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Steroidogenic Enzyme Inhibitors and Hormone Dependent Cancer

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Summary

Objectives—To improve treatment for patients with breast and prostate cancer.

Methods—A number of novel inhibitors of steroidogenic enzymes have been developed. Their biological effects have been evaluated in a variety of preclinical models. Aromatase (estrogen synthetase) inhibitors have now been extensively tested in clinical trials in breast cancer patients. Inhibitors of 17α-hydroxylase/lyase have also been studied in preclinical models and are beginning trials in prostate cancer patients.

Results—The enzyme aromatase (CYP19) has proved to be an important therapeutic target. Inhibitors of aromatase (AIs) as are showing greater benefit than antiestrogens in the treatment of breast cancer. Although effective in other conditions in both women and men, AIs have not been useful in benign prostatic hypertrophy or prostate cancer. However inhibitors of 17αhydroxylase/lyase (CYP17) to block synthesis of androgens may be effective for prostate cancer. Recent clinical trials with abiraterone and preclinical studies with other novel CYP17 inhibitors which also interact with the androgen receptor and cause its down regulation could provide a new approach for treating this disease.

In further studies we optimized treatment with aromatase inhibitors and antiestrogens utilizing an intratumoral aromatase xenograft model. AIs were more effective and sustained growth inhibition longer than antiestrogens. However, inevitably tumors eventually began to grow despite continued treatment. Analysis of breast tumors from mice treated with letrozole revealed upregulation of HER-2 and MAPKinase signaling proteins and downregulation of the estrogen receptor. Our studies showed that tumors adapt to AI treatment by activating alternate signaling pathways, thus enable them to proliferate in absence of estrogen. When mice bearing resistant tumors were treated with trastuzumab the anti-HER-2 antibody (Herceptin), HER-2 was decreased in the tumor but the estrogen receptor and aromatase were restored. Tumor growth was significantly inhibited by treatment with trastuzumab in addition to letrozole.

Conclusions—Aromatase inhibitors are proving to be an effective new class of agents for the treatment and breast cancer. Compounds inhibiting 17αhydroxylase/lyase have potential for the treatment of prostate cancer. Our results suggest that strategies to overcome resistance to these types of agents can restore sensitivity of the tumors to hormone therapy.

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Keywords

breast cancer; xenograft models; letrozole; anastrozole; Her2; trastuzumab

Introduction

Aromatase (P-450$_{arom}$) has a key role in development and reproduction. Androgens (namely, androstenedione and testosterone) are converted to estrogens (estrone and estradiol) in a rate limiting reaction catalyzed by aromatase. In humans, the enzyme is expressed in the granulosa cells of the ovarian follicle, the syncytiotrophoblasts of the placenta during pregnancy [1,2] and in the Leydig cells of the testes. Estrogens produced by aromatase have a number of important physiology and pathologic functions in men and women. One of the most important is the stimulatory role of estrogens breast cancer in women.

While the ovaries are the principal source of circulating estrogens in premenopausal women, the majority of patients with BC are postmenopausal women. About 75% of these patients have estrogen dependent tumors [3]. After menopause, the ovaries cease to produce estrogens but these steroids continue to be synthesized in non-ovarian tissue [4] including the breast tissue [5], albeit in much smaller amounts. Important sites of non-ovarian estrogen synthesis are adipose tissue and muscle [6], where production increases with age and is the primary source of circulating estrogen in postmenopausal women [7]. However, breast tissue has been found to have several-fold higher levels of estrogen than those in serum and are equivalent to levels in premenopausal women [8,9]. A number of reports indicate that aromatase activity as well as aromatase mRNA is present in normal breast tissue and breast tumors [10-16]. Approximately 60% of breast tumors express aromatase [16,17] and have aromatase activity [18]. Although gonadal aromatase is regulated by follicle-stimulating hormone, aromatase in extragonadal sites is regulated by other factors including glucocorticoids, cyclic adenosine monophosphate, and prostaglandin E2 [19]. For example, cycooxygnease-2 expression has been correlated with aromatase expression, suggesting that local production of prostaglandin E2 may stimulate aromatase [17,20]. Thus, in postmenopausal breast cancer patients, estrogen synthesis is independent of feedback regulation between the pituitary and the ovary and involves tissue-specific regulation of P-450$_{arom}$ using alternative promoters [19].

Peripherally synthesized estrogens act locally in an intracrine or paracrine manner without being released into the circulation and act by binding to specific estrogen receptor α (ERα) in the tumor cells thereby mediating transcription of genes regulating proliferation.

Aromatase Inhibitors for Breast Cancer Treatment

Tamoxifen blocks the action of estrogens by binding to the ER. For the past 30 years tamoxifen has proved to be effective first-line or adjuvant endocrine treatment for advanced breast cancer. However, there were concerns initially that it might not be completely effective in inhibiting the tumor as it was a partial agonist or weak estrogen. In addition, its estrogenic actions in some tissues such as the uterus and vasculature could result in increased risk of endometrial cancer [21] and stroke [22]. Moreover, tamoxifen's efficacy proved to be limited to 5 years of treatment and it was considered to be without further benefit beyond that time [22]. Therefore to overcome these problems a different strategy was initiated. Compounds selectively targeting aromatase and lacking estrogenic activity were envisaged as being more effective than the antiestrogens. For the same reason, they were likely to cause fewer side-effects in patients. As one of the first successful targets for

therapeutic inhibition, aromatase has several important attributes. Aromatization of androgens to estrogens is the last in the series of reactions in steroid biosynthesis and is rate-limiting for estrogen synthesis. Therefore, there are no steroids produced downstream to be affected by inhibition of aromatase. Also, although aromatase shares common features with other P-450 enzymes, the unique characteristics of the aromatization reaction, involving loss of the C-19 carbon and conversion of the steroidal A ring to an aromatic ring, provide the opportunity to develop inhibitors selective for $P450_{arom}$. A number of selective aromatase inhibitors of were identified [23] in the early 1970. Among these, 4-OHA (formestane) was developed [24] and became the first selective AI for the treatment of BC [25,26]. Today, three aromatase inhibitors have been approved by the FDA exemestane (Aromasin), a steroidal inhibitor similar to formestane and two non-steroidal inhibitors anastrozole (Arimidex) and letrozole (Femara). All have high potency and specificity for aromatase. The latter two inhibitors were based on inhibition of P-450 enzymes and were derived from drugs used as antifungal agents such as ketoconazole, an inhibitor of fungal P-450 enzymes. Triazole and imidazole compounds possess a heteroatom, such as nitrogen-containing heterocyclic moiety. This interferes with steroidal hydroxylation by binding with the heme iron of cytochrome P-450. These nonsteroidal compounds are reversible inhibitor of aromatase whereas the steroidal inhibitors cause irreversible inactivation of the enzyme. Aromatase inhibitors are proving to be superior to tamoxifen in the advanced setting [27] and now are replacing tamoxifen as first-line therapy.

Aromatase and Steroid Synthesis Inhibitors in Men

In men, aromatase inhibitors may be useful for conditions associated with estrogen excess, such as gynecomastia and oligospermia. It had also been suggested that aromatase inhibitors might be of value in prostatic cancer and benign prostatic hypertrophy (BPH) [28].

While androgens are of primary importance in the growth of normal prostate, benign prostatic hypertrophy (BPH) and prostatic cancer, several lines of evidence suggested that estrogens may also have a role. In BPH, estrogen receptors have been identified and higher than normal concentrations of estradiol have been detected in stroma of the prostate [29]. Based on these findings, we investigated whether aromatase is present in prostatic tissue and whether aromatase inhibitors might have a role in BPH and prostatic cancer treatment. Our results indicated that aromatase is absent or, at the most, very low in the human prostate [30], although detection of aromatase in prostate stroma has recently reported [31]. However, estrogen synthesized in other tissues, such as adipose tissue as well as the testis could influence the prostate. In studies in the cynomolgus monkey [32], 4-OHA and another aromatase inhibitor, 1-methylandrosta-1,4-diene-3,17-dione (atamestane; Schering AG) have been shown to reverse estrogen-induced hyperplastic changes in the prostate. However, atamestane was without effect in a clinical trial of patients with BPH. We, and others, had found that 4-OHA inhibits 5α-reductase *in vitro* although with less potency than it inhibits aromatase [30]. Because of these two activities, the possibility that 4-OHA might be effective in prostatic cancer was explored in a small group of men with advanced disease. Subjective responses were observed in 80% of these patients, although there was no clear evidence of objective remissions [33]. Estrogen levels were reduced as expected but dihydrotestosterone concentrations were unchanged in the patients. This latter finding in addition to the weak androgenic activity of the compound may have determined the lack of objective responses. The other AI studies yielded negative results [34, 35].

We therefore turned to a different approach. We have designed and synthesized some novel inhibitors of androgen biosynthesis with the aim of providing more effective treatment for patients with prostatic cancer. The majority of patients initially respond to hormone ablative therapy although they eventually relapse, as is typical with all cancer treatments.

Nevertheless, more strategic use of androgen ablative approaches could also improve outcome. In an ECOG trial, where patients receiving radical prostatectomies were given immediate or no ablation therapy, survival after 7 years was 17% with treatment versus 30% without [36]. Current treatment by orchidectomy or GnRH agonists result in reduced androgen production by the testis but does not interfere with androgen synthesis by the adrenals. In fact, increased adrenal DHEA and DHEAS (androgen precursors) have been observed in patients treated with GnRH implants [37]. Following 3 months of treatment with a GnRH agonist, testosterone and DHT concentrations in the prostate were found to remain at 25% and 10% respectively, of pretreatment levels [38]. Similarly, about 20% of castrated patients in relapse had significant levels of DHT in their prostatic tissue [39]. These findings suggest that the adrenals may contribute precursor androgens to the prostate. This is supported by clinical studies of patients receiving combined treatment with either GnRH or orchidectomy and an antiandrogens, such as flutamide, to block the actions of androgens via the androgen receptor, including adrenal androgens which are unaffected by GnRH treatment and castration alone. Such patients have increased progression-free survival time compared to patients treated with GnRH agonist or orchidectomy alone [40,41]. While androgen ablation is an effective treatment, patients eventually relapse and their tumors progress despite continued treatment. It has been reported that AR are not lost in “hormone insensitive” prostate cancer. In patients with recurring tumors treated with endocrine therapy, high level androgen receptor (AR) amplification was found in about 30% of cases [42,43]. This suggests that AR amplification may facilitate tumor cell growth in low androgen concentrations. Mutations in the androgen receptor have also been found in a number of human prostatic cancers [44-46].

Further support for the role of AR and androgens in PC is the recent report of increased expression of genes of androgen converting enzymes and persistence of androgen regulated genes in androgen-independent PC [47-49]. These observations suggest that therapies that inhibit production of androgens and target multiple points in the AR signaling cascade could offer a more effective approach for prolonging remission of PC. Thus, total androgen blockade using more potent agents as first line therapy may be more effective than conventional androgen deprivation by achieving maximum suppression of androgen (and estrogen) concentrations and inhibition of androgen action [50]. Furthermore, new agents which act by different mechanisms could produce second responses in a portion of relapsed patients. Although the percentage of patients who respond to second-line hormonal therapy may be relatively low, a substantial number of patients may benefit because of the high incidence of prostatic cancer. Recent studies in breast cancer patients treated with the aromatase inhibitor letrozole (Femara®) raise the possibility that in estrogen-responsive neoplasms, approaches that remove estrogen's presence are a successful subsequent maneuver in patients with progression of disease while receiving estrogen-receptor antagonist treatment [51]. Currently no analogous highly efficient means of accomplishing the same end with expected favorable side effect profile exists to influence the androgenic axis in patients with prostate cancer. The novel androgen synthesis inhibitors we are developing may provide such an opportunity. They may address this truly unmet medical need, and accomplish for patients with prostate cancer what aromatase inhibitors are accomplishing for patients with breast cancer. Androgen synthesis inhibitors may allow clinical investigators to manipulate the “androgen axis” in a way not currently encompassed by any current single agent. The eventual use of our inhibitors might not only occur in patients relapsing form LHRH inhibitors or castration plus androgen receptor antagonists, but it could afford a new dimension for hormonal modulation in newly diagnosed patients.

We have identified a number of novel compounds which inhibit CYP17 and are also potent antiandrogens and inhibit growth of human prostate cancer cells [52,53]. The 17α-hydroxylase/$C_{17,20}$-lyase (CYP17) is a key enzyme in the biosynthesis of androgens and

converts the C_{21} steroids (pregnenolone and progesterone) to the C_{19} androgens, dehydroepiandrosteone (DHEA), 5-androstenediol (A-diol), testosterone, and androstenedione in the testis and adrenals. Currently, ketoconazole, an imidazole fungicide, is the only inhibitor used to reduce testosterone biosynthesis in the treatment of patients with advanced prostatic cancer [54,55]. However, ketoconazole is not very potent or specific. Despite its draw backs, careful scheduling of treatment can produce prolonged responses in otherwise hormone-refractory patients [56]. Also, in a study by Small et al. [57], 62.5% of patients with advanced prostate cancer who had progressed following antiandrogens (flutamide) withdrawal, were found to have greater than 50% decrease in PSA values, while 48% had greater than 80% decrease. These findings suggest that more potent and selective inhibitors of this enzyme could provide useful agents for treating this disease, even in advanced stages and in some patients who may appear to be hormone refractory. It is possible that these tumors would be amenable to more potent inhibitors of androgen synthesis or second line therapy with differently acting agents.

We have reported the effects of a number of novel steroidal inhibitors of CYP17. Some have been shown to be strong inhibitors of androgen production and tumor growth in rodent models [52,58-61]. Jarman and colleagues recently described the effects of a similar steroidal CYP17 inhibitor, abiraterone, in patients with PC [62, 63]. Interestingly, our most effective CYP17 inhibitors possess several activities, such as inhibition of 5α-reductase and/or are antiandrogens with potent antitumor efficacy [64,53]. VN/124-1 exhibits potent AR antagonism and downregulates the AR. This compound causes tumor growth suppression in LAPC4 xenografts [Sean, 66] that is significantly greater than due to castration. Development of this compound is in progress for clinical trials in prostate cancer patients.

Effects of Long-Term Treatment with Aromatase Inhibitors

Clinical evidence indicates that AIs are more effective than tamoxifen and provide significant benefit to breast cancer patients. Nevertheless, tumors of some patients are initially refractory while others inevitably become resistance to treatment. Therefore, we have developed a xenograft model suitable for investigating the mechanisms of resistance in tumors that are no longer responsive to treatment with AIs. Our finding parallels our studies in xenograft models of prostate cancer [67] and suggest that tumors adapt and survive treatment by activating alternate signaling pathways such as the HER-2/MAPK pathway [67,68]. Similarly in the LNCap prostate cancer model, loss of androgen dependency was associated with activation of mTOR [67]. We propose that blocking signaling pathways associated with resistance as well as estrogen signaling may restore sensitivity of the tumors to aromatase or CYP17 inhibitors.

In our intratumor aromatase xenograft model letrozole treatment caused marked inhibition of tumor growth for an extended period of time and reduced estrogen levels by 90%. In this model human ER positive breast cancer cells (MCF-7) stably transected with aromatase [69] are inoculated into athymic immune suppressed mice [70]. These tumor cells (MCF-7Ca) served as an autocrine source of estrogen by aromatizing androstenedione. The model simulates the post-menopausal situation where breast and tumor aromatase are the main source of estrogen and not under gonadotropin feedback regulation. Since the tumor cells express ERα as well as aromatase, antiestrogens as well as aromatase inhibitors could be studied in tumors formed from these cells [70,71]. This intratumoral aromatase xenograft model has predicted the outcome of several clinical trials [71]. It has provided data showing that the AI letrozole was more effective than tamoxifen in suppressing breast tumor growth (Figure 1) and for a longer period of time. Also, our findings that combining an AI with tamoxifen was consistent with the ATAC trial where BC patients did not benefit significantly more from the combined treatment of anastrozole and tamoxifen than

tamoxifen alone [72]. We have also shown that tumors progressing on tamoxifen remained sensitive to second-line therapy with the AI letrozole compared with those remaining on tamoxifen [72]. In clinical trials where patients received tamoxifen for 2-5 years switched to letrozole showed significant survival benefit (MA-17) [51] or exemestane showed improved disease-free survival [73]. In contrast, switching from letrozole treatment to second-line therapy with the antiestrogens fulvestrant or tamoxifen was less beneficial than remaining on letrozole [74]. Analysis of tumors from the mice in these studies provides a better insight into current trials that are examining the correct sequences of AIs and AEs [75].

Despite marked and sustained suppression of tumor growth, tumors eventually acquired the ability to grow in the presence of letrozole and were unresponsive to subsequent treatment with AEs [74]. Tumors were collected from the mice at specific times (4, 28 and 56 weeks) during the course of treatment and analyzed for changes associated with resistance to treatment (Figure 1A) [75]. Tumors at 4 weeks had regressed in response to treatment. At 28 weeks, tumors had almost doubled in size, whereas those at 56 weeks had increased 6-fold compared to their initial size even though letrozole treatment was continued. Immunoblot analysis revealed that during transition from a responsive to an unresponsive state, the level of the ER was decreased whereas the Her2/Raf/MAPK signaling pathway was activated (Figure 1B) [75]. At 4 weeks of letrozole treatment, when tumors were regressing, expression of Her-2 in the tumor was increased two-fold (Figure 1B). Greater increases in Her-2 expression were seen at 28 and 56 weeks when tumors were growing on letrozole treatment (Figure 1A). The activation of Shc (p-Shc) (Figure 1B) as well as increases in Grb-2 and p-MAPK were also observed (6-fold) (Figure 1B) [75] at these later times.

For further studies, a cell line was isolated from the long term letrozole treated tumors (LTLTCa cells) (Figure 1A) [74]. As in the tumors, the cells showed upregulation of Her-2, Grb-2, p-Shc, p-Raf, p-MEK1/2 and p-MAPK (Figure 2A). In addition, downstream kinesis that are targets for MAPK, such as p90RSK and Elk were also activated by phosphorylation (Figure 2A). It has been shown previously that MAPK can phosphorylate ER directly or indirectly via these downstream targets Elk-1 and p90RSK [76-78]. We found that at 4 weeks when tumors were regressing ERα levels were increased but when they became insensitive to letrozole and were actively growing, ER-α expression was decreased below control levels at 28 and 56 weeks (Figure 2B). Although the total amount of ER-α was reduced, the levels of phosphorylated ER-α were significantly greater than the levels of phosphorylated ER-α seen in letrozole responsive tumors. Furthermore, the level of expression of ER regulated progesterone receptor (PgR) during 56 weeks of treatment was unchanged evenwhen the tumors progressed from a responsive state to a refractory state (Figure 2A). Consistent with findings in the tumors, the LTLTCa cells also have decreased expression of ER-α (Figure 2B) whereas PgR was similar to levels in parental MCF-7Ca cells (Figure 2A). These results suggest that the growth of letrozole resistant tumors becomes independent of estrogen stimulation due to decrease in ER expression via interaction between Her-2/Raf/MAPK and ER that ultimately causes constitutive activation of ER by phosphorylation. These findings are consistent with previous reports that the interaction between growth factor receptor and ER-α results in an increased ER-related transactivation in a ligand-independent manner [79]. In our model, the increase in the phosphorylation of the ER promoted its transactivation even in the absence of the ligand as indicated by the steady expression of the estrogen-regulated PgR (Figures 1B).[75] Using LTLTCa cells we have shown that letrozole resistance is similar to resistance to other types of hormonal therapies such as anastrozole, exemestane, tamoxifen and fulvestrant (Figures 3A and 3B).

Consistent with the above findings, letrozole resistant LTLTCa cells were also unresponsive to E_2 [75] (Figure 3C). Due to the constitutive activation of the ER, the LTLTCa cells

became insensitive to proliferative concentrations of E_2 and to reduction in E_2 levels caused by AIs. The estrogen independence of the LTLTCa was further elucidated when they were transplanted into mice. These cells proliferated and formed tumors in the animals without any hormonal supplements. Thus the tumors grew at the same rate as those receiving androstenodione (Δ4A) or a combination of Δ4A and letrozole.

Overcoming resistance to AI therapy

Tumors that overexpress Her-2 are likely to be ER negative and PgR negative [80]. Our studies suggest that Her-2 causes downregulation of ER expression resulting in resistance to letrozole. We therefore investigated whether inhibition of key proteins in the Her-2 pathway would restore ER expression to normal levels and reverse resistance to AIs. When LTLTCa cells were treated with the clinically used Her-2 antibody, trastuzumab (Herceptin) (Figures 5) or the MAPK inhibitor PD98059 (Figure 2B) there was a marked increase in expression of ER to levels observed in the parental MCF-7Ca cells and much higher than the levels of the untreated LTLTCa cells [75]. In addition, Her-2 and MAPK inhibitors effectively inhibited proliferation of LTLTCa cells indicating their dependency on Her-2/MAPK signaling for stimulating growth. Importantly, treatment of LTLTCa cells with trastuzumab was also very effective in restoring the antiproliferative effect of letrozole treatment in LTLTCa cells (Figure 3A) [68]. Trastuzumab (100μg/mL) showed a synergistic inhibition of proliferation when combined with letrozole. Furthermore, trastuzumab not only reversed the resistance of LTLTCa cells to letrozole but also to tamoxifen, and other AIs exemestane and anastrozole (Figure 3B) [68]. As shown in Figure 3c, LTLTCa cells are unresponsive to estradiol (E_2). However, when pre-treated with 100μg/mL of herceptin, they became as sensitive to the effects of E_2 as MCF-7Ca cells. Trastuzumab (100μg/mL) also normalized the levels of p-Elk1, p-MAPK and p-p90RSK expression in LTLTCa cells to those of MCF-7Ca cells (Figure 4) [68]. These results indicate that Her-2 is a negative regulator of ER-α and that blockade of the Her-2 signaling pathway restores ER-α expression to normal levels observed in hormone sensitive MCF-7Ca cells. Furthermore, the results indicate that control over tumor growth is achieved by blocking growth factor receptor similarly as well as ER-mediated signaling [68].

We investigated the hypothesis that combining agents that inhibit estrogen mediated signaling and growth factor receptor signaling will block their interaction and overcome resistance to hormone therapy. When MCF-7Ca tumors reached a measurable size (~300 mm^3) mice in the control group were divided into groups. One group continued as control another was treated with trastuzumab (5 mg/kg/week) and a third group with letrozole (10μg/day). The tumors in the trastuzumab group grew at the same rate as the controls. Tumors in the letrozole group, initially regressed for several weeks but eventually began to grow and had doubled in volume by 18 weeks. The mice were then re-grouped and continued on letrozole, switched to trastuzumab treatment or treated with letrozole plus trastuzumab (5mg/kg/week). Tumors switched to trastuzumab were significantly reduced by the Her-2 blocker for a few weeks but subsequently grew to the same size as control tumors. The addition of trastuzumab to letrozole treatment caused significant regression compared to the tumors that remained on letrozole and all other treatments (Fig 5) [68]. This finding shows that resistance to letrozole can be overcome by adding trastuzumab to the letrozole treatment regime and could be effective in BC patients whose tumors have developed resistance to this AI. A number of clinical trials are in progress with tyrosine kinase inhibitors that will test the hypothesis.

Conclusions

Targeting steroidogenic enzymes such as aromatase (CYP19) has proved an effective strategy for treating breast cancer. Although aromatase inhibitors are having utility in other conditions in both women and men, aromatase inhibitors have not been successful in treating either benign prostatic hypotrophy (BPH) or prostate cancer. New strategies to develop inhibitors of 17α-hydroxylase/lyase (CYP17) have potential on this regard. Clinical trials with abiraterone are currently in progress. Some of these CYP17 inhibitors are of particular interest as they are also androgen receptor antagonists and cause down regulation of this receptor. A lead compound is anticipated to begin clinical trials in the near future. Strategies to meet the challenge of inevitable resistance to these treatments are in progress. Results indicate that tumors adapt by activating alternate signaling pathways. Inhibiting these pathways as well as estrogen/androgen signaling is effective in model systems in restoring and extending the benefits of therapy with these hormonal agents.

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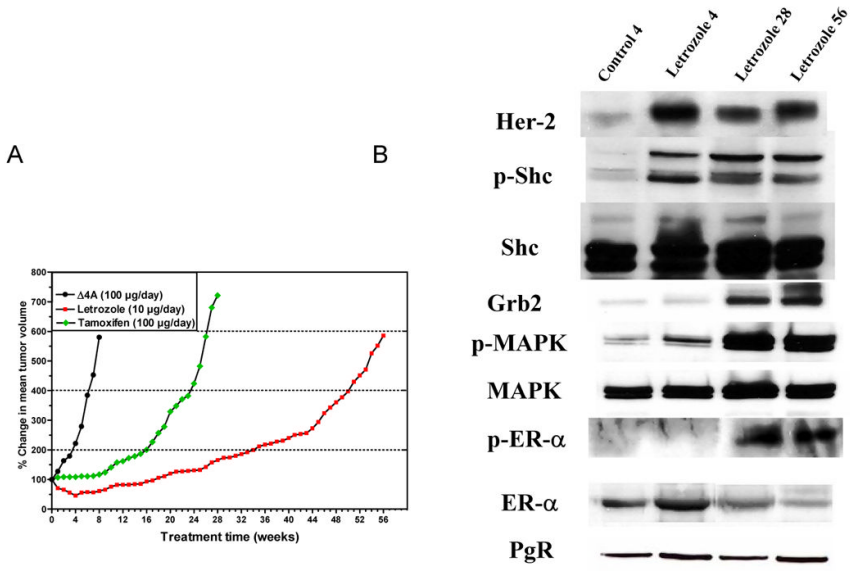

Figure 1. The effects of long term treatment with letrozole on the growth of MCF-7Ca xenografts

A) Animals were inoculated with MCF-7Ca cells at two sites on each flank and were supplemented with androstenedione (100 mg/day) for the duration of experiment [75]. When the tumors reached a measurable size (~300 mm3), animals were assigned to 3 groups (n = 20 per group), and injected sc daily with vehicle (control), or tamoxifen (100 mg/day), or letrozole (10 mg/day). Tumor volumes were measured weekly and were expressed as the percent change relative to the initial tumor volume. Two mice per group were sacrificed and tumors were collected for analysis at 4, 28, and 56 weeks as indicated on the graph. B) The effect of letrozole treatment on Her-2, p-Shc, Shc, Grb2, p-MAPK, MAPK, ER-a, p-ER-a and PgR expression in MCF-7Ca tumor xenografts [75]. Letrozole treated tumors collected at 4 weeks, 28 and 56 weeks (above), were analyzed by Western immunoblotting, and were compared to vehicle treated tumors collected at Week 4 (control). Tumors were homogenized in lysis buffer as described previously [75].

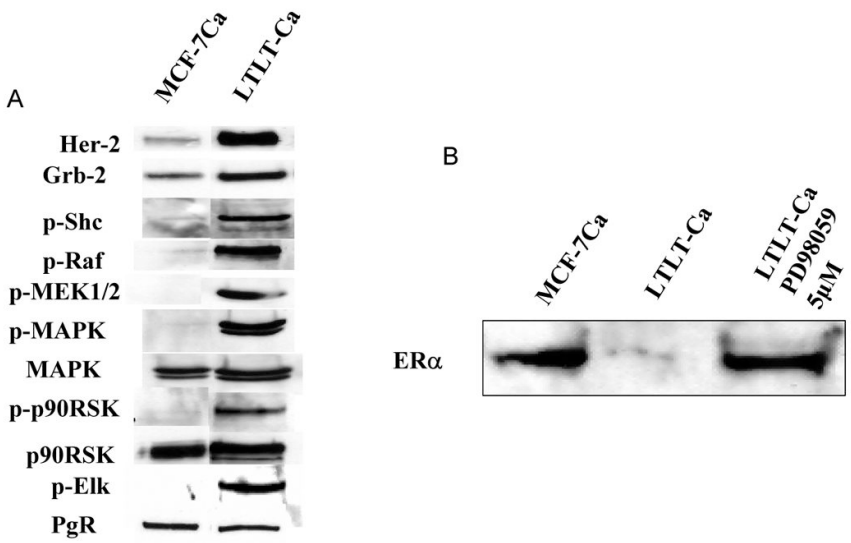

Figure 2. Profile of protein expression on LTLTCa cells compared to MCF-7Ca cell

A) Expression of Her-2, p-Shc, Grb2, p-MEK1/2, p-p90RSK, p90RSK, p-Elk, p-MAPK, MAPK, and PgR in MCF-7Ca (letrozole sensitive) and LTLT-Ca (letrozole resistant) cells. Cell lysates were prepared and used for Western Blot as described [75]. B) Effect of 5 mM of PD98059 on increasing ER-a expression in LTLTCa cells compared to MCF-7Ca cells. LTLT-Ca cells were transferred into steroid-free medium for 3 days before plating. The next day cells were washed and treated with steroid-free medium containing androstenedione (25nM) and the indicated concentration of PD 98059. The medium was changed every 3 days, and the cells were lysed 9 days later [75].

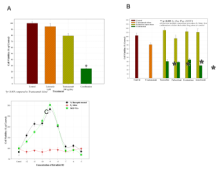

Figure 3. Restoration of hormonal sensitivity to LTLTCa cells with trastuzumab (Herceptin)

LTLTCa cells were transferred to IMEM without phenol red before they were plated into 96-well plates (1000 cells/well) and allowed to attach overnight. The next day, they were treated with the indicated agents for a total of 6 days. The cell viability was measured using the MTT assay as described [68]. A) *Effect of combination of letrozole and trastuzumab in LTLT-Ca cells*. Combination of letrozole *plus* trastuzumab was significantly better than single drug treatment or control, $p < 0.0001$ (10-12M-10-9M), $p < 0.05$ (10-8M-10-5M). B) *Effect of combining trastuzumab with AEs tamoxifen, fulvestrant and AIs exemestane, anastrozole in LTLT-Ca cells*. The cell viability was found to be significantly lower in groups treated with the combination of trastuzumab plus AI or AE versus control *($p<0.0001$)* or trastuzumab alone *($p<0.0001$)* or the endocrine agent alone *($p<0.0001$)*. C) *Effect of estradiol (E2) on proliferation of MCF-7Ca and LTLT-Ca cells in presence or absence of trastuzumab (Herceptin) pretreatment*. When pre-treated with herceptin (100μg/mL), proliferation of LTLT-Ca cells was significantly stimulated in response to E2 at concentrations of 10-12M - 10-7M when compared to E2 alone *($p <0.0001$)*. When MCF-7Ca cells were pretreated with herceptin, E2 stimulated proliferation was increased at concentrations 10-11M - 10-10M *($p = 0.02$ and 0.03 respectively)* [68].

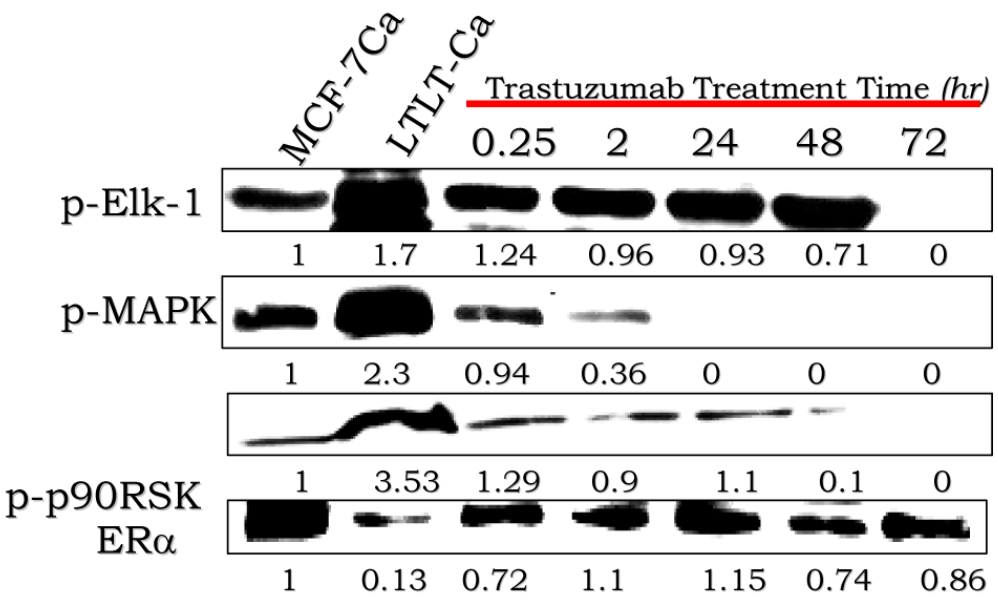

Figure 4. The effect of trastuzumab treatment on ER-a expression

LTLTCa cells were transferred to IMEM without phenol red 24 hours before the beginning of the experiment. The next day, they were treated with the indicated agents for the specified amount of time. The cell lysates were prepared as described [75]. The effect of trastuzumab treatment at various time points on protein expression of p-ELK-1, p-MAPK, p-p90RSK and ER-a in LTLT-Ca cells is shown. Protein expression was examined using western immunoblotting. Blot shows phospho-Elk-,1 at 60 kDa, phospho-p90RSK at 90 kDa, p-MAPK at 42-44 kDa and ER-a at 66 kDa. The blots show a single representative of three independent experiments.

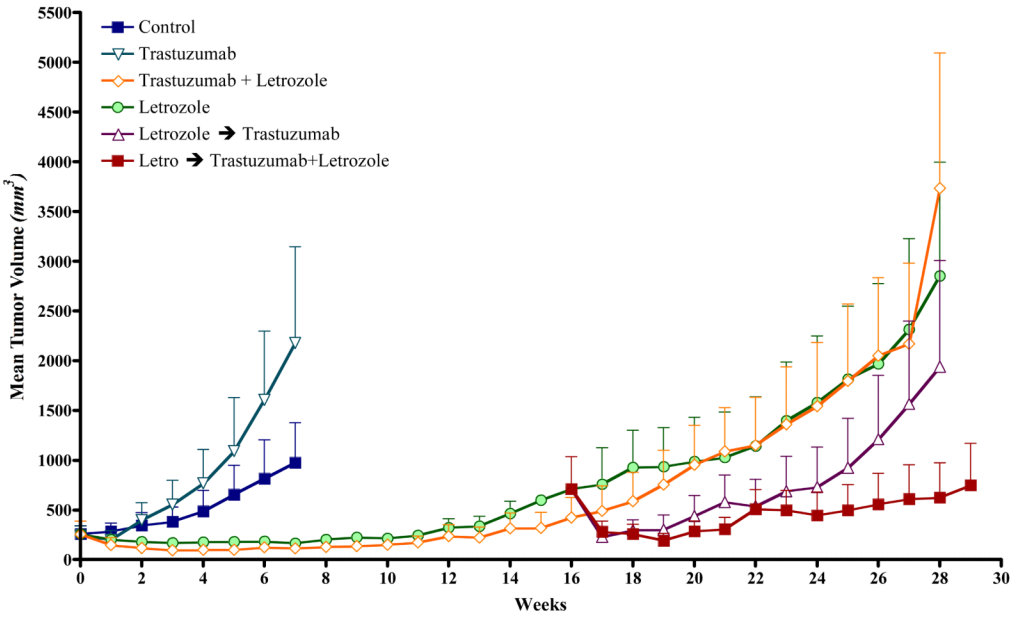

Figure 5. The Effect of trastuzumab alone or in combination with letrozole on the growth of MCF-7Ca xenografts

Tumors of MCF-7Ca cells were grown in mice following procedures described in Fig 1 and treated with vehicle, trastuzumab or letrozole. At week 16, the group receiving letrozole was divided into two groups and received letrozole plus trastuzumab or continued on letrozole. Trastuzumab alone (5mg/kg/week) did not inhibit the growth of MCF-7Ca tumors. The difference in the exponential parameter governing the growth rate of letrozole versus letrozole switched to letrozole *plus* trastuzumab was 0.21 ± 0.08, $p = 0.008$. The difference in the exponential parameter governing tumor growth rate of letrozole *plus* trastuzumab versus letrozole switched to letrozole *plus* trastuzumab was 0.39 ± 0.09, $p < 0.0001$. The difference in the exponential parameter governing rate of letrozole switched to trastuzumab versus letrozole switched to letrozole *plus* trastuzumab was 0.2 ± 0.08, $p = 0.011$, over weeks 15-28. When compared through week 29, the difference in the exponential parameter governing growth rate of letrozole versus letrozole switched to trastuzumab was 0.005 ± 0.08, *p value = 0.97* [68].