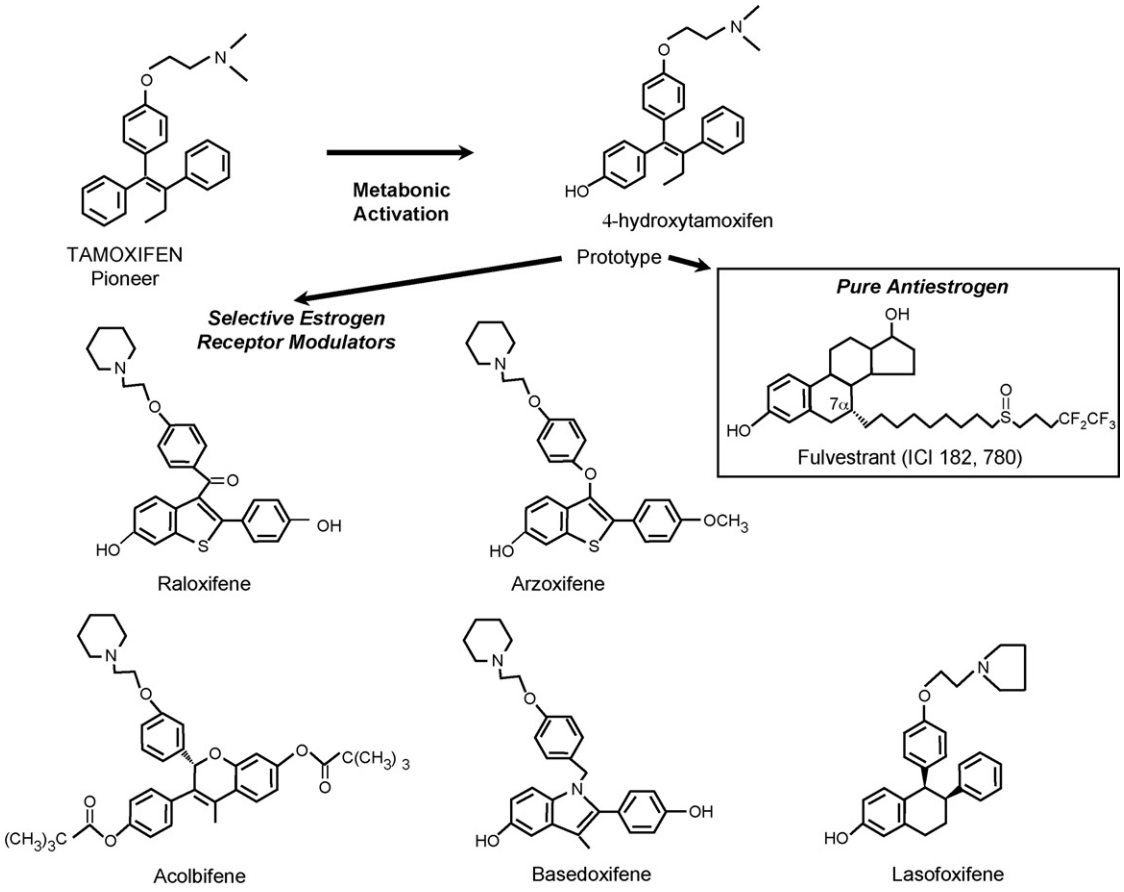

Fig. 1 – The activation of the pro drug tamoxifen to 4-hydroxytamoxifen, which has a high binding affinity for ER [103]. This knowledge resulted in the development of numerous new agents for use as selective estrogen ER modulations (SERMs) for the prevention of breast cancer and osteoporosis or the pure antiestrogen fulvestrant used as a treatment for ER positive advanced breast cancer following the failure of either tamoxifen treatment or an aromatase inhibitor.

in the treatment and prevention of breast cancer during the 1980/1990s.

1.1. *Translational research with tamoxifen*

A scientific strategy for the appropriate clinical application of tamoxifen was developed in the laboratory during the 1970s to target the drug to the tumors that were the most likely to respond [13,14]. Tamoxifen blocked the binding of estradiol to human breast and rat mammary tumor ERs and prevented the induction and growth of ER positive carcinogen-induced rat mammary carcinomas [10,15–17] These early studies raised the question of whether tamoxifen could prevent the majority of breast cancers, i.e.: ER positive breast cancer. However, the finding that long term tamoxifen treatment in animals with early mammary cancer, i.e., a low tumor burden [18–20] could create a tumor-free state suggested longer was going to be better than shorter durations of adjuvant therapy. The laboratory observations and pilot clinical studies [21,22] were to prove remarkably effective as an approach to treat women with early node positive and node negative ER positive breast cancer. However, the original clinical strategy in the 1970s for the evaluation of tamoxifen was to use 1 year of adjuvant treatment after surgery. The reason for this was that tamoxifen was only effective for the treatment of advanced breast cancer for about a year and there was a sincere concern that longer adjuvant treatment durations would result in premature drug resistance. This approach was to change.

An enormous advance in medicine is the introduction of meta-analysis or Overview analysis of small randomized clinical trials that individually show little or no benefits for agents under investigation but together provide a statistically secure result. The Overview analysis of breast cancer clinical trials was first conducted at Heathrow airport in 1984 [23]. The results when they were published in full in 1988 demonstrated a significant advantage for postmenopausal patients receiving tamoxifen [24]. Most importantly, a Consensus Conference held in Bethesda, MD recommended that tamoxifen should be used as an adjuvant therapy for postmenopausal ER positive, node positive patients with breast cancer [25]. The year 1985 was a good time for ICI Pharmaceuticals Division (now AstraZeneca) to be awarded a use patent for tamoxifen from the US Court of Appeals. The award of a patent for tamoxifen in 1985 started a 17 year exclusivity use patent in the US just at the time when the patent for tamoxifen had expired worldwide and just at the time that tamoxifen was poised to change healthcare. Thus, the accumulative 40-year patent for tamoxifen was to be the financial engine that facilitated

the development of a whole range of cancer therapies including aromatase inhibitors from AstraZeneca and subsequently other companies.

2. Angela M.H. Brodie—aromatase inhibitors: developing 4-hydroxyandrostenedione

I had received my PhD degree from Manchester University and was awarded an NIH Postdoctoral Training Fellowship, which brought me to the Worcester Foundation in 1962. The exciting atmosphere of cutting edge research enticed me to remain there after my fellowship. By the early 1970s, I had married a fellow scientist, Harry Brodie, and joined his lab working on the biochemistry of aromatase, the key enzyme in the biosynthesis of estrogens. Harry, an organic chemist, had begun developing inhibitors of aromatase as potential contraceptive agents and reported the first of these compounds in 1973 [26].

With my background in breast cancer at the Christie Hospital in the UK and the death of the previous lab director from the disease still on my mind, I was very interested in the possibility that aromatase inhibitors might be of value in the treatment of breast cancer. Clinical trials with ICI 46,474 (tamoxifen) had begun about this time. Although the antiestrogen was effective, it clearly did not yet have the impact on the disease that eventually brought it to the important position it has today. However, at that time, tamoxifen and other antiestrogens were known to be partial estrogen agonists as well as antagonists [6], raising concerns that they may not be optimally effective against breast cancer and may have adverse estrogenic effects. We reasoned that by using a different approach, compounds that blocked the production of estrogen without having significant estrogenic activity themselves might be identified. Results showed this to be the case. For the same reason, the possibility existed that aromatase inhibitors might also be more effective in treating breast cancer than antiestrogens. A number of laboratory studies were carried out which demonstrated the efficacy of the most potent aromatase inhibitor, 4-hydroxyandrostenedione (4-OHA) [27]. Some time later, we found that this compound acts not only by rapid competitive inhibition but also by inactivation of the enzyme. This effect is long lasting or irreversible (Fig. 2); see further below on steroidal inhibitors [28]. With some help from Craig, who had become proficient in developing mammary tumors with DMBA in rats, we showed that 4-OHA was effective in suppressing ovarian estrogen levels and causing regression of rodent mammary tumors [27]. In contrast to tamoxifen, 4-OHA was not estrogenic on other tissues such as the rat uterus. 4-OHA also inhibited peripheral (non-ovarian) estrogen synthesis in non-human primates in studies carried out in collaboration with Chris Longcope at the Worcester Foundation [29].

2.1. *Efforts to bring 4-OHA into the clinic*

Although these studies demonstrated that 4-OHA is highly effective, clinical studies were difficult to initiate, despite encouragement from the Decision Network Group at NCI who set aside funds to carry out toxicity studies. By 1978, Harry had left research, and I had moved to the University of Maryland in 1979. I was hopeful that the Cancer Center would be interested in bringing 4-OHA into the clinic. Tamoxifen had by then been shown to be effective and there was a lack of enthusiasm to investigate other approaches, not only at the University of Maryland but also in pharmaceutical companies. One approach, however, that did help the cause of aromatase inhibitors was the use of aminoglutethimide (AG) (Fig. 3) in treating breast cancer patients. Aminoglutethimide was a drug developed for treating epilepsy. However, it was found to cause adrenal suppression by inhibiting multiple cytochrome P-450 enzymes [30]. As adrenalectomy had been shown to be effective in treating breast cancer by Charles Huggins, Richard Santen and colleagues in the late 1970s began using aminoglutethimide as a medical approach to suppressing adrenal steroids in breast cancer patients [31–33]. Because aminoglutethimide inhibited a number of steroidogenic P-450 enzymes including CYP11, patients were given cortisol replacement. Santen was able to show that the main beneficial effect of aminoglutethimide then was inhibition of estrogen synthesis. However, aminoglutethimide had a number of significant side effects. Thus, it was my good fortune that some oncologists experienced with using aminoglutethimide were receptive to testing our selective aromatase inhibitor, 4-OHA. In the fall of 1981, I was invited to a conference in Rome to give a presentation about my research. Afterwards, an oncologist from London, Charles Coombes expressed interest in testing 4-OHA in breast cancer patients. Soon after my return to Maryland, a letter arrived from the Royal Marsden Hospital in London suggesting collaboration to bring 4-OHA, the first selective aromatase inhibitor into the clinic. In my laboratory at the University of Maryland we were able to produce a kilogram of 4-OHA by combining several batches of material. The toxicology was carried out through the Cancer Research Campaign in the UK. Paul Goss joined Charles Coombes as a PhD student and cared for the first patients that were treated with 4-OHA. Mitch Dowsett was also an important part of the team and measured estrogen and drug levels in the patients. Significant responses were seen in these first series of patients many of whom had relapsed from tamoxifen treatment. With these exciting results, Charles Coombes and I traveled to Horesham to Ciba-Geigy with the proposition that they take on 4-OHA

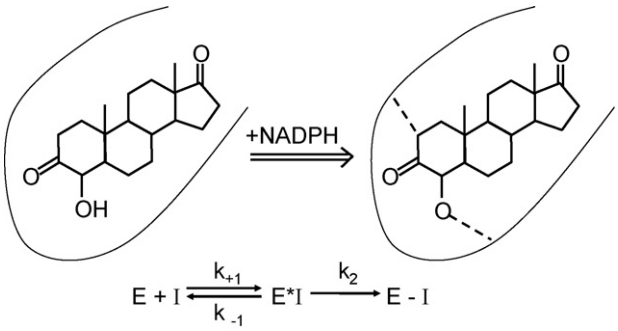

Fig. 2 – Aromatase inactivators: several steroidal inhibitors have been demonstrated to bind irreversibly or very tightly to the active site of aromatase e.g., 4-hydroxyandrostenedione (4-OHA).

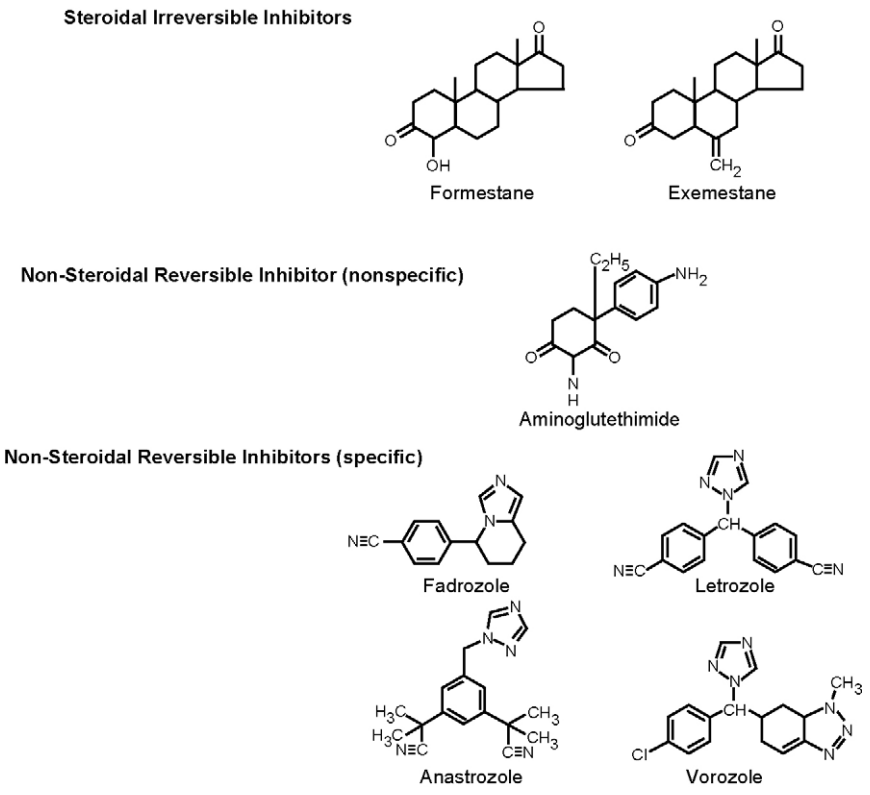

Fig. 3 – The structures of various aromatase inhibitors tested clinically for the treatment of breast cancer. The compounds are classified based on their mode of action and specificity for the aromatase enzyme.

and expand the clinical trials. Ciba-Geigy produced AG and it was quickly appreciated by the late Stuart Hughes that selective aromatase inhibitors such as 4-OHA would have distinct advantages. Clinical trials proceeded and Formestane (4-OHA) was the first selective aromatase inhibitor to become available and was the first new treatment of breast cancer in 10 years at that time.

As 4-OHA was of benefit in patients who had relapsed on tamoxifen, interest gradually grew in the possible benefits of using additional "hormonal" agents that are well tolerated. Before long, a number of pharmaceutical companies began producing aromatase inhibitors. Several of these were highly effective in inhibiting estrogen synthesis and some were more potent than 4-OHA. Although several US companies had produced excellent inhibitors, these did not come to clinical trials, largely due to internal company decisions. The field eventually thinned to three companies who had developed highly potent inhibitors.

One of these, exemestane, was a steroidal compound similar to formestane and developed by Farmitalia. The company had a history of making steroid and androgenic compounds mostly for anabolic activity. Exemestane (Fig. 3) has proved to be potent and effective in patients and is now approved by the FDA for breast cancer treatment. The two other FDA approved aromatase inhibitors came from pharmaceutical companies who investigated existing drug types for example, antifungal agents that inhibited cytochrome P-450 enzymes. The challenge was to modify such agents to be selective for aromatase. The result of these endeavors initially included vorazole (Fig. 3) also an inhibitor in this class. It was later discontinued despite good efficacy in breast cancer patients. However, the third generation agents letrozole and anastrozole (Fig. 3) were shown to be highly selective, yet reversible inhibitors of the aromatase enzyme.

2.2. *The estrogen ER signal transduction pathway as a model for molecular targeting*

Because of the importance of estrogen as a stimulus to the development and progression of breast cancer, estrogen synthesis (via aromatase) and action (via ER) continue to be exceptional targets for the treatment and chemoprevention of breast cancer [34]. Thirty years ago the idea of targeting and blocking estrogen action to treat breast cancer with tamoxifen or the idea of blocking the estrogen synthetase (aromatase) enzyme appeared to be simple and straightforward concepts. Today, these simple approaches have become multifaceted with many layers of complexity that are being explored to enhance tissue selectively, address intrinsic resistance and block the development of acquired antihormonal resistance.

2.3. *Mechanism of estrogen synthesis*

Aromatase mediates the conversion of the steroidal C-19 androgens to C-18 estrogens, which is the critical step in the biosynthesis of estrogens. This enzyme, therefore, has important functions in female development and reproduction. In the human, aromatase is expressed primarily by the

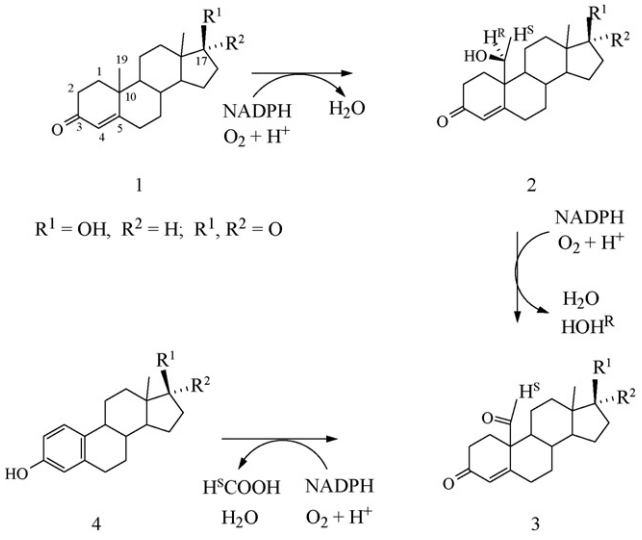

Fig. 4 – Aromatase mediates conversion of androgens to estrogens. Three hydroxylation steps are postulated.

ovary in premenopausal women [35]. However, central aromatization is necessary for the manifestations of many sex behavioral, neuroendocrine and developmental responses of several species [36,37]. In addition, aromatase is expressed in a number of other tissues throughout the body. The most important sites of non-gonadal estrogen synthesis are muscle and adipose tissue [38], where production increases with age in both sexes. Peripheral aromatization is the main source of estrogens in postmenopausal women [39] with significant production equivalent to premenopausal levels occurring in the breast [40].

Aromatization of androgens to estrogens occurs via a series of reactions (Fig. 4). An understanding of the mechanisms involved is important to the development of effective aromatase inhibitors. The aromatase complex consists of a cytochrome P450 hemoprotein and a flavoprotein, NADPH-cytochrome P450 reductase. The latter is common to most cell types and functions to donate electrons to the cytochrome P450. The P450 aromatase (P450 arom) binds the C-19 androgen substrates, androstenedione and testosterone and catalyzes their conversion to estrone and estradiol. This reaction is thought to involve three steps, each utilizing 1 M equiv. of NADPH and oxygen [41]. The first step is hydroxylation at the C-19 of the androgen substrate. This appears to be a characteristic cytochrome P450 hydroxylation [42,43]. Based on site-directed mutagenesis studies of the enzyme [44–46], it is suggested that hydrogen bonding of the 19-hydroxylation intermediate to an acidic side-chain residue Glu-302 is of critical importance in the aromatization process [46]. Hydrogen bonding of the 3-ketone may also occur at a polar active site (His-128 residue). This anchors the intermediate and assures stereospecific removal of the C-19 pro-R hydrogen by a heme iron-oxo species during the second hydroxylation step. Because of the high electrophilicity of the aldehyde, the usual ferric peroxide breakdown may be circumvented and the normal hydroxylation cycle altered. A number of theories have been postulated to explain the mechanisms involved in the last step. The C10–C19 bond is cleaved resulting in aromatization of the steroid A-ring and release of formic acid. Recently, Hackett et al. [46] showed that the 1β-hydrogen atom removal by an iron-oxo intermediate from the substrate in the presence of the 2,3-enol meets little resistance (5.3–7.8 kcal/mol), whereas in the keto tautomer, this same process encounters barriers of 17.0–27.1 kcal/mol. Although the residues involved in the enolization of C-3 toward C-2 have not yet been identified, they would be essential for the final catalytic step.

Aromatization is a unique reaction in steroid biosynthesis and may therefore be inhibited by selective compounds that do not interfere with other P450 enzymes. Since aromatization is the last step in the biosynthetic sequence of steroid production, blockade of aromatization should not affect production of other steroids. For these reasons, aromatase is a particularly suitable target for inhibition (Table 1).

2.4. *Steroidal aromatase inhibitors*

The first selective aromatase inhibitors were reported in 1973 and were a number of C-19 steroids [26]. These compounds were substrate analogs and exhibited properties typical of competitive inhibitors. They included 1,4,6-androstatriene-3,17-dione [47] 4-hydroxyandrostenedione (4-OHA) [48] and 4-acetoxyandrostenedione [49]. Interestingly, some of these inhibitors were later found to cause inactivation of the enzyme [28] and appear to be functioning as mechanism-based inhibitors. While not intrinsically reactive, inhibitors of this type are thought to compete rapidly with the natural substrate and subsequently interact with the active site of the enzyme (Fig. 2). They bind either very tightly or irreversibly to the enzyme, thus causing its inactivation [50]. Because they bind to the active site, these inhibitors should be quite specific and should also have lasting effects *in vivo* as a result of

Table 1 – Randomized Phase III trials of aromastase inhibitors vs. tamoxifen as first-line therapy in metastatic breast Cancer

Efficacy results, A/I tamoxifen	ORR (%)	Clinical benefit (%)	TTP (months)
Anastrozole, N = 1021 (pooling)	29/27	57/52	8.5/7.0
Letrozole, N = 907 (1 trial)	30/20[a]	49/38[a]	9.4/6.0[a]
Exemestane, N = 382 (randomized Phase II/III trial)	44/29[a]	72/66	10.9/6.7[a]

Data from: Bonneterre et al. [86], Mouridsen et al. [87], Paridaens et al. [89] and Pritchard [90].

[a] Statistically significant; ORR = overall response; TTP = time to progression.

inactivating the enzyme. Thus, the continued presence of the drug to maintain inhibition is not necessary and the chance of toxic side effects, therefore, will be low.

Dr. Chen and co-workers [51] have expressed a structurally stable and functionally active human aromatase in *E. coli*. Using this purified preparation, molecular features of the interaction of androstenedione (substrate) and exemestane (steroidal inhibitor) with aromatase have been studied by UV/vis spectral analysis. In addition, proteomic studies combined with MOLDJ-TOF MS revealed a 3-D overall folding of human aromatase, similar to that of the recently published 3-D theoretical computer model [52]. Proteomic results suggest that aromatase forms a symmetric dimer in solution through the interaction of the F helix and the F-G loop. The B and C helices and the B–C loop of aromatase appear to undergo major conformational changes when the enzyme binds to substrate or steroidal inhibitors. Reaction intermediate analysis suggested that residues E134, D309, T310, 5478, and 1-1480 are involved in enzyme catalysis. From inhibitory profile analysis and time-dependent inhibitory studies, residues E302, D309, and 5478 are thought to participate in the mechanism of the suicide inhibition of aromatase with exemestane.

A number of steroidal aromatase inhibitors in addition to 4-OHA and exemestane have been shown to cause inactivation. Brueggemeier et al [53,54] studied a number of 7α-substituted androstenedione derivatives, several of which cause inactivation of aromatase. The 1-methylandrosta-l,4-diene-3,17-dione (SH 489) [55] was shown to cause inactivation *in vitro*. Metcalf et al. [56] reported l0-(2-propynyl)estr-4-ene-3, 17-dione (MDL 18962) as the most potent aromatase inhibitor in their series. Two compounds with demonstrated biological activity are 6-methylen-androsta-1,4-diene-3,I7-dione (Exemestane, FCE 24304) and 4-aminoandrosta-l,4,6-triene-3,l7-dione (FCE 24928), [57] also cause inactivation of aromatase.

In rats, a single oral dose of 25 mg exemestane was found to cause a long-lasting reduction in plasma and urinary estrogen levels. Maximal suppression of circulating estrogens occurred 2–3 days after dosing and persisted for 4–5 days [58]. The lengthy duration of estrogen suppression is thought to be related to the irreversible nature of the drug-enzyme interaction rather than pharmacokinetic properties of the compound. Exemestane causes a marked decrease in serum and urine estrogen levels, and has no effect on other endocrine factors [59–61].

3. Non-steroidal aromatase inhibitors

Non-steroidal aromatase inhibitors contain a heteroatom (e.g., N, S, O) possessing a free electron pair for coordination with the heme iron (Fe^{3+}) and a substituent for interaction with other regions of the enzyme (Fig. 5). This type of binding is reflected in Soret band changes (usually bathochromic with respect to Type I inhibitors). Compounds that carry a nitrogen heteroatom have been the most studied and their binding with cytochrome P-450 enzymes give rise to a Type II difference spectrum with Soret maximum at 421–430 nM and minimum at 390–410 nM [62,63]. Intrinsically, nonsteroidal inhibitors are likely to be less enzyme specific than steroidal substrate analogs and can inhibit other cytochrome P450-mediated

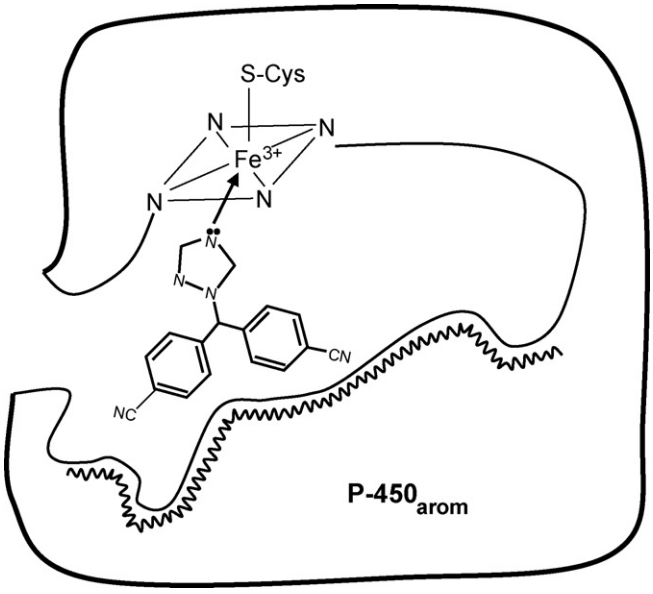

Fig. 5 – Interaction of non-steroidal inhibitors with aromatase.

hydroxylations as was the case for aminoglutethimide [30,64]. The newer non-steroidal inhibitors, anastrozole and letrozole, are triazole derivatives are potent, reversible inhibitors with high specificity. As they do not interact significantly with other P450 enzymes, they have few side effects in patients and have low toxicity.

3.1. *Selective modulation of aromatase*

The value of aromatase inhibitors as chemopreventives for postmenopausal women would be enhanced by tissue targeting. Remarkably, the regulation of aromatase appears to be different in different tissues. Several tissue-specific promoter regions have been identified upstream from the CYP19 gene [65–67]. Promoter PI.1 is the major promoter used in placental tissues and is the farthest upstream. Promoter II is utilized in the ovary. PII contains a cAMP response element and is predominant in breast cancer tissue as a result of a switch in promoters. Thus, aromatase can be stimulated by prostaglandin PGE_2 by increasing cAMP levels. Promoters PI.3, PI.4, PI.6, and PI.7 are the promoters used in other extraglandular sites. Promoter PI.3 is also present in adipose tissues such as normal breast tissue, and is increased in breast cancer tissue. Promoter PI.4 is the main promoter used in normal adipose tissue and responds to glucocorticoids and cytokines (e.g., IL-1β, IL-6 and TNFα). Because of this tissue specific regulation, there is interest in the possibility of identifying aromatase inhibitors that are selective for breast cancer by acting via promoter regulation [68]. Safi et al., [68] reports that orphan nuclear receptor liver receptor homolog-1 (LRH-1) is a specific transcriptional activator of aromatase in human breast preadipocytes and proposes LRH-1 as a target for selective inhibition of aromatase in the breast. Recently, Bruggemeier and colleagues found that a novel series of sulfonilide analogs could suppress aromatase activity and transcription indepen-

dent of Cox-2 [69]. This separation of activities may provide a basis for developing tissue specific aromatase inhibitors.

3.2. *The estrogen receptor signal transduction pathway*

A model of the current thinking about estrogen action is illustrated in Fig. 6. There are two ligand activated ER signal transduction molecules (ER alpha and ER beta) located at sites through the body. The assumption that is made is that specific receptors can modify the activation or suppression of genes in a particular target site thereby creating cellular homeostasis. The developmental and growth of breast cancer appears to be mediated through a balance of ER alpha and ER beta molecules. There is evidence to suggest that in an environment with an excess of ER beta this will create a growth inhibiting state and modifying the action of tamoxifen by enhancing antiestrogenic properties of the complex. In contrast, when ER alpha dominates the equation, as in breast cancer, estrogen can enhance breast cancer cell survival and tumor growth. Simply stated, the development of drugs to block estrogen regulated tumor growth (tamoxifen, raloxifene [SERMS]) or aromatase inhibitors (4-hydroxyandrostenedione) was predicted to prevent tumor growth, but the molecular biology of the transduction system has now provided a wealth of new information about drug resistance (see later section) and receptor modulation that is currently being applied to develop new medicines. It is clear from the investigation of the "nonsteroidal antiestrogens" tamoxifen and raloxifene that the receptor is modulated to enhance or suppress gene activation in different target tissues [70]. One proposed mechanism [71] is the balance of corepressor or coactivator proteins that can bind to the SERM ER protein complex to switch off (corepressor) or switch on (coactivator) the transcriptional potential of the complex. Alternatively in an estrogen-deprived environment, the unliganded ER would initially remain inactivated bound to a corepressor molecule.

The natural turnover of ER is accomplished by ubiquitinilation and destruction through the proteosome system. Fulvestrant, a pure antiestrogen is a derivative of the natural hormone estradiol with a long hydrophobic side chain substituted at the 7α position on the B ring of the steroid nucleus [72]. The steroid binds to the ligand binding domain of the ER in an inverted configuration with the hydrophobic side chain disrupting the ER shape by binding with the grove that can be occupied by Helix 12 of the ER [73] As a result of the unusual shape of the ER complex, it is rapidly ubiquitinilated and destroyed by the proteosome [74]. Pure antiestrogens cause a down regulation of ER so no genes can be activated. In contrast, a promiscuous SERM-like tamoxifen binds to ER

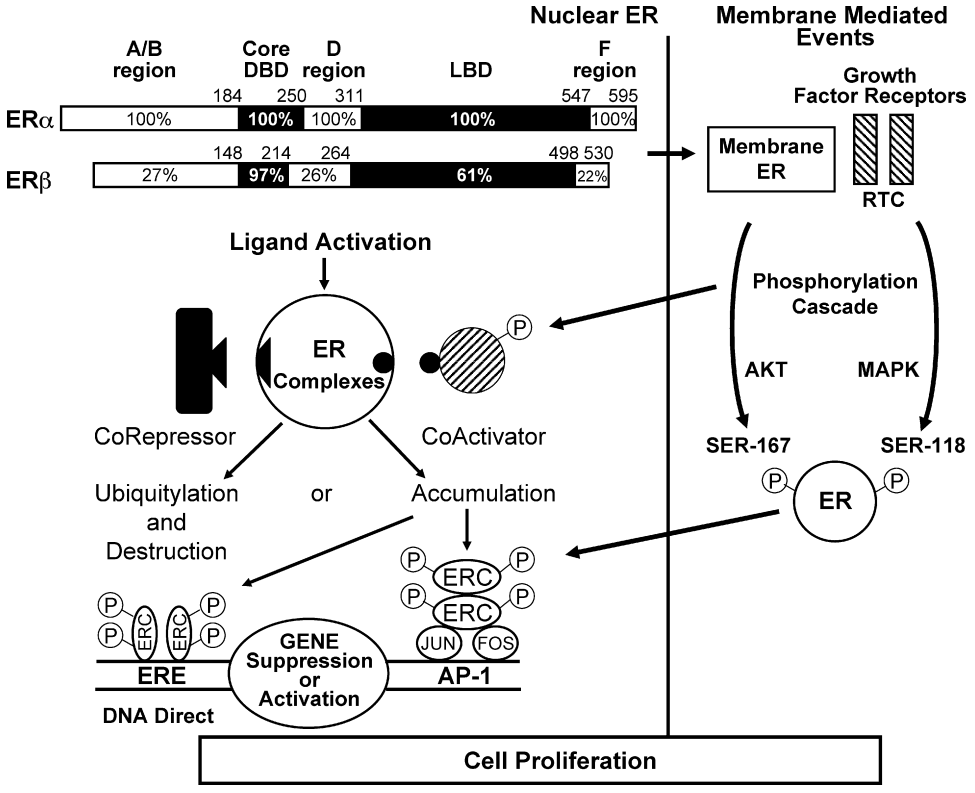

Fig. 6 – A diagramatics representation of the estrogen signal transduction pathway. The ligand estrogen receptor (either alpha or beta) (ER) complex is modulated in its actions by coactivators (CoA) or the unoccupied receptor is neutralized by corepressors (CoR). Overall the pathway can be further modified by phosphorylation of the ER or modulator molecules via growth factor receptor signaling pathways at the cell membrane (and membrane bound ER). These phosphorylation pathways are enhanced in antihormone resistance to allow the ER to promote unregulated gene activation through genomic and tethered mechanisms. This aids survival by preventing cancer cell apoptosis. Previously published in [14] and reprinted and adapted with permission.

and the complexes can accumulate thereby activating multiple genes via genomic and tethered mechanisms with the promoter region of target genes (Fig. 6).

The simple signal transduction pathway of estrogen action is also modified in cancer to cause either intrinsic or acquired resistance to antihormonal agents. The ER can be phosphorylated in cells with high levels of growth factor (HER2, EGFR, IGFR) signaling and redeploying from the nucleus to interact with protein kinases at the cell membrane. The ER then becomes part of the phosphorylation signal cascade that auto activates the ER and coactivators. It is essential to appreciate that a current understanding of the integration of the cross talk between ER and growth factor signal transduction pathways is important in antihormonal drug resistance and can be addressed logically in the treatment of breast cancer. This knowledge now provides a window of opportunity to apply new treatment approaches to control tumor growth. Unfortunately, the ER system eventually becomes redundant and the tumor is unresponsive to further antihormonal therapies. This survey of therapeutic target for the treatment of breast cancer can now be melded into a description of the advance in breast cancer treatment and prevention that, 30 years ago, would have seemed unlikely.

3.3. *Adjuvant therapy with tamoxifen*

Based on the successive analysis of accumulative randomized worldwide clinical trials, it is possible to summarize the main conclusions for tamoxifen therapy. At the time 20 years ago, when the Overview analysis first occurred, tamoxifen was the only universally used antihormonal agent. With no other competition, tamoxifen became the "gold standard" and established the principles of tumor targeting and identified the appropriate treatment strategy to aid survivorship [24,75–77].

- Five years of adjuvant tamoxifen enhances disease free survival. There is a 50% decrease in recurrences observed in ER positive patients 15 years after diagnosis.
- Five years of adjuvant tamoxifen enhances survival with a decrease in mortality 15 years after diagnosis.
- Adjuvant tamoxifen does not provide an increase in disease free or overall survival in ER negative breast cancer.
- Five years of adjuvant tamoxifen alone is effective in premenopausal women with ER.
- The benefits of tamoxifen in lives saved from breast cancer, far outweighs concerns about an increased incidence of endometrial cancer in postmenopausal women.
- Tamoxifen does not increase the incidence of second cancers other than endometrial cancer.
- No non-cancer related overall survival advantage is noted with tamoxifen when given as adjuvant therapy.

It is known that cytotoxic chemotherapy causes ovarian ablation by destroying the supply of follicles. However in young women the follicles are too numerous for complete destruction and menses continue after chemotherapy. Although tamoxifen is known to increase ovarian steroidogenesis in women with intact ovaries, [78,79] the antiestrogen is effective and approved for the treatment of premenopausal patients. That being the case, the question arises "should women without ovarian failure following chemotherapy receive antihormonal therapy?" The evidence suggests that this is a reasonable course of action and tamoxifen profoundly increases the control of recurrences in young premenopausal women following chemotherapy [80].

3.4. *The road to adjuvant treatment with aromatase inhibitors*

Two important observations stimulated further clinical development of aromatase inhibitors. The first was the meta-analysis discussed above showing the benefits of tamoxifen treatment [24] in ER+ breast cancer patients. The second was that it was later determined that after 5 years of adjuvant tamoxifen, no further benefit was observed [81]. This opened the door for the introduction of other treatment approaches. Interest was already turning towards aromatase inhibitors to fill the void.

The first clinical studies with formestane (4-OHA) were in patients who had relapsed from all available treatment. This included tamoxifen and second-line treatment with megace (medroxyprogesterone acetate) and aminoglutethimide. Although patients were often heavily pre-treated, response to 4-OHA was equal to or better than that of other agents. In addition, the compound was well tolerated and had fewer side effects than either Megace or aminoglutethimide. Eventually, 4-OHA was tested against tamoxifen as first-line treatment and was found to have equivalent efficacy [82]. The compound, 4-OHA was approved in most countries for treating patients with advanced breast cancer. Although 4-OHA was more effective in inhibiting peripheral aromatase and better tolerated than aminoglutethimide, subsequent aromatase inhibitors were able to block estrogen synthesis almost completely. Nevertheless, 4-OHA led the way for more potent aromatase inhibitors.

By the early 1990s, clinical trials had begun with aromatase inhibitors produced by several pharmaceutical companies. Novartis had developed oral non-steroidal aromatase inhibitors, fadrozole and letrozole, in addition to having 4-OHA. Letrozole proved especially potent. Oral doses as low as 0.1 and 0.25 mg in patients with metastatic breast cancer caused marked suppression of plasma estradiol, estrone and estrone sulfate levels and were observed within 24 h of the first administration [83]. These results suggest that this compound is a very powerful and selective aromatase inhibitor *in vivo*.

All three FDA approved compounds inhibited peripheral aromatization between 97 and 99%. Two randomized, double-blind studies demonstrated that anastrozole (1 mg daily) was slightly more effective than tamoxifen (20 mg daily) as first-line therapy in postmenopausal women with advanced breast cancer. Among those with ER+ tumors [84–86], the benefit was significant in terms of partial and complete responses including stable disease as well as time to progression. In a multicenter, randomized, double-blind study in advanced breast cancer, letrozole proved to be significantly better than tamoxifen in response rate, clinical benefit, time to progression, and time to treatment failure [87]. Exemestane was also significantly more effective than tamoxifen as first-line therapy in postmenopausal women with advanced breast cancer [88].

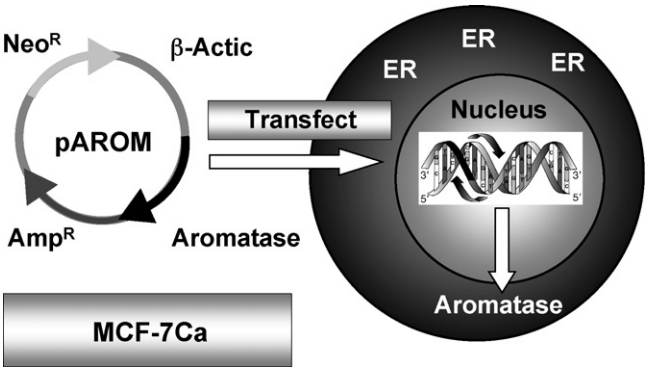

Fig. 7 – A human breast cancer cell line stably transfected with aromatase is sensitive to aromatase inhibitors and antiestrogens.

Once it was evident that aromatase inhibitors were more effective than tamoxifen, the focus of clinical trials soon moved to the use of the agents in the adjuvant setting for the treatment of early breast cancer [91,92]. The trials studied the effectiveness of aromatase inhibitors following tamoxifen, of aromatase inhibitors alone, and/or of the combination of aromatase inhibitors and tamoxifen in adjuvant therapy. These trials had their foundations in preclinical studies. We had developed a xenograft model that simulated the postmenopausal breast cancer patient. In this model, tumors are grown in ovariectomized, immunodeficient mice from MCF-7 human breast cancer cells stably transfected with the aromatase gene (MCF-7Ca) (Fig. 7). The possibility that blockade of estrogen action and estrogen synthesis may be synergistic was explored by treating mice with the aromatase inhibitor letrozole and the antiestrogen tamoxifen alone and in combination. However, the results in the model indicated that letrozole alone was better than tamoxifen or combined treatments [93]. This result was analogous to results later reported for the ATAC trial [94]. In addition, when tamoxifen treatment was no longer effective, tumor growth was significantly reduced in mice switched to letrozole treatment. Similar conclusions were reached in the MA-17 trial with letrozole [95] and the IES trial with exemestane following tamoxifen in early breast cancer [96]. Based on data from these and other multiple, large randomized trials, it was recommended by the American Society of Clinical Oncology (ASCO) technology assessment panel [97] that optimal adjuvant hormonal therapy for a postmenopausal woman with receptor-positive breast cancer include an aromatase inhibitor as initial therapy or after treatment with tamoxifen.

The aromatase inhibitors are all well tolerated. Patients experienced less gynecologic symptoms such as endometrial cancer, vaginal bleeding, and vaginal discharges. There were fewer cerebrovascular and venous thromboembolic events in patients receiving aromatase inhibitors than in those on tamoxifen. However, a low incidence of bone toxicity and musculoskeletal effects are associated with aromatase inhibitors. The latter includes small but significant increases in arthritis, arthralgia, and/or myalgia with aromatase inhibitors compared to tamoxifen. Fractures were increased with all three aromatase inhibitors compared to tamoxifen or placebo. The ATAC trial reported a fracture incidence of 7.1% in the anastrozole arm and 4.4% in the tamoxifen arm [94,98]. The fracture rate of letrozole-treated patients in the MA-17 trial was 3.6% versus 2.9% in placebo, following 5 years of tamoxifen treatment [95]. Similar small increases in osteoporosis and/or fractures (7.41%) were associated with the steroidal aromatase inhibitor, exemestane compared to tamoxifen (5.7%) in the IES trial [96].

It is suggested that patients are evaluated for baseline bone mineral density and receive bisphosphonate therapy if indicated [99]. No significant changes in serum cholesterol, HDL cholesterol, LDL Cholesterol, triglycerides or Lp(a) occur in non-hyperlipidemic postmenopausal women treated for 3 years following 5 years of adjuvant tamoxifen [100].

Other studies are on-going to compare the three aromatase inhibitors and/or combination therapies in early stage breast cancer or in the chemoprevention setting. A randomized, Phase III, double-blind trial (BIG 1-98) of the Breast International Group is comparing several adjuvant endocrine therapies in postmenopausal women with ER+ breast cancer. Letrozole versus tamoxifen treatment was compared in the first analysis of the monotherapy arms of the BIG1-98 study. After a median follow-up of 25.8 months, adjuvant treatment with letrozole was found to reduce the risk of recurrences significantly compared with tamoxifen [101]. The MA-27 study is a Phase III adjuvant trial in postmenopausal women with primary breast cancer comparing exemestane to anastrozole, with or without celecoxib, a COX-2 inhibitor. Overall, the aromatase inhibitors are proving to be superior agents to tamoxifen in the treatment of postmenopausal women with all stages of breast cancer. However, the pharmacology of tamoxifen and other non-steroidal antiestrogens was to provide new therapeutic opportunities for improving women's health.

3.5. *Selective estrogen receptor modulation and chemoprevention*

Twenty years ago, tamoxifen was classified as a nonsteroidal antiestrogen [102]. Of interest, however, was the observation that tamoxifen was metabolically activated to 4-hydroxytamoxifen, a nonsteroidal antiestrogen with a high binding affinity for the ER [103,104] (Fig. 1). This observation would provide a scientific foundation for future structure activity studies and subsequent drug development in this area. Now a whole range of new compounds with high affinity for the ER are available as therapeutic agents (Fig. 1).

In pharmacological terms tamoxifen was described as a partial agonist (estrogen-like) in target tissues such as the immature rat uterus but it was antiestrogenic because it blocked the full action of estradiol alone. In 1986, it was plausible that if estrogen was necessary to fend off osteoporosis and coronary heart disease the long-term administration of an antiestrogen to node negative women could eventually have a deleterious effect on bone density and produce a potential increase in the incidence of coronary heart disease for the majority of women. The potential side effects would be even worse for women only at high risk to develop breast cancer. Only a small minority of women would have a reduced risk of

breast cancer, but all women would be exposed to potential "antiestrogenic" toxicities. However, the classification of nonsteroidal antiestrogens was to change just after 1986. Today the concept is known as selective ER modulation (SERM).

In 1986, virtually nothing was known about the actions of nonsteroidal antiestrogens on bone density. A single report from NASA scientists showed that clomiphene, an impure isomeric mixture of a nonsteroidal estrogen and antiestrogen used for the induction of ovulation, would preserve bone density in ovariectomized rats [105]. Clearly there were efforts to prevent osteoporosis during space flight but the choice of experimental compound was flawed and frankly, a little bizarre. Since nonsteroidal antiestrogen such as tamoxifen reduce libido in men maybe that was the rationale!

However, the interpretation of the NASA results was not that simple. If clomiphene is an impure mixture of estrogenic and antiestrogenic isomers, which isomer is affecting bone? The consistent laboratory finding that tamoxifen the pure *trans* antiestrogenic isomer of a triphenylethylene maintained bone density in ovariectomized rats [106–108] seemed to translate to postmenopausal women [109], but would prospective clinical studies really show benefit? The Wisconsin Tamoxifen Study was started in 1986 to explore the potential toxicity of tamoxifen on bone density. The study demonstrated, in a double blind placebo controlled clinical trial, that tamoxifen could preserve bone in the postmenopausal woman [110]. Bone building would clearly be an advantage for chemoprevention studies, thereby enhancing the possibility that the worth of tamoxifen to prevent breast cancer could be tested safely. In the same studies, tamoxifen lowered low density lipoprotein [111,112] and, by inference, would appear not to increase the risk of coronary heart disease. These results were good. The bad was the laboratory discovery that although tamoxifen prevented the estrogen-stimulated growth of human breast cancers, the drug stimulated the growth of human endometrial cancers grown in the same athymic mouse [113]. This again was selective ER modulation. Stimulate one target site to produce growth and block the growth of another target site.

There was a very quick response from the clinical community to the warnings [113] that long-term tamoxifen treatment could be associated with an increase in the incidence of endometrial cancer [114–116] but not all reported studies [117,118] found increases in endometrial cancer associated with tamoxifen treatment. These studies were either too small or data was just not collected. There was also a question of whether the high dose of tamoxifen (40 mg. daily) used by Fornander and coworkers [116] was responsible for their findings but the report by Fisher [119] neutralized the argument because NSABP studies all use 20 mg. tamoxifen daily. Endometrial cancer again became an issue during recruitment to the pioneering tamoxifen chemoprevention study by Fisher and the NSABP when it was suggested that extremely dangerous endometrial cancer could be caused by tamoxifen treatment [120]. Nevertheless, results from the prospective chemoprevention study with tamoxifen actually showed that only postmenopausal women developed an excess of early stage mainly grade one endometrial cancers. There were no fatalities from endometrial cancer associated with tamoxifen in the study. [121,122].

The recognition that the so called "nonsteroidal antiestrogens" had estrogenic and antiestrogenic actions at different sites in the ovariectomized female rat and that these data translated to women to prevent bone loss and breast cancer created a new dimension in drug development. The fact that tamoxifen and the failed breast cancer drug keoxifene (LY156,758) [123] both prevented the development of carcinogen-induced rat mammary carcinomas [124] and maintained bone density in ovariectomized rates [106] indicated that this was a class effect. The significance of these observations for public health and chemoprevention of breast cancer was immediately recognized. At the first International Chemoprevention Meeting hosted by Dr. Ezra Greenspan, a group of scientists and clinicians were invited to New York in 1987 to share their vision of the possibilities and potential of chemoprevention [125]. The future of drug development was clear.

"The majority of breast cancer occurs unexpectedly and from unknown origin. Great efforts are being focused upon the identification of a population of high-risk women to test "chemopreventive" agents. But are resources being used less than optimally? An alternative would be to seize upon the developing clues provided by an extensive clinical investigation of available antiestrogens. Could analogs be developed to treat osteoporosis or even retard the development of atherosclerosis? If this proved to be true then a majority of women in general could be treated for these conditions as soon as menopause occurred. Should the agent also retain anti-breast tumor actions then it might be expected to act as a chemosuppressive. A bold commitment to drug discovery and clinical pharmacology will potentially place us in a key position to prevent the development of breast cancer by the end of this century" [125]. This blueprint to improve healthcare was subsequently restated at the annual meeting of the American Association of Cancer Research in San Francisco, 1989 [126].

Compounds of the keoxifene class (LY117018 and LY156758) were obvious candidates for study despite the fact that the program to develop the drugs to treat breast cancer had been abandoned by Eli Lilly in 1988. The compounds were known to be less uterotrophic than tamoxifen in rodents [127] but they were short acting [128], which could explain their poor antitumor properties when compared with tamoxifen. Interestingly enough, keoxifene was already known to partially inhibit the growth of tamoxifen-stimulated human endometrial tumors under laboratory conditions [129].

Keoxifene, an estrogen that had failed to be developed as a drug to treat breast cancer [123] was reinvented in the early 1990s as raloxifene, a SERM. A use patent for the treatment and prevention of osteoporosis was filed by Eli Lilly in 1992. Raloxifene has now been available for the treatment and prevention of osteoporosis in postmenopausal women since 1999 based on the prospective clinical trials demonstrating an approximately 40% decrease in spinal fractures [130] with the advantage over hormone replacement therapy of causing a 70% decrease in the incidence of breast cancer [131,132]. The anticipated result in reducing the risk of breast cancer as a beneficial side effect of treating osteoporosis propelled raloxifene into clinical trial versus tamoxifen for the prevention of breast cancer as the primary endpoint. The results

from the study of tamoxifen and raloxifene (STAR)[1] in high risk postmenopausal women show that tamoxifen and raloxifene are equivalent in reducing the incidence of breast cancer but there is a decrease in the incidence of endometrial cancer, pulmonary emboli, deep vein thrombosis and endometrial hyperplasia noted with raloxifene. Raloxifene and tamoxifen, as would be expected for two SERMs, both have equivalent activity in preventing fractures [133].

Raloxifene also causes decreases in circulating low density lipoprotein cholesterol [134] and for this reason was evaluated as a preventive for coronary heart disease in the study named raloxifene use for the heart (RUTH) (see footnote 1). Although raloxifene prevents an increase in breast cancer incidence in the RUTH trial, there is no benefit in protecting against coronary heart disease and myocardial infarction [135]. Clearly, further studies with different agents will be needed to rethink the SERM strategy for a multifunctional drug that can prevent cancer (breast/uterus), osteoporosis and coronary heart disease.

Nevertheless, results with raloxifene are part fulfillment of the predicted promise [125,126] of the SERMs as medicines to prevent cancer and osteoporosis. As a result, there are now numerous new SERMs (e.g. lasofoxifene, basedoxifene, arzoxifene, etc.) being evaluated [136,137] (Fig. 1). Additionally, the concept is being applied throughout the steroid receptor super family so the impact on medicine, in the years to come with selective androgens, glucocorticoids or progestins will be considerable.

4. Aromatase inhibitors as chemopreventive agents

Aromatase inhibitors have potential for chemoprevention in women with increased risk of developing breast cancer for many of the same reasons as tamoxifen. Thus, reducing the number of proliferative events by inhibiting the stimulatory effects of estrogen will reduce the number of mutations that would otherwise occur. Evidence to support the value of aromatase inhibitors in the prevention setting comes from the adjuvant clinical trials that compare and contrast tamoxifen with an aromatase inhibitor. All studies show that an aromatase inhibitor is more effective than tamoxifen at preventing the development of contralateral breast cancer [95,101,138]. Based on the recent findings from the STAR trial [133], there is active interest in comparing an aromatase inhibitor with raloxifene. The National Surgical Adjuvant Breast and Bowel Project will compare raloxifene with letrozole in their next clinical trial in postmenopausal women at high risk for breast cancer. The success of such chemoprevention trials will depend on not only reduction in tumor incidence but also the long-term tolerability of the agents. The International Breast Cancer Intervention Group is currently comparing anastrozole versus placebo in a prevention study, and the accompanying DCIS study is comparing tamoxifen versus anastrozole in women with locally excised ductal carcinoma *in situ* (DCIS) [139]. A three-arm prevention study organized by the National Cancer Institute of Canada, will compare placebo versus exemestane versus exemestane and celecoxib [139]. Although increased risk of stroke and endometrial hyperplasia have not been associated with aromatase inhibitors, other potential side effects such as increased osteoporosis will also be important criteria in considering aromatase inhibitors for preventing breast cancer. Bisphosphonates are currently used by many postmenopausal women to prevent osteoporosis and could be used with aromatase inhibitors if bone loss is a concern.

The possibility that aromatase inhibitors can prevent breast cancer by an additional mechanism has been proposed by several investigators [140]. This hypothesis suggests that estrogens are metabolized to catechol estrogens, 2-hydroxy-estradiol, and 4-hydroxy-estradiol. Evidence exists that 4-hydroxy-estradiol but not 2-hydroxy-estradiol is potentially carcinogenic by forming depurinating estrogen-DNA adducts. Therefore, preventing the formation of estrogens and of their subsequent metabolism to catechol estrogen 3,4-quinones, may provide better protection from breast cancer than targeting the estrogen receptor. Recent studies by Russo et al., [141] demonstrated that exposure of the MCF-10F ER negative cell line to four 24 h alternate periods of 70 nM estradiol induced anchorage-independent growth, loss of ductulogenesis in collagen, invasiveness in Matrigel, and loss of 9p11-13. Only invasive cells that exhibited a 4p15.3-16 deletion were tumorigenic in nude mice. Tumors were poorly differentiated ER-α and PR-negative adenocarcinomas and expressed keratins, EMA, and E-cadherin. The complete transformation of the ER negative MCF-10F cells *in vitro* that resulted in tumor formation *in vivo* supports the concept that estrogen may act as an initiator of breast cancer in women.

4.1. *Drug resistance to antihormones*

Overall, the long-term, or perhaps indefinite use of antihormonal therapy will result in the development of antihormone resistance. To investigate the mechanisms of resistance to aromatase inhibitors, one approach is to use the xenograft model with tumors of human breast cancer MCF-7Ca cells stably transfected with aromatase [142,143] (Fig. 7). Mice were treated with letrozole until tumors eventually began to grow. The expression of signaling proteins was determined in tumors during the course of letrozole treatment compared to tumors of control mice. Tumors initially upregulated the ER while responding to treatment, but subsequently receptor levels decreased in tumors unresponsive to letrozole. Nevertheless, p-ER (Ser167) was increased suggesting that ligand independent activation of ER may enhance proliferation in tumors treated with letrozole. Tyrosine kinase receptor protein HER-2 and adapter proteins (p-Shc and Grb-2) as well as the signaling proteins in the MAPK cascade (p-Raf, p-Mekl/2, and p-MAPK), but not in the Pl3/Akt pathway, were all increased in tumors no longer responsive to letrozole, suggesting the possibility that ER may be phosphorylated by MAPKinase in the absence of ligand. To investigate whether sensitivity to

[1] The STAR trial compared and contrasted the efficacy of tamoxifen and raloxifene to reduce the incidence of breast cancer in postmenopausal women at high risk for breast cancer. Side effect profiles were a secondary endpoint. The RUTH trial is a placebo-controlled study to determine whether raloxifene would reduce the risk of coronary heart disease in high risk women.

letrozole could be regained, cells were isolated from the letrozole resistant tumors (LTLT) and treated with inhibitors of the MAPKinase pathway (P098059 and U0126). These compounds reduced MAPK activity and increased ER expression. EGFR/HER-2 inhibitors, gefitinib and AEE78S although not effective in the parental MCF-7Ca cells, also restored the sensitivity of LTLT cells to letrozole. Since there appears to be cross-talk between the ER and the tyrosine kinase receptor, the hypothesis was tested that degrading the ER with fulvestrant may prevent development of resistance. In xenografts, beginning treatment with letrozole *plus* fulvestrant to down regulate the ER prevented increases in HER-2, activation of MAPK, and was highly effective in inhibiting tumor growth throughout 29 weeks of treatment. These results suggest that disrupting the ER or blocking growth factor-mediated transcription may delay development of resistance to aromatase inhibitors and maintain growth inhibition of ER+ breast cancer by hormonal agents.

Numerous studies have been published on the estrogen deprivation of ER positive breast cancer cells *in vitro*. Two types of cellular response can occur based on the organization of the ER system [144]. In MCF-7 cells, ER is upregulated during conditions of estrogen deprivation whereas T47D cells undergo ER down regulation. As a result MCF-7 cells eventually grow spontaneously in the absence of estrogen [144–146], remain unresponsive to estrogen, but retain ER. In contrast T47D cells loose ER and become hormone independent [147].

There have been extensive laboratory investigations of the development of drug resistance to SERM (tamoxifen and raloxifene) treatment [148]. These studies have established some general principles that not only have applications in the treatment of breast cancer but also provide some unanticipated insights into the potential failure of second or third line treatment with the pure antiestrogen fulvestrant. Additionally, long-term antihormone action has also exposed a vulnerability of the breast cancer cells. Although estrogen is considered to be a survival signal for breast cancer cells, physiologic estrogen can cause rapid apoptosis of estrogen deprived breast cancer cells [149,150], tamoxifen resistant breast cancer cells [151] and raloxifene resistant breast cancer cells [152]. Most interestingly, combinations of physiologic estrogen and fulvestrant can cause a reversal of the apoptotic action of estrogen and robust tumor growth. [153]. Clearly, these observations can have potential implications for the treatment of advanced breast cancer once multiple antihormonal therapies have failed. There are current proposals to use physiological estrogen to reduce tumor burden in sensitive tumors that have responded and failed two consecutive antihormonal therapies. The clinical basis for estrogen responsiveness has already been demonstrated in this patient group [154] but the use of a 12 week course of low dose estrogen followed by the reintroduction of aromatase inhibition plus fulvestrant would not only incorporate the current laboratory results for the design of a clinical trial but also improve patient disease control prior to chemotherapy [155].

4.2. *Perspective*

We have described our personal experiences over the past 35 years with the investigation and development of antihormones for the treatment and prevention of breast cancer. We have been fortunate to have both been at the right places at the right times to be able to contribute to the advance in therapeutics. However, it should be clear from our narrative that we have also been unfashionable and proposed solutions to treatment at the wrong times. Nevertheless fashions in cancer research often take a decade to change. In our case, the philosophy that cytotoxic combination chemotherapy would cure breast cancer was tested by the clinical community and failed. Unfortunately, the clinical trials community can only test what is provided by the pharmaceutical industry and the idea of killing cancer cells seemed "a good idea at the time". Fortunately, when alternatives to chemotherapy were sought and changes in fashion were occurring, new agents and treatment strategies were in place to fill a potential treatment void. We independently target the same signal transduction pathway but at different points to provide an alternative approach to cytotoxic chemotherapy. Today, the concept of drug targeting has come of age as every new agent being developed by the pharmaceutical industry is designed to target the tumor specifically thereby potentially reducing toxicity for the patient. It remains for a future generation to integrate the new menu of targeted medicines into the treatment plan and to ensure that the appropriate affordable drug is made available to the appropriate woman.

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